

Reassembly of *Synechocystis* sp. PCC 6803 F₁-ATPase from its over-expressed subunits

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Abstract Subunits α , β , and γ of the F₁-part of cyanobacterial F₀F₁-ATPase have been cloned into expression vectors. Over-expressed subunit β was found soluble in the cytoplasmic fraction of *Escherichia coli* cells under appropriate culture and induction conditions and was purified from cell extracts. Recombinant α and γ subunits precipitated into inclusion bodies and had to be solubilized, purified and refolded. The correct folding and functional integrity of the α and β subunits was monitored by their ability to bind nucleotides. Active cyanobacterial F₁-ATPase was assembled from its purified subunits α , β , γ , δ and ϵ . The reassembled enzyme reconstituted ATP synthesis in F₁-depleted thylakoid membranes of *Synechocystis* sp. PCC 6803 and hydrolyzed ATP.

Key words: ATPase; F₀F₁; Purification; Expression; *Synechocystis*

1. Introduction

F-ATPases play a central role in the energy supply of many organisms, utilizing transmembrane electrochemical potential differences of a coupling ion for the synthesis of ATP. These enzymes are composed from two large entities: The F₀-part consists of subunits a , b , and c in bacteria (additionally b' in photosynthetic bacteria) and allows for passage of mostly protons, in a few species sodium-ions, across the coupling membrane. The F₁-part, carrying the catalytic and regulatory centers of the enzyme, consists of subunits α , β , γ , δ , and ϵ , named in order of decreasing molecular masses. Transfer of ions through F₀ is probably linked via conformational changes to ATP hydrolysis or synthesis on F₁ [1]. Various models for this coupling process and the participating subunits at the interface between F₀ and F₁ have been presented, suggesting participation of at least the small F₁ subunits δ and ϵ in conjunction with b and perhaps b' in F₀ [2–5]. The γ subunit has been proposed to function as a proton gate [6], perhaps also implicating interactions with the proton conductor. A direct interaction of the two large α and β subunits of F₁ with the F₀ part has been

controversially discussed. On one hand, a couple of studies yielded evidences suggesting physical contact of α and/or β with F₀ [7–9]. On the other hand, electron-micrographs show distances in the range of 50 Å between the bulk of F₁ and the membrane surface [10].

Since thylakoids allow for the fast and controlled generation of proton gradients by light, they have been the preferred system for studies aiming at proton conduction through F₀. Further progress in these studies towards the interaction of proton transport with subunits and events in F₁ was hampered by the fact that individual F₁-subunits were not available so far for binding and reconstitution studies. Subunit preparations obtained by fragmentation of native CF₁ were argued to be contaminated by traces of other proteins [11], and most over-expressed single subunits of F₁ could not be correctly refolded from the denatured state and used in reconstitution experiments with F₁-depleted membranes. A recent study described the reassembly of ATPase from recombinant, urea-solubilized CF₁ subunits α , β , and γ and indicated a synergistic refolding of the core complex only in the presence of several chloroplast chaperones [12], without the possibility to obtain individually refolded subunits.

Here we describe the expression of the F₁ subunits α , β , and γ of the cyanobacterium, *Synechocystis* sp. PCC 6803, in *Escherichia coli*. Purified single subunits have been tested for biological activity, and reassembly of the cyanobacterial F₁ ATPase from its purified and renatured subunits is demonstrated.

2. Materials and methods

Engineering, amplification and maintenance of expression vectors (pET system, purchased from Novagen, Basel) was carried out in *Escherichia coli* DH5 α (BRL). For expression of subunits, constructs were transferred into *Escherichia coli* BL21 (DE3), or in some cases into the same strain additionally harbouring plasmid pLysS. All molecular biology procedures were as described [13]. Sequencing was carried out by the dideoxynucleotide chain termination method [14]. For the cloning of cyanobacterial genes into pET vectors, we amplified the respective stretches of cyanobacterial genomic DNA by means of the polymerase chain reaction [15]. PCR products were subcloned into pUC18 (Pharmacia SureClone Kit), and restriction sites introduced by PCR primers were used to transfer the inserts into pET. Final constructs were sequenced in order to certify the correct insertion of the respective genes. Optimized expression conditions are summarized in Table 1. Purification procedures for the particular subunits are presented in the following:

Subunit α : recombinant α that contained ten additional Histidine residues at the N-terminus was purified from inclusion bodies after solubilization in 6 M guanidinium hydrochloride (GdnHCl) by metal-chelate chromatography on 'His-Bind' Resin (Novagen). All centrifugations were done at 15°C, all other steps at room temperature, if not otherwise stated. After induction over night, cells were harvested by 5 min centrifugation at 5,000 $\times$ g and resuspended in 1/10 vol of Novagen's Binding buffer without GdnHCl. Cells were broken by sonication in a Branson B15 Cell disruptor and subsequently centrifuged

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; Chl, Chlorophyll; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; GdnHCl, guanidinium hydrochloride; Mega 9, nonanoyl-N-methyl-glucamide; PCR, polymerase chain reaction; PMS, phenazinemethosulfate; SDS-PAGE, sodiumdodecylsulfate-polyacrylamide-gel electrophoresis; TNP-ATP, trinitrophenyl-adenosinetriphosphate; tricine, N-Tris-(hydroxymethyl) methyl glycine; Tris, N-tris-(hydroxymethyl)-aminomethane.

15 min at $20,000 \times g$. Supernatants were discarded and the pelleted inclusion bodies were washed twice with 1/20 vol of Binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Pellets were then solubilized in 5 ml Binding buffer with 6 M GdnHCl added and incubated 1 h on ice. Insoluble material was removed by 20 min centrifugation at $39,000 \times g$. A 'His-Bind' column (Novagen, Basel) was charged according to the manufacturer's procedure and equilibrated with Binding buffer, containing 6 M GdnHCl as all buffers used for subsequent washing and developing the column. The solubilized material was loaded to the column in 10 column volumes Binding buffer. The column was then washed with 6 volumes wash buffer (20 mM Imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9). The protein was eluted with 6 vol Elution buffer (1 M Imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Refolding was subsequently achieved by gelfiltration against 25 mM Tris-HCl pH 7.8, 3 mM MgCl₂, 1 mM ATP on NAP 10 columns (Pharmacia).

Subunit β : was purified from soluble fractions by differential (NH₄)₂SO₄-precipitation, followed by anion-exchange chromatography and gelfiltration. Cells were harvested as before and washed in 1/10 vol buffer A (50 mM Tris-HCl pH 7.8, 2 mM EDTA). They were then resuspended in 1/10 vol buffer A, sustained with 40 mM Mega 9 and 0.1% (w/v) Lysozyme, and incubated 1 h on ice. Subsequently, cells were sonified on ice until the solution completely lost viscosity. Insoluble material was removed by 30 min centrifugation at $30,000 \times g$. The clear supernatant was stepwise precipitated with 10, 20, and 50% (w/v) (NH₄)₂SO₄. The final pellet was resuspended in 10 ml of 50 mM ethanolamine-HCl, pH 9.5, and centrifuged 1 h at $10,000 \times g$. The supernatant of this centrifugation was applied to a Mono Q column (HR 5/5, Pharmacia) and eluted with a NaCl-gradient in 50 mM ethanolamine-HCl, pH 9.5. After inspection by SDS-PAGE, peaks containing the β subunit were pooled and pressure dialyzed on Amicon YM 10 membranes. The sample volume was reduced to 5 ml, brought to 50 ml by addition of 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, and again reduced to 3 ml. After centrifugation for 1 h at $150,000 \times g$, the sample was loaded onto a Superdex 200 column (Hi-Load 2.6 $\times$ 60 cm, Pharmacia) and eluted with 25 mM Tris-HCl, pH 8.0, 100 mM NaCl at 2 ml/min. Fractions from 130–150 ml contained pure β and were pooled.

Subunit γ : was expressed similar to α with ten additional Histidine residues at its N-terminus. Purification and solubilization of inclusion bodies as well as loading the sample to a 'His-Bind' column was carried out as described with α . In contrast to α , pure γ eluted already with the wash buffer at 20 mM Imidazol. Refolding however was unsuccessful with γ , since this subunit precipitated upon gelfiltration or dilution into GdnHCl-free buffer. Therefore, the precipitated sample was centrifuged at $30,000 \times g$, and the pellet was resuspended in 10 ml of 25 mM Tris-HCl, pH 7.8, 0.5 mM DTT, 8 M urea. By dilution with the same buffer, but without urea, the sample was brought to 4 M urea and stored at 4°C.

***Synechocystis* thylakoid membranes:** were prepared according to Lubberding et al. [16]. Measurement of ATP synthesis and hydrolysis were carried out according to published procedures [17]. For the preparation of F₁, *Synechocystis* cells from 1.5 liters of culture grown in BG11 at an OD₇₃₀ of 1.0 were pelleted and resuspended in a buffer, containing 10 mM tricine-KOH at pH 7.8, 0.5 M mannitol, 5 mM

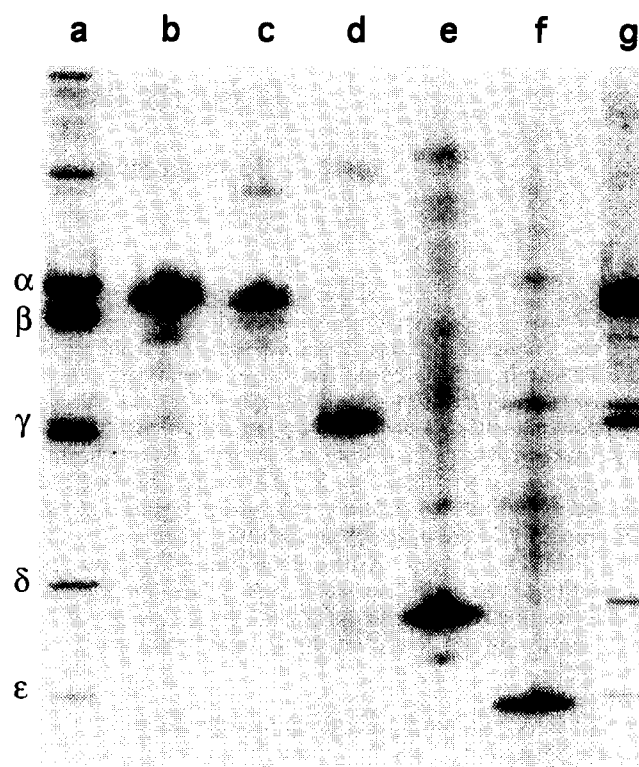


Fig. 1. SDS-PAGE of purified recombinant *Synechocystis* F₁ subunits. The figure shows a silver stained SDS-PAGE gel (Pharmacia Phastgel 8–25%) of the following samples: lane a, 1 mg/ml spinach CF₁; lane b, 1.25 mg/ml α ; lane c, 0.9 mg/ml β ; lane d, 0.032 mg/ml γ ; lane e, 0.95 mg/ml δ ; lane f, 0.75 mg/ml ϵ ; lane g, 1 mg/ml *Synechocystis* F₁.

NaH₂PO₄, 10 mM MgCl₂, 1 mM PMSF, and 1 mM Benzamidine. Chlorophyll concentration was adjusted to 0.1 mg/ml in the same buffer, and cells were incubated 3 h at room temperature after addition of 0.1% (w/v) Lysozyme. The following procedures were carried out on ice or at 4°C. Cells were washed in 25 ml of 10 mM Tricine-KOH at pH 7.8, 5 mM MgCl₂, 1 mM PMSF, and 1 mM Benzamidine and resuspended to 1 mg/ml chlorophyll in the same buffer. To remove phycobilisomes, this suspension was diluted to 0.2 mg/ml chlorophyll in 10 mM Na₂P₂O₇ and centrifuged another 30 min at $10,000 \times g$. F₁ was extracted by resuspension of the pellet to 0.1 mg/ml chlorophyll and 30 min incubation in 50 mM Sucrose, 2 mM Tris, adjusted to pH 7.5 by addition of solid Tricine. After another 30 min centrifugation at $100,000 \times g$, the supernatant was loaded on a Resource Q column (Pharmacia). The column was equilibrated with 25 mM Tris-HCl and developed with 1 M NaCl in the same buffer. F₁ eluted around 260 mM NaCl as judged by ATPase measurements and SDS PAGE. TNP-ATP (Molecular Probes, Eugene, OR) titration data were fitted as in [18].

3. Results and discussion

After over night induction, expressed subunits were purified from soluble fractions or inclusion bodies as detailed in section 2 or, for δ and ϵ , in [19]. Fig. 1 shows samples of the purified subunits run on SDS-PAGE. The gel documents that all five subunits are appreciably free from contaminating proteins. Biological activity of recombinant cyanobacterial δ and ϵ was already demonstrated by their ability to functionally reconstitute ATP synthesis together with CF₁(- δ , ϵ) in spinach thylakoids and by inhibition of ATP hydrolysis by spinach CF₁(- ϵ) through cyanobacterial ϵ [19].

The location of nucleotide binding sites on α and β in F₁

Table 1
Expression of *Synechocystis* F₁ subunits in *Escherichia coli*

Subunit	Vector	pLysS	mM IPTG	Additions
α	pET-16b	–	1.0	1% ethanol
β	pET-3a	–	0.4	–
γ	pET-16b	+	1.0	2.5 mM betaine 0.5 M sorbitol

Expression conditions are summarized for the *Synechocystis* F₁ subunits α , β , and γ . 5 ml of overnight cultures of cells harbouring respective plasmids were used to inoculate 0.5 l of fresh LB-medium, containing 50 μ g/ml carbenicillin, and in case of γ additionally 34 μ g/ml chloramphenicol. Cultures were grown to OD₆₀₀ = 0.8 at 37°C and transferred to 20°C. After about 30 min, IPTG was added and the induced cultures were kept at 20°C overnight.

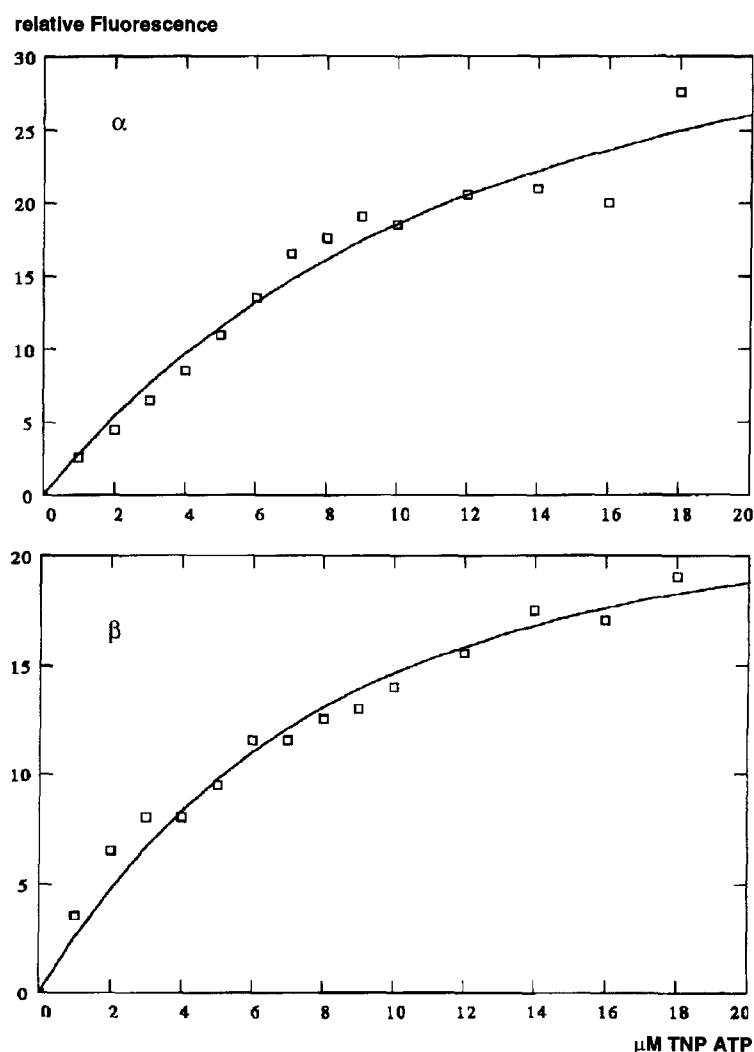


Fig. 2. Titration of α and β subunits with TNP-ATP. A cuvette containing 1 ml of 25 mM Tris-HCl, pH 7.8 with 3.1 μ M α (upper panel) or 2.9 μ M β (lower panel) was placed in a fluorimeter at room temperature. Proteins were gel-filtrated (NAP 10 columns, Pharmacia) against the same buffer. Excitation was set to 405 nm, Emission was recorded at 520 nm. TNP-ATP was added from a 2 mM stock solution in H₂O. The fluorescence of unbound TNP-ATP was also determined in protein-free solution for every concentration and subtracted. Solid lines represent fits done as in [18]. For the resulting fit parameters, see text.

allows for a convenient test of the biological activity of purified subunits. It has been shown previously that either biochemically prepared or recombinant nucleotide binding proteins, including F₁ subunits α [20] and β [21], are still capable of binding

Table 2
Reconstitution of ATP synthesis in F₁-depleted *Synechocystis* thylakoid membranes

Sample	Addition	U/(mg chl)	%
Thylakoids		146	100
	5 μ M Venturicidin	7	5
	5 mM NH ₄ Cl	12	8
F ₁ -depleted		35	24
	F ₁ -extract, 45 μ g protein	82	56
	$\alpha\beta\gamma\delta\epsilon$, 10 μ g protein	87	60

Synechocystis thylakoid vesicles have been prepared according to [16]. Depletion of F₁ was achieved by washing the membranes in a buffer, containing 50 mM sucrose, 2 mM Tris, adjusted to pH 7.5 by addition of solid tricine. Membranes and proteins were incubated 1 h on ice prior to measurement. 1 U = 1 μ mol ATP synthesized/h.

nucleotides or modified analogues. We used TNP-ATP for monitoring nucleotide binding to recombinant α or β . This ATP analog signals binding to proteins by a massively enhanced fluorescence. Fig. 2 shows titrations of α and β with increasing concentrations of TNP-ATP. Upon fitting of graphs to the data points according to [18], K_d values of 9.84 μ M for α and 5.04 μ M for β were obtained, well within the range of figures found in the literature [18,21]. The molar ratio of nucleotide bound per subunit was found to be 0.99 with α , and 0.83 with β , indicating specific binding of the ATP analog at one binding site. We concluded that subunit α , which was purified and refolded from inclusion bodies, as well as β , purified from the cytoplasmic fraction, were present in the correct conformation.

Subunit γ had to be stored solubilized in 4 M urea, since it precipitated upon dilution into urea-free solution. Hence, stoichiometric amounts of the four soluble subunits and of γ were mixed, and the competence of this mixture to reconstitute ATP synthesis in F₁-depleted cyanobacterial thylakoid membranes

Table 3
ATPase activity of native *Synechocystis* F₁ and of mixed recombinant subunits

Sample	$\mu\text{mol P}_i/(\text{mg protein} \cdot \text{min})$
F ₁	3.20
$\alpha\gamma\delta\epsilon$	0.03
$\beta\gamma\delta\epsilon$	0.01
$\alpha\beta$	0.02
$\alpha\beta\gamma$	0.06
$\alpha\beta\gamma\delta\epsilon$	0.15

Methanol-stimulated ATPase activity was determined as before [17]. 2–10 μg F₁ were used in the assay. The following amounts of subunits were mixed: 500 μl purified α with 0.25 mg/ml; 682 μl β with 0.18 mg/ml; 833 μl urea-solubilized γ with 0.032 mg/ml; 80 μl δ with 0.19 mg/ml; 75 μl ϵ with 0.15 mg/ml. The mixture was gel filtrated against 25 mM Tris-HCl, 1 mM DTT, pH 7.8. Aliquots of 5–20 μg protein were then assayed for ATPase activity. All values were determined 4 times in independent experiments.

was examined. As shown in Table 2, the mixture of recombinant subunits was equally active in reconstitution of ATP synthesis as was the native enzyme. This finding did not unequivocally demonstrate reassembly of native F₁ complexes though, since a correct refolding of only a part of the enzyme interacting with F₀ could be sufficient for the observed effect. Therefore, we also examined ATPase activity in the mixture. As evident from Table 3, we found reconstitution of 5% of the hydrolytic activity of native *Synechocystis* F₁. This low, but significant and reproducible activity was dependent on the presence of both of the large subunits, and it was also dependent on the presence of the smaller subunits, since less than half of the ATPase activity was found without δ or ϵ . These subunits were not necessary in the reconstitution of ATPase activity from isolated subunits of F₁ from a thermophilic *Bacillus* [22]. We attribute this difference to the fact that γ had to be added solubilized in urea, and that apparently only a minor fraction of the subunit refolded and assembled correctly. The presence of the small subunits in the assembly process probably was essential because they assisted in the folding of γ since at least for ϵ , direct interactions with γ have been demonstrated [23]. We are currently examining conditions for the expression of γ as a soluble protein in *Escherichia coli* and for an improved procedure for refolding of urea-solubilized γ .

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