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Remarkable stability of the proton translocating F_1F_O-ATP synthase from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1

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Abstract

For functional characterization, we isolated the F_1F_O-ATP synthase of the thermophilic cyanobacterium *Thermosynechococcus elongatus*. Because of the high content of phycobilisomes, a combination of dye-ligand chromatography and anion exchange chromatography was necessary to yield highly pure ATP synthase. All nine single F_1F_O subunits were identified by mass spectrometry. Western blotting revealed the SDS stable oligomer of subunits c in *T. elongatus*. In contrast to the mass archived in the database (10,141 Da), MALDI-TOF-MS revealed a mass of the subunit c monomer of only 8238 Da. A notable feature of the ATP synthase was its ability to synthesize ATP in a wide temperature range and its stability against chaotropic reagents. After reconstitution of F_1F_O into liposomes, ATP synthesis energized by an applied electrochemical proton gradient demonstrated functional integrity. The highest ATP synthesis rate was determined at the natural growth temperature of 55 °C, but even at 95 °C ATP production occurred. In contrast to other prokaryotic and eukaryotic ATP synthases which can be disassembled with Coomassie dye into the membrane integral and the hydrophilic part, the F_1F_O-ATP synthase possessed a particular stability. Also with the chaotropic reagents sodium bromide and guanidine thiocyanate, significantly harsher conditions were required for disassembly of the thermophilic ATP synthase.

Keywords: ATP synthase; Cyanobacteria; Thermo stability; Chaotropic reagent

1. Introduction

Cyanobacteria have been tremendously important in shaping the course of evolution and ecological change throughout earth's history. The oxygen atmosphere was generated by cyanobacteria [1]. The other fundamental contribution of the cyanobacteria is the origin of chloroplasts. Cyanobacteria and plants share numerous photosynthesis-related genes that are missing in genomes of other phototrophes [2]. We studied the thermophilic cyanobacterium *Thermosynechococcus elongatus*, which can be found in freshwater hot springs at an optimal growth temperature of 55 °C. The knowledge of the complete genome structure [3] facilitates mass spectrometric analyses. For survival in extreme environments thermophilic organisms need heat-adapted proteins and enzymes. The higher thermodynamic stability of thermophilic proteins is probably based on a decreased rate of unfolding [4] but depends also on the Gibbs free energy [5]. By Jaenicke and Böhm [6] several potential interactions are described, that seem to render proteins extremely thermo stable. For example, thermophilic proteins show an increase in the number of hydrogen bonds, additional or improved electrostatic interactions caused by salt bridges, optimized hydrophobic interactions, association to oligomers and reduction of the content of thermally labile amino acids. Beside increased thermo stability, thermophilic enzymes exhibit a decreased catalytic activity at lower temperatures. Thermophilic enzymes seem to be stable up to 70 °C or 90 °C, mesophilic enzymes only up to 40 °C or 60 °C [7].

The F_1F_O-ATP synthase is one of the most widespread and important enzymes both in relation to oxidative phosphorylation and photosynthesis. ATP synthesis by the F_1F_O-ATP

Abbreviations: BN-PAGE, Blue-native polyacrylamide gel electrophoresis; CF_1F_O, chloroplast ATP synthase; chl a, chlorophyll a; DCCD, dicyclohexylcarbodiimide; DDM, *n*-dodecyl-β-D-maltoside; ESI-MS, electrospray ionisation mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

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synthase is energized by an electrochemical proton gradient (proton-motive force, Δp) across the thylakoid membrane [8]. The CF_1F_O-ATP synthase of higher plants is found in chloroplasts, whereas the F_1F_O-ATP synthase of cyanobacteria is embedded in thylakoid membranes, which lie parallel to each other or strongly curved in the peripheral protoplasm. The F_1F_O-ATP synthase consists of two parts. The hydrophilic F_1 part, composed of the subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ [9], catalyzes the formation of ATP. F_O is the membrane integral proton-conducting complex, in cyanobacteria made up of the subunits $abb'c_x$. The flow of protons propels the rotation of a transmembrane entity comprised of identical subunits c. The number of subunits in this proton turbine determines the H^+/ATP ratio and therefore the efficiency of energy conversion. Stoichiometries identified vary between 10 and 15 and seem to be specific for the respective organism. Ten c subunits were determined for the thermophilic bacterium *Bacillus* sp. strain PS3 [10], *Saccharomyces cerevisiae* [11] and *E. coli* [12], 11 subunits for *Ilyobacter tartaricus* [13], *Propiogenium modestum* [14] and the thermoalkaliphilic bacterium *Clostridium paradoxum* [15], 13 for the cyanobacterium *Synechococcus elongatus* SAG 89.79 and *Bacillus* sp. strain TA2.A1 [16,17], 14 for the spinach chloroplast ATP synthase [18] and 15 for the mesophilic cyanobacterium *Spirulina platensis* [19]. This variation could be a strategy for adaptation to different environmental conditions.

Several isolation procedures for cyanobacterial ATP synthase are described in literature, but they lead only to a crude purification of the complete ATP synthase or solely isolation of certain subunits. The first purification method for cyanobacterial ATP synthases was published by Binder and Bachofen [20]. The F_1 part of the ATP synthase of the thermophilic cyanobacterium *Mastigocladus laminosus* was isolated and purified by chloroform extraction and density gradient centrifugation. Lubberding et al. [21] modified the procedure for the ATPase complex from membrane vesicles [22] to isolate the F_1 part of the ATP synthase of the thermophilic cyanobacterium *Synechococcus* 6716. Detergent extraction and ammonium sulfate precipitation followed by chromatographic purification on a Sepharose 6B column were applied. The ATP synthase-enriched fractions were free of phycocyanin but still contaminated with carotenoids and only contained the subunits α, β and γ. Van Walraven et al. [23] modified the purification method of Lubberding et al. [21]] by replacing the Sepharose 6B column with a Sephacryl S-300 column. Subunits α, β, δ and ε were identified, but the SDS gel indicated that other contaminants were also present. Lill et al. [24] applied a linear sucrose gradient after detergent extraction and ammonium sulfate precipitation. The c-oligomers of the ATP synthase of the cyanobacteria *Spirulina platensis* and *Synechococcus elongatus* SAG 89.79 were isolated by treatment with *N*-lauroylsarcosine and subsequent ammonium sulfate precipitation [17,19]. There is still no purification method published that results in highly pure and intact ATP synthase. We describe a method for the isolation and purification of highly pure F_1F_O-ATP synthase of *T. elongatus* and characterize functional properties.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Thermosynechococcus elongatus strain BP-1 was grown at 55 °C on BG11-medium [25], bubbled with CO_2-enriched air (5%). The volume of the transparent, airlift tube fermenter was 20 L. The light intensity was increased from 50 to 100 μmol photons/s m^2 during the first 12 h and after 24 h set to 150–200 μmol photons/s m^2. Cells were harvested after 3 days.

2.2. Isolation of thylakoid membranes

Whole culture was centrifuged for 10 min at 10,850 ×*g*, 4 °C and resuspended in 150 ml decomposition buffer (20 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 6.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$,) containing lysozyme. Cells were stirred for 1 h at 4 °C in the dark. After a centrifugation step (10 min at 10,850 ×*g*, 4 °C) the cells were resuspended in 30 ml decomposition buffer containing freshly added protease inhibitors (0.5 mM Pefabloc (Biomol GmbH; Hamburg, Germany), 0.5 mM iodoacetamide, 5 mM sodium fluoride) and a small amount (tip of a spatula) DNase I. Cells were disrupted using a Parr bomb according to Kuhl et al. [26]. Membranes were separated by centrifugation (10 min, 16,270 ×*g*). Approximately 30 g thylakoid membranes were isolated from the 20 L culture. The chlorophyll a (chl a) content of the membrane suspensions was determined according to Arnon [27]. The membranes were flash frozen in liquid nitrogen and stored at −80 °C.

2.3. Isolation of F_1F_O

15 g thylakoid membranes were incubated for 10 min on ice in a freshly prepared buffer containing 10 mM sodium pyrophosphate and 0.5 mM ATP (pH 7.5) at a chl a concentration of 0.2 mg/ml and then centrifuged for 10 min at 12,000 ×*g*, 4 °C. To remove phycobilisomes quantitatively, this washing procedure was repeated twice. Finally obtained pellets were resuspended at 2 mg/ml chl a in solubilization buffer (200 mM sucrose, 3 mM KCl, 3 mM $MgCl_2$, 50 mM Tricine, pH 8.0) and dithiothreitol (DTT) was added up to 0.5 mM. After 15 min stirring at 4 °C, 1.5% (w/v) *n*-dodecyl-β-D-maltoside (DDM) (Calbiochem®, EMD Biosciences; La Jolla, CA), 0.5% (w/v) sodium cholate and 12.5% (v/v) suspension buffer (0.8 M sucrose, 12 mM $MgCl_2$, 12 mM KCl) were added. Solubilization took place at a final concentration of 1 mg/ml chl a. Ammonium sulfate was set to a final concentration of 7% (w/v) and DTT to 25 mM. The suspension was sonified in interval mode (60% duty cycle, position 4) with cell disruptor B15 (Branson Sonic Power Company; Danbury, CO) four times 2.5 min at 4 °C. After stirring 10 min at 4 °C, non-solubilized material was spun down for 1 h at 181,564 ×*g*. To partially separate the ATP synthase from contaminating proteins and lipids a fractionated ammonium sulfate precipitation followed. In the first step, the concentration was adjusted to 30% saturation. After centrifugation for 10 min at 12,000 ×*g*, the pellet was discarded. The supernatant was then adjusted to 50% saturation with ammonium sulfate solution (pH 8.0). After centrifugation as described before, the pellet was resuspended in a minimum volume of centrifugation medium. This medium for rate-zonal centrifugation contained 30 mM Tris pH 6.5, 30 mM succinic acid, 8 mM DDM, 0.5 mM EDTA and 1 mg/ml soybean asolectin (FLUKA, Buchs, Switzerland). The resuspended pellet was layered on a continuous sucrose gradient (15–50% (w/v)). Gradients were centrifuged at 105,624 ×*g* for 15 h at 4 °C (Beckman VTI 50 rotor). Proteins separated in the gradient were fractionated and analyzed by SDS-PAGE and Western blotting. F_1F_O was found between 35% and 40% sucrose.

2.4. Chromatographic purification of F_1F_O

Fractions containing the ATP synthase were pooled and desalted by passage through 10 ml Handee™ spin centrifugation columns (PIERCE; Rockford, IL) (2 min at 500 ×*g*) filled with Sephadex® G-25 medium (GE Healthcare; Uppsala, Sweden). Columns were rinsed with equilibration buffer (20 mM Tris/HCl, pH 8.0, 20% (w/v) glycerol, 5 mM $MgSO_4$ and 4 mM DDM). The protein suspension was then loaded on a Reactive Red 120 column (40 ml resin; Sigma; Steinheim, Germany). ATP synthase did not bind to the dye ligand. Fractions

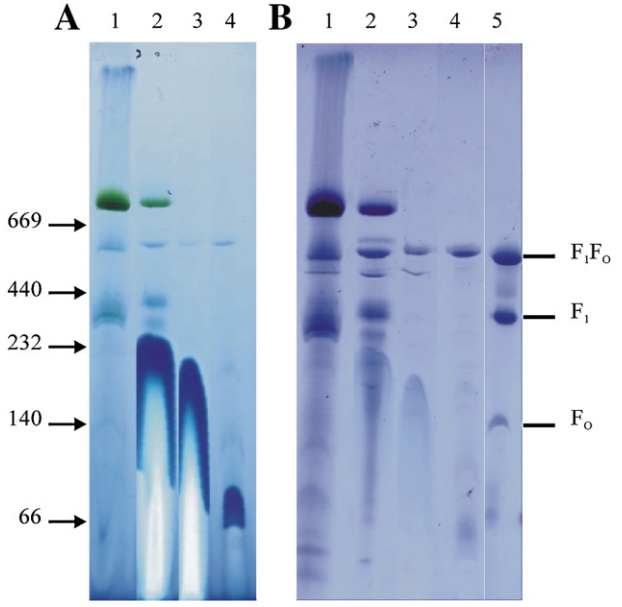

Fig. 1. BN-PAGE (3.5–16% gradient of polyacrylamide) of the purification steps of F_1F_O from thylakoid membranes of *Thermosynechococcus elongatus*. (A) unstained gel; (B) same gel but additionally Coomassie R-250-stained. Lane 1, suspended pellet after 50% ammonium sulfate precipitation; lane 2, typical fraction of the density gradient containing 38% sucrose; lane 3, F_1F_O after Reactive Red 120 – chromatography; lane 4, F_1F_O after anion exchange – chromatography; lane 5 shows the complete CF_1F_O of *Spinacea oleracea* and its two parts F_1 and F_O. As standard a high molecular mass marker (in kDa) is indicated on the left side. Purification is demonstrated by decreasing protein contamination in comparison to the F_1F_O content.

containing the ATP synthase were eluted with three column volumes of equilibration buffer at a flow rate of 2 ml/min. Afterwards, the proteins that bound to the dye ligand were eluted with three column volumes of elution buffer (equilibration buffer including 1 M NaCl). For further purification an anion exchange chromatography with a HiTrapQ column (1 ml resin volume; GE Healthcare) follows. ATP synthase solution was loaded on the column and elution started with 10 column volumes of equilibration buffer at 1 ml/min. Subsequently, a linear gradient of 20 column volumes equilibration buffer/elution buffer up to 100% elution buffer was applied to yield highly pure ATP synthase.

2.5. Reconstitution of F_1F_O into liposomes

Reconstitution of F_1F_O was carried out according to Poetsch et al. [28] with the lipids phosphatidyl choline/phosphatidic acid (9:1 (w/w)). As modification the F_1F_O/detergent/lipid-mixture (600 µl) was dialyzed against 600 ml of reconstitution buffer (10 mM Tricine, pH 8.0, 25 mM $MgCl_2$, 0.2 mM EDTA, 0.25 mM DTT) at 4 °C for 5 h. Dicyclohexylcarbodiimide (DCCD) inhibition of F_1F_O [29] was performed by adding 1 µl 5 mM DCCD in methanol to 100 µl of proteoliposomes in reconstitution buffer. The mixture was incubated 30 min at 20 °C and then dialyzed as described before.

2.6. Activity of ATP synthesis

Two procedures were used to measure ATP synthesis. With the "one-step method", ATP was determined during its production by a slightly modified procedure of Fischer et al. [30] as described previously [28]. The activity was calculated from the initial slope of the ATP synthesis kinetics.

During the "two-step method", proteoliposomes were incubated 30 min at various temperatures (4 °C, 23 °C, 42 °C, 55 °C, 65 °C, 75 °C, 85 °C and 95 °C). Then ATP production was triggered and stopped after a certain time. The amount of ATP was determined according to Gremminger [31]. 10 µl proteoliposomes, pre-incubated for 30 min at the selected temperature, were mixed with 50 µl buffer LI (20 mM sodium succinate, 5 mM sodium dihydrogenphosphate, 0.6 mM KCl, 2.5 mM $MgCl_2$, 1 µM valinomycin (freshly added), pH 4.7). After incubation for 2 min at the different temperatures, 60 µl buffer LII (200 mM Tricine, 5 mM sodium dihydrogenphosphate, 120 mM KCl, 2.5 mM $MgCl_2$, 200 µM ADP (freshly prepared), pH 8.5) was added. ATP synthesis was stopped after 10 s with 100 µl of a 4% (w/v) trichloroacetic acid (TCA) solution. The samples were stored at 4 °C until the amount of ATP generated was determined. For ATP quantification, 200 µl luciferase-buffer (100 mM Tris, 2 mM EDTA, pH 7.75) was mixed with 50 µl luciferin–luciferase-solution (ATP-Monitoring Kit; Thermo Labsystems, Vantaa, Finland) at room temperature. The baseline was recorded and thereafter 10 µl of the sample was used to determine the ATP signal with a luminometer (Luminometer 1250; BioOrbit, Turku, Finland). Finally 5 µl of a 1 µM ATP solution was added for calibration. To analyze the stability of the F_1F_O-ATP synthase, the ATP synthesis activity was measured after incubation at 55 °C over a time period of 6 h.

2.7. Electrophoresis

SDS-PAGE was performed as described [32] with 14% acrylamide gels ($80 \times 75 \times 0.75$ mm^3). Protein samples were incubated in SDS sample buffer [32] at room temperature before electrophoretic separation, in order to maintain the integrity of the c-oligomer of F_O [33]. BN-PAGE was carried out according to [34,35] on 3.5% to 16% acrylamide gradient gels ($80 \times 75 \times 1.5$ mm^3). The cathode buffer with only 0.002% (w/v) Serva blue G was used to prevent the dissociation of the F_1F_O holoenzyme in its F_1 and F_O parts. Coomassie R-250 and silver staining were performed according to [36,37].

2.8. Western blot analysis

After electrophoresis the gel was incubated in SDS-buffer (400 mM glycine, 50 mM Tris, 3.5 mM SDS) for 10 min. Proteins were transferred to PVDF membranes at 1 mA/cm^2 using Roti®-Blot 2 anode and cathode buffers (Carl Roth GmbH; Karlsruhe, Germany). Membranes were blocked for 1 h in a ten fold dilution of Roti®-Block (Carl Roth GmbH) and subsequently incubated for 1 h with polyclonal antibodies raised against monomer III from *Spinacea oleracea* (anti-III 492; 1:2000 dilution in a ten fold dilution of Roti®-Block) and against subunit α from spinach (anti-α 464; 1:500 dilution in a ten fold dilution of Roti®-Block) respectively (both antibodies were obtained from Prof. Dr. Berzborn, Ruhr-University Bochum, Germany). PVDF membranes were washed five times with phosphate buffered saline (PBS) (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% (w/v) Tween-20 (PBST). Thereafter anti-rabbit secondary antibodies (1:2000 dilution in a ten fold dilution of Roti®-Block) (GE Healthcare) linked to horseradish peroxidase were applied for 1 h. After five washing steps with PBST and one washing step with PBS, ECL detection was performed according to Haan and Behrmann [38].

Table 1
Purification of the F_1F_O-ATP synthase of *Thermosynechococcus elongatus*

Purification step	ATP synthase activity (nmol ATP/min per mg of protein)	Total protein (mg)	Activity (%)	Purification (fold)
Solubilized thylakoid membranes	37	542	100	1
Supernatant 30% (w/v) ammonium sulfate	56	350	98	1.5
Pellet 50% (w/v) ammonium sulfate	81	219	88	2.2
Rate-zonal centrifugation	109	122	66	3.0
Chromatography	486	1.1	3	15

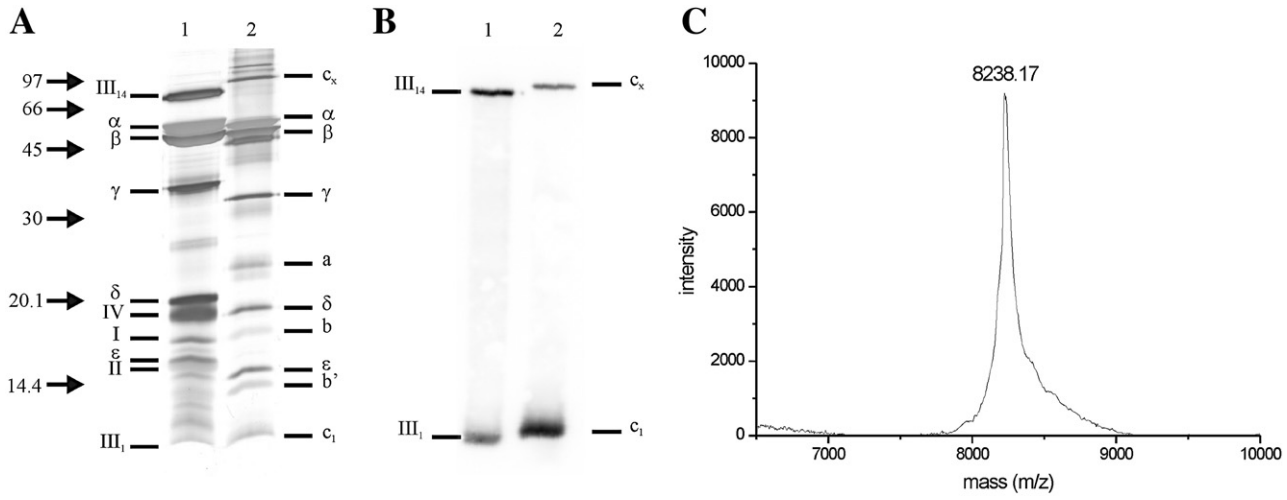

Fig. 2. (A) Silver-stained SDS-PAGE (14%) of purified CF_1F_O of *Spinacea oleracea* (lane 1) and F_1F_O of *Thermosynechococcus elongatus* (lane 2). All subunits of F_1F_O except subunits c and a were identified with MALDI-TOF-MS PMF. Subunit a was identified with ESI-MS/MS. (B) Western blot of a SDS-PAGE with polyclonal antibodies raised against monomer III from *Sp. oleracea*. Lane 1, oligomer and monomer of the subunit III of CF_1F_O of *Sp. oleracea*; lane 2, oligomer and monomer of subunit c of *T. elongatus*. (C) MALDI-MS analysis of intact c monomer. The identified mass is indicated. Subunit III of CF_1F_O of spinach was used as external standard.

2.9. MALDI-TOF-MS PMF

Protein and peptide samples were analyzed with a Voyager-DE PRO (Applied Biosystems) matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) [39]. For peptide mass fingerprinting, tryptic peptides were obtained from destained silver-stained gels [40] and in-gel digestion [41]. Peptides were analyzed in the reflector mode using a 5 mg/ml solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid [37]. The "Mascot" software (http://www.matrixscience.com) was used to match detected masses against the NCBInr database. The search included one possible missing cleavage site and the possible oxidation of methionine.

2.10. Molecular mass determination of subunit c

SDS gel pieces with the c monomer and the oligomer were incubated in water at least for 2 h to elute proteins by diffusion. After loading on ZipTips® C4 (Millipore Corporation; Bedford, USA) the proteins were washed three times with 0.5% trifluoroacetic acid and then eluted with 75% acetonitrile/0.1% trifluoroacetic acid. For dried droplet target preparation 5 mg/ml solutions of 2-(4-hydroxyphenylazo-)benzoic acid (HABA) and ferulic acid in 75% acetonitrile/0.1% trifluoroacetic acid were used. Mass spectra were obtained in the linear extraction mode.

2.11. ESI-MS/MS

Subunit a could not be identified by MALDI-TOF-MS PMF and was therefore analyzed by nLC-ESI-MS/MS systems generally as described previously [42,43], except that mass spectra were recorded on an LTQ-Orbitrap instead of an LTQ. The linear ion trap and orbitrap were operated in parallel, i.e., during a full MS scan on the orbitrap at a resolution of 60,000, MS/MS spectra of the four most intense precursors were detected on the ion trap. Further instrument settings were used as described by Fischer et al. [44]. MS/MS spectra were interpreted by the SEQUEST algorithm (UniProtKB/Swiss-Prot database) using Bioworks v3.3 (Thermo Fisher) with a precursor mass tolerance of 10 ppm. Results were evaluated on the basis of a conservative criteria set, i.e., only results with ΔC_n scores greater than 0.1 were accepted, all fragments had to be tryptic and the cross-correlation scores (Xcorr) of single charged, double charged or triple charged ions had to be greater than 1.5, 2.5 or 3.5 respectively.

2.12. Stability studies of F_1F_O

To compare the stability of the F_1F_O-ATP synthase with a typical mesophilic ATP synthase, CF_1F_O from spinach purified according to [45] was used. For stability studies of the F_1F_O-ATP synthase in presence of Coomassie, purified F_1F_O was mixed with different volumes of BN-loading buffer (5% (w/v) Coomassie Serva blue G, 500 mM ε-aminocaproic acid, 50 mM Bis–Tris; pH 7.0) and incubated for 15 min at room temperature. Additionally, the stability of F_1F_O-ATP synthase against the dissociation inducing salts sodium bromide and guanidine thiocyanate was determined. Pure CF_1F_O- and F_1F_O-ATP synthases were incubated for 15 min in 1 M sodium bromide, 1.5 M sodium bromide and 250 mM guanidine thiocyanate at different temperatures (4 °C, 23 °C, 37 °C and 56 °C). For desalting, sodium bromide treated samples were

Table 2
Subunits of the ATP synthase of *Thermosynechococcus elongatus* identified with MALDI-TOF-MS PMF after tryptic digestion

Subunit	Protein description	NCBInr [a]	Score [b]	M_r (Da) [c]	p*I* theoretic [c]
α	ATP synthase subunit A	NP 681225	256	54,271	5.14
β	ATP synthase subunit B	NP 681315	163	51,795	4.89
γ	ATP synthase subunit C	NP 681175	180	35,013	8.54
δ	ATP synthase subunit D	NP 681224	74	20,621	6.84
b	ATP synthase subunit B	NP 681223	149	19,617	6.64
b′	ATP synthase subunit B	NP 681222	74	15,457	4.60
ε	ATP synthase subunit epsilon	NP 681316	81	14,741	5.04

[a] NCBInr database accession number.
[b] Scores obtained by "Mascot". Scores of 70 and above are considered as significant.
[c] Theoretic values from mature proteins, i.e., without transit peptides.

Table 3
Identification of the ATP synthase subunit a of *Thermosynechococcus elongatus* by nLC-ESI-MS/MS after tryptic digestion

Matched peptides of subunit a [a]	Observed mass [b]	Charge state	Xcorr [c]	ΔC_n [d]	Ions [e]
R.VPSGIQNLMEYALEFIR.D	1980.03	2	5.99	0.75	25/32
K.YIEPTPVLLPIAILEDFTK.P	2172.22	2	3.81	0.89	24/36
K.YIEPTPVLLPIAILEDFTKPLSLSFR.L	2972.67	3	3.44	0.99	31/100
R.VPSGIQNLMEYALEFIRDLTK.S	2437.28	3	3.85	0.88	31/80

[a] The amino acid residues appearing before and after the dot correspond to residues preceding and following the peptide in the protein sequence.
[b] Monoisotopic mass.
[c] Cross-correlation score is based on comparison of the MS/MS data to the theoretical distribution of ions produced for the peptide.
[d] Calculated difference between the top Xcorr values for the given peptide.
[e] Total number of b and y ions identified/theoretical.

dialyzed 15 min in agarose gels as described by Freifelder and Better [46]. Reference ATP synthases without the addition of chaotropic reagents were incubated at the respective temperatures. Samples were analyzed with BN-PAGE and Western blotting as described.

3. Results

3.1. Isolation and purification of the F_1F_O-ATP synthase of Thermosynechococcus elongatus

To remove interfering phycobilisomes, the thylakoid membranes of *T. elongatus* were washed three times with a buffer containing sodium pyrophosphate. The removal of the phycobilisomes resulted in a blue color of the supernatant after centrifugation. This color diminished with each washing step yielding finally in a colorless supernatant. Washed thylakoid membranes were solubilized with DDM and cholate. The subsequent precipitation partially separated the ATP synthase from contaminating proteins and lipids. According to SDS-PAGE, the pellet of 30% ammonium sulfate saturation was slightly contaminated with ATP synthase (data not shown), but this was caused by individual subunits. F_1F_O was precipitated by adjusting the supernatant to 50% ammonium sulfate. The pellet after centrifugation included most of the ATP synthase (Fig. 1, lane 1). Rate-zonal centrifugation separated several different colored layers. Gel electrophoretic analysis revealed the F_1F_O-ATP synthase in the 35% to 40% sucrose region. As indicated by the green color, the main contaminants of the sample are phycobilisomes and other chromophore containing protein complexes (Fig. 1, lane 2). Therefore, the ATP synthase suspension was loaded onto a Reactive Red 120 column. As described previously for the spinach CF_1F_O [45], the ATP synthase did not bind to the dye ligand in contrast to contaminating proteins. As a consequence, the flow-through contained the F_1F_O. But still, the ATP synthase suspension was colored light green and the BN-PAGE showed additional protein bands (Fig. 1, lane 3). Finally, an anion exchange chromatography served as polishing step. F_1F_O eluted at approximately 0.4 M NaCl in colorless fractions containing pure F_1F_O (Fig. 1, lane 4). The results of a typical preparation are summarized in Table 1. SDS-PAGE of the purified F_1F_O revealed the presence of all ATP synthase subunits (Fig. 2A).

3.2. Identification of ATP synthase subunits

For unambiguous identification of all subunits of F_1F_O mass spectrometry and Western blotting were employed. MALDI-MS enabled the allocation of all bands belonging to the F_1 part of the ATP synthase (Table 2). Additionally the two different b subunits could be identified. In spite of numerous attempts, subunits a and c could not be identified by MALDI-TOF-MS PMF. However, subunit a could be identified with ESI-MS/MS (Table 3). For further identification of the subunit c monomer and its oligomer, a Western blot of a SDS gel with polyclonal antibodies raised against monomeric III from spinach was performed (Fig. 2B). CF_1F_O of *Sp. oleracea* and *T. elongatus* F_1F_O samples exhibited bands of the c monomer and the c oligomer, whereas the apparent masses of the monomer and the oligomer of *T. elongatus* F_1F_O appeared to be slightly higher than the masses of the respective spinach homologues. Due to the fact that there are different masses for *Synechococcus* subunit c in the databases, this subunit was analyzed by MALDI-TOF-MS. For molecular mass determination, SDS gel pieces of both the c monomer and the oligomer band were cut out and incubated in water. During purification of the extracted proteins with ZipTips® C4, bound proteins were eluted with 75% acetonitrile/0.1% trifluoroacetic acid. Elution with 50% acetonitrile/0.1% trifluoroacetic acid resulted in low protein yields (data not shown), demonstrating the strong hydrophobicity of subunit c. The matrices 2-(4-hydroxyphenylazo-)benzoic acid (HABA) and ferulic acid were compared for mass spectrometric measurements. A better resolution could be obtained with ferulic acid. The mass spectra revealed a single peak of the subunit c monomer of 8238 Da (m/z) (Fig. 2C). Subunit III of CF_1F_O with 8002 Da (m/z) served as external standard [47]. Calibration with subunit III as internal standard led to the same resulting mass for the subunit c (data not shown). Although samples of the oligomers were used for measurements, no additional peak besides the monomer could be identified in the region from 5000 to 120,000 Da.

3.3. Measurement of ATP synthesis activity

Proton-driven ATP synthesis can only be performed by an intact enzyme. Therefore ATP synthesis activity is a proof of success in the purification of intact F_1F_O. The F_1F_O-ATP synthase was

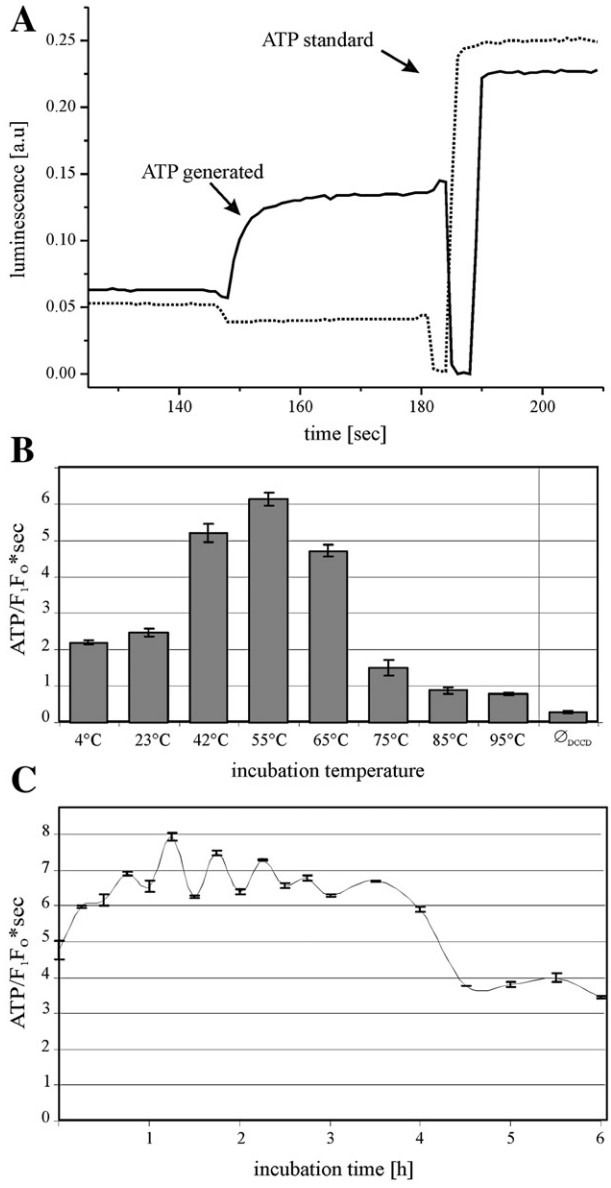

Fig. 3. (A) ATP synthesis activity of F_1F_O of *Thermosynechococcus elongatus* measured with the "one-step method". The graph reflects the change in ATP produced, initiated by the established $\Delta pH/\Delta\Psi$. The steep increase of the curve after 190 s is caused by adding the ATP standard. The curve of the DCCD inhibited sample is shown as dotted line, demonstrating that no ATP is produced. (B) ATP synthesis rate of F_1F_O of *T. elongatus* at different temperatures measured with the "two-step method". For calculation of the ATP synthesis rate, the total amount of ATP produced within 10 s was quantified. Highest ATP synthesis rate could be observed at 55 °C. The average ATP synthesis rate of the DCCD inhibited ATP synthases incubated at 75 °C, 85 °C and 95 °C is shown ($Ø_{DCCD}$). (C) ATP synthesis rate of F_1F_O of *T. elongatus* at 55 °C over a time period of 6 h measured with the "two-step method". The ATP synthesis rate remains constant at a high level for 4 h of incubation. Longer incubation leads to a decrease of the ATP synthesis activity.

reconstituted into lipid vesicles. An electrochemical proton gradient enabled ATP synthesis. Within the "one-step method" ATP synthesis and the determination of the produced ATP occurred simultaneously. Fig. 3A shows the kinetics of ATP synthesis. The initial slope of the curve was used to calculate ATP synthesis activity. With DCCD inhibited samples a flat base line was present, demonstrating that no ATP was produced. Therefore, F_1F_O was inhibited completely.

In a second experiment the amount of ATP produced within 10 s upon sample incubation for 30 min at different temperatures was determined at room temperature by the "two-step method". The liposome embedded ATP synthase utilized the electrochemical proton gradient to produce ATP. After 10 s the enzyme was denatured with TCA. Since the optimal working temperature for the luciferase is 20 °C to 25 °C, measurement

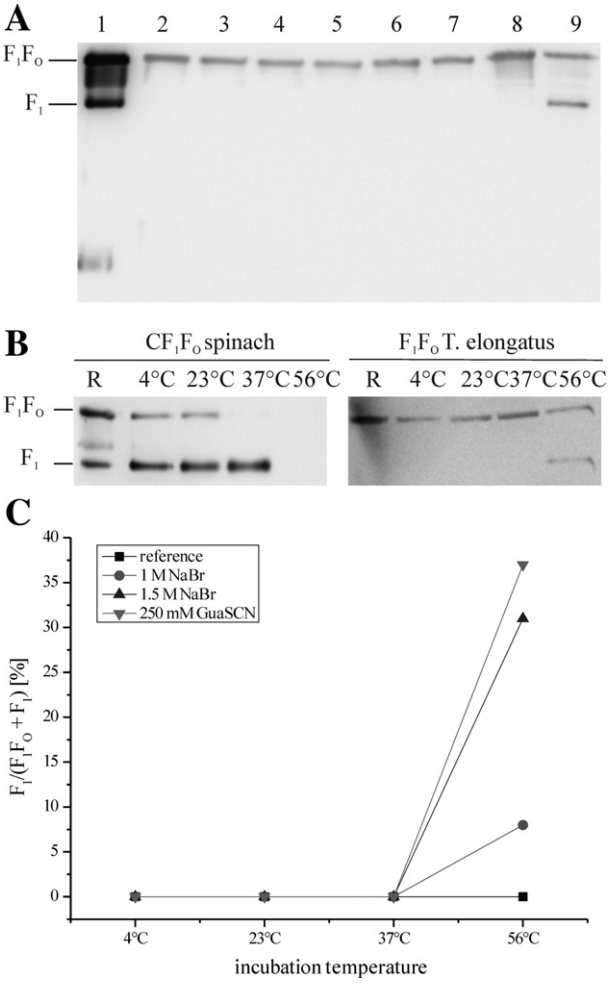

Fig. 4. Stability of F_1F_O of *Thermosynechococcus elongatus*. (A) Western blot of a BN-gel showing the stability of the F_1F_O-ATP synthase of *Thermosynechococcus elongatus* towards Coomassie dye. Lane 1 CF_1F_O of *Spinacea oleracea*, lanes 2–9 F_1F_O with different concentrations of Coomassie Serva blue G (2) 0% (w/v), (3) 1.0% (w/v), (4) 1.5% (w/v), (5) 2.0% (w/v), (6) 2.5% (w/v), (7) 3.0% (w/v), (8) 3.5% (w/v), (9) 4.0% (w/v). The stability of the F_1F_O-ATP synthase was not strongly affected by the Coomassie dye. In contrast to the reference CF_1F_O, F_1F_O is stable up to 3.5% Coomassie dye. Only the highest dye concentration of 4.0% induced the disaggregation of the F_1F_O-ATP synthase in its F_1 and F_O part. (B) Stability of F_1F_O of *Thermosynechococcus elongatus* towards sodium bromide and guanidine thiocyanate at various temperatures. Western blot of spinach CF_1F_O and F_1F_O incubated in 250 mM guanidine thiocyanate at the different temperatures. The Western blots demonstrate the higher stability of the F_1F_O-ATP synthase at higher temperatures compared to CF_1F_O. R, reference ATP synthases. (C) Effect of chaotropic reagents on the stability of the F_1F_O-ATP synthase at different temperatures. The percentage of F_1 compared to the total amount of ATP synthase is shown. The fraction of the F_1 part increases at 56 °C, indicating the starting disassembly of F_1F_O.

of the total amount of produced ATP took place at room temperature. A dependence on the incubation temperature could be demonstrated (Fig. 3B). With increasing temperature the activity also increased. The highest ATP synthesis rate was obtained at 55 °C. However the synthesis rate at 65 °C was as high as at 42 °C. F_1F_O activity was detected until 95 °C. ATP synthesis rates of samples incubated with DCCD were reduced to approximately 15% to 20 % of the synthesis rate of the untreated sample (data not shown). Even at higher temperatures ATP synthesis rate is reduced by DCCD inhibition (Fig. 3B). Incubation of the F_1F_O-ATP synthase at 55 °C for 6 h and interval measurements of the ATP synthesis rate indicated constant high rates over a time range of 4 h (Fig. 3C). Afterwards the synthesis rate decreased.

3.4. Stability of the F_1F_O-ATP synthase against Coomassie dye and chaotropic reagents

Coomassie dye induces the disassembly of the ATP synthase holoenzyme at a concentration of 0.02 % (w/v) [48]. To trace the stability of *T. elongatus* F_1F_O concerning Coomassie dye, pure F_1F_O was incubated with different Coomassie concentrations from 0% up to 4.0% (w/v). BN-PAGE combined with Western blotting was employed to demonstrate the disassembly of F_1F_O into its F_1 and F_O part. Surprisingly, F_1F_O was still stable at a Coomassie concentration of 3.5% (w/v) (Fig. 4). In contrast, the spinach CF_1F_O, which was not incubated with additional Coomassie, showed a typical disaggregation pattern. The content of 0.002% Coomassie blue in the cathode buffer was sufficient to disassemble CF_1F_O. With antibodies raised against subunit α from *Sp. oleracea* CF_1F_O and CF_1 were visible (Fig. 4, lane 1).

Sodium bromide and guanidine thiocyanate were suggested to induce the disaggregation of the ATP synthase [49,50]. Pure CF_1F_O- and F_1F_O-ATP synthases were incubated with 1.0 M and 1.5 M sodium bromide or 250 mM guanidine thiocyanate at different temperatures. At room temperature the ATP synthases were stable for 15 min at these salt concentrations. Higher concentrations of guanidine thiocyanate affect the stability of the ATP synthase stronger than 4 or 6 fold concentrations of sodium bromide. Therefore the former was only used in a concentration of 250 mM. For the BN-gel electrophoresis it was necessary to desalt the sodium bromide treated samples to prevent streaking of the band pattern in the gel. The spinach CF_1F_O-ATP synthase showed the typical disaggregation pattern caused by the Coomassie dye used during BN-PAGE (Fig. 4B). When the spinach ATP synthase was incubated with the chaotropic reagents solely the CF_1 part was visible at 37 °C, which means that no holoenzyme was present (Fig. 4B). At 56 °C no CF_1F_O specific bands could be determined. In contrast to the spinach ATP synthase, the thermophilic F_1F_O-ATP synthase was stable at 37 °C (Fig. 4B). A F_1 band at an incubation temperature of 56 °C indicated the starting disaggregation (Fig. 4B and C).

4. Discussion

The initial aim of the study presented here was to obtain highly pure F_1F_O-ATP synthase of *T. elongatus* for functional studies. A combination of rate-zonal centrifugation and two chromatographic purification steps led to pure and intact ATP synthase. This purification is a combination of two purification steps described previously for the CF_1F_O-ATP synthase of *Sp. oleracea* [45,28]. A particular problem was the high content of phycobilisomes in thylakoid membranes of *T. elongatus*. Lubberding et al. [21] and Van Walraven et al. [23] described that their ATP synthase preparations were still contaminated after purification. Using Reactive Red 120 chromatography combined with anion exchange chromatography we obtained highly pure F_1F_O that can be employed for further functional and structural investigations. The combination of these two chromatography steps seems to be very important to achieve highly pure and active F_1F_O (Table 1). The SDS gel of the purified ATP synthase revealed all nine single subunits and the c-oligomer. All subunits of the F_1F_O-ATP synthase were identified by mass spectrometry. Former purification methods [23,21] only enabled the identification of the subunits α, β, γ, δ and ε. In contrast, in our preparation the presence of all membrane integral subunits was unambiguously shown.

The mass of intact subunit c was analyzed by MALDI-TOF-MS. During the measurements, purification with ZipTips® C4 proved to be invaluable to obtain well resolved spectra. ZipTips® with long chain alkyl residues (C18) bound the very hydrophobic subunit c too strongly (data not shown). The mass spectra revealed a mass of the subunit c monomer of 8238 Da (m/z). In the UniProtKB/TrEMBL database the mass of the c subunit of *T. elongatus* is archived with 10,141 Da (Access number Q8DLP7_SYNEL). According to our results, we conclude that the first 17 amino acids of the database entry (MISGNFVLSY YVSRKST) are a presequence not belonging to the mature protein or that the start codon was predicted incorrectly.

An artificial electrochemical proton gradient enabled the reconstituted F_1F_O-ATP synthase to synthesize ATP. The highest synthesis rate was obtained at natural growth temperature around 55 °C. During measurements with the two-step method, samples incubated with the inhibitor DCCD showed only approximately 15% to 20% of the ATP synthesis rate of untreated samples. This background may result from ATP that was still present in the sample. Such directly released ATP can be distinguished easily from the generated ATP by the kinetics with the "one-step method". As described previously, thermophilic enzymes are often barely active at room temperature, but are as active as their mesophilic counterparts at the corresponding physiological temperatures [6]. Our activities for F_1F_O at 55 °C are similar to activities of CF_1F_O at room temperature. Maximal activity of *Synechococcus* 6716 ATP synthase and *Mastigocladus laminosus* ATP synthase was observed at 50 °C [51,20]. ATP hydrolysis activity was increased after trypsin treatment [52]. In contrast to the *Spirulina platensis* ATP synthase, the ATP synthase of *Synechococcus* 6716 was not activated by methanol [53]. Furthermore, Krenn et al. [52] demonstrated that although the ATP synthase was purified by gel-filtration chromatography, no further increase of the specific activity was obtained by the purification. But as mentioned before, these ATP synthases containing fractions were still contaminated with other proteins. During our purification procedure, the specific

activity increased with every step (Table 1). Lubberding et al. [21] supposed that the rather low measured P_i/ATP exchange activity from purified *Synechococcus* 6716 ATP synthase possibly was caused by the contamination with phycocyanin. We showed that the purity of the ATP synthase sample is essential for good activity measurements (Table 1). Long time activity measurements with incubation at 55 °C demonstrated that the ATP synthesis activity of F_1F_O remained constant for several hours. Concerning the ATP synthesis activity and the stability of the holoenzyme, only few studies can be found in literature. Cook et al. [54] demonstrated that 80% of the ATP hydrolysis activity of the F_1F_O-ATP synthase of the thermoalkaliphilic *Bacillus* sp. was retained after 7 days at 4 °C. The irreversible thermal denaturation of the F_1 part of the chloroplast ATP synthase was described by Wang et al. [55]. With bovine heart submitochondrial particles the thermal denaturation of respiratory chain complexes was demonstrated [56]. This thermal denaturation proceeded in multiple stages. Reconstituted F_1F_O vesicles exhibited inactivation temperatures for the ATP/P_i exchange (49 °C), proton pumping (49 °C) and ATP hydrolysis (58 °C). Purified cyanophycin synthetase of the thermophilic *Synechococcus* sp. strain MA19 was active at 50 °C and retained 90% of its activity after 2 h [57]. The thermal stability of photosystem II isolated from *T. elongatus* was demonstrated by Zimmermann et al. [58].

Neff and Dencher [34] described the influence of Coomassie dye on the intactness of the spinach CF_1F_O-ATP synthase. At a Coomassie concentration of 0.002% CF_1F_O remained intact, whereas at a ten fold higher concentration the ATP synthase disaggregated in its CF_1 and CF_O parts. Even for preparative isolation of CF_O, this procedure was employed [48,59]. Applied to the thermophilic F_1F_O-ATP synthase, this enzyme showed a much higher stability against the dye. Disaggregation started at a 200 fold Coomassie concentration, i.e. 4% (w/v). For further investigations of this increased stability, the chaotropic salts sodium bromide and guanidine thiocyanate were employed. Grotjohann and Gräber [49] were able to separate CF_1 and CF_O in proteoliposomes with a treatment of 2 M sodium bromide at 0 °C. The same concentration was used to deplete the CF_1-content in a chloroplast suspension [60]. The effect on solubilized ATP synthase is expected to be more pronounced. Guanidine thiocyanate efficiently denatures water-soluble proteins like CF_1 [50,61] and also guanidine hydrochloride is applied for a long time to remove mitochondrial MF_1 [62]. Our studies demonstrated that the effect of sodium bromide and guanidine thiocyanate on the holoenzyme is influenced by the incubation temperature. During incubation at different salt concentrations, the F_1F_O-ATP synthase proved a higher stability than spinach ATP synthase. At 37 °C mostly all CF_1F_O was disassembled whereas complete F_1F_O could be found at 56 °C. This confirms the studies of Yoshida et al. [63], who describe the higher stability of the TF_1-part of the ATP synthase of the thermophilic bacterium PS3 against dissociating agents and organic solvents. Finally, it indicates that the thermophilic ATP synthase has special adaptations for survival in extreme environments.

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