Reassembly of *Synechocystis* sp. PCC 6803 F_1-ATPase from its over-expressed subunits

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Received 31 January 1995

Abstract **Subunits α, β, and γ of the F_1-part of cyanobacterial F_0F_1-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 F_1-ATPase was assembled from its purified subunits α, β, γ, δ and ϵ. The reassembled enzyme reconstituted ATP synthesis in F_1-depleted thylakoid membranes of *Synechocystis* sp. PCC 6803 and hydrolyzed ATP.**

Key words: ATPase; F_0F_1; Purification; Expression; *Synechocystis*

1. Introduction

F-ATPases play a central role in the energy supply of many organisms, utilizing transmembraneous electrochemical potential differences of a coupling ion for the synthesis of ATP. These enzymes are composed from two large entities: The F_0-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 F_1-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 F_0 is probably linked via conformational changes to ATP hydrolysis or synthesis on F_1 [1]. Various models for this coupling process and the participating subunits at the interface between F_0 and F_1 have been presented, suggesting participation of at least the small F_1 subunits δ and ε in conjunction with *b* and perhaps *b'* in F_0 [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 F_1 with the F_0 part has been controversially discussed. On one hand, a couple of studies yielded evidences suggesting physical contact of α and/or β with F_0 [7–9]. On the other hand, electron-micrographs show distances in the range of 50 Å between the bulk of F_1 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 F_0. Further progress in these studies towards the interaction of proton transport with subunits and events in F_1 was hampered by the fact that individual F_1-subunits were not available so far for binding and reconstitution studies. Subunit preparations obtained by fragmentation of native CF_1 were argued to be contaminated by traces of other proteins [11], and most over-expressed single subunits of F_1 could not be correctly refolded from the denatured state and used in reconstitution experiments with F_1-depleted membranes. A recent study described the reassembly of ATPase from recombinant, urea-solubilized CF_1 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 F_1 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 F_1 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* DH5α (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 × *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-gelelectrophoresis; TNP·ATP, trinitrophenyl-adenosinetriphosphate; tricine, *N*-Tris-(hydroxymethyl) methyl glycine; Tris, *N*-tris-(hydroxymethyl)-aminomethane.

SSDI 0014-5793(95)00238-3

15 min at 20,000 × 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. Unsoluble material was removed by 20 min centrifugation at 39,000 × 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 $MgCl_2$, 1 mM ATP on NAP 10 columns (Pharmacia).

Subunit β: was purified from soluble fractions by differential $(NH_4)_2SO_4$-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. Unsoluble material was removed by 30 min centrifugation at 30,000 × g. The clear supernatant was stepwise precipitated with 10, 20, and 50% (w/v) $(NH_4)_2SO_4$. The final pellet was resuspended in 10 ml of 50 mM ethanolamine·HCl, pH 9.5, and centrifuged 1 h at 10,0000 × 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 × g, the sample was loaded onto a Superdex 200 column (Hi-Load 2.6 × 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 aditional 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 pricipitated sample was centrifuged at 30,000 × 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 F_1, *Synechocystis* cells from 1.5 liters of culture grown in BG11 at an OD_{730} 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 NaH_2PO_4, 10 mM $MgCl_2$, 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 $MgCl_2$, 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 $Na_2P_4O_7$ and centrifuged another 30 min at 10,000 × g. F_1 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 × 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. F_1 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].

Table 1
Expression of *Synechocystis* F_1 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* F_1 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 μg/ml carbenicillin, and in case of γ additionally 34 μg/ml chloramphenicol. Cultures were grown to OD_{600} = 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.

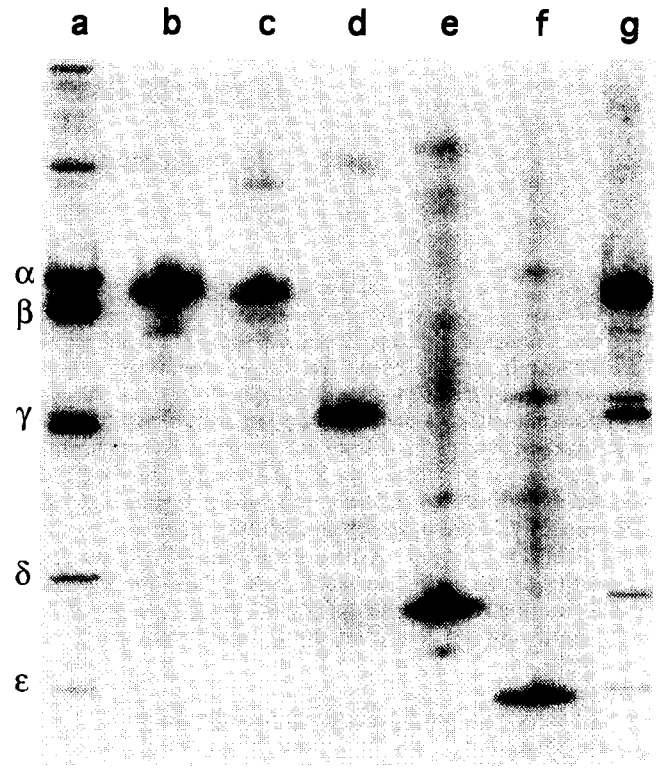

Fig. 1. SDS-PAGE of purified recombinant *Synechocystis* F_1 subunits. The figure shows a silver stained SDS-PAGE gel (Pharmacia Phastgel 8–25%) of the following samples: lane a, 1 mg/ml spinach CF_1; lane b, 1.25 mg/ml α; lane c, 0.9 mg/ml β; lane d, 0.032 mg/ml γ; lane e, 0.95 mg/ml δ; lane f, 0.75 mg/ml ε; lane g, 1 mg/ml *Synechocystis* F_1.

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 $CF_1(-\delta,\varepsilon)$ in spinach thylakoids and by inhibition of ATP hydrolysis by spinach $CF_1(-\varepsilon)$ through cyanobacterial ε [19].

The location of nucleotide binding sites on α and β in F_1

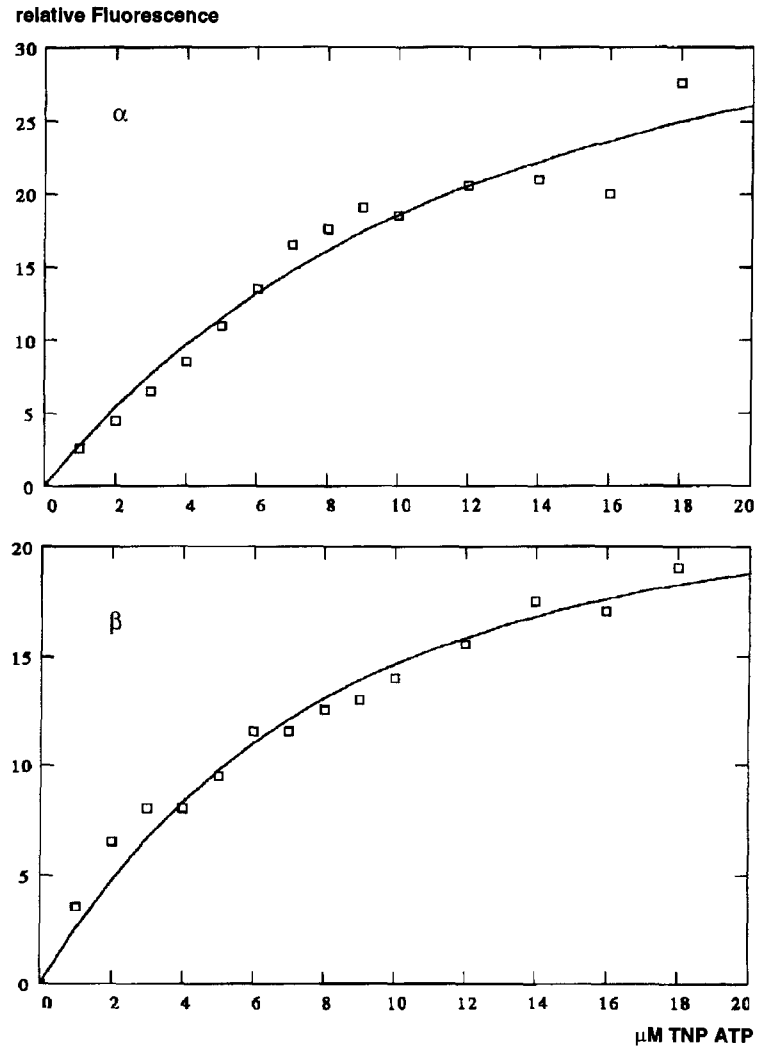

Fig. 2. Titration of α and β subunits with TNP·ATP. A cuvette containing 1 ml of 25 mM Tris-HCl, pH 7.8 with 3.1 μM α (upper panel) or 2.9 μM β (lower panel) was placed in a fluorimeter at room temperature. Proteins were gelfitrated (NAP 10 columns, Pharmacia) against the same buffer. Excitation was set to 405 nm, Emission was recorded at 520 nm. TNP·ATP was added from a 2 mM stock solution in H_2O. 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 F_1 subunits α [20] and β [21], are still capable of binding 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 mM for a and 5.04 μM 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.

Table 2
Reconstitution of ATP synthesis in F_1-depleted *Synechocystis* thylakoid membranes

Sample	Addition	U/(mg chl)	%
Thylakoids		146	100
	5 μM Venturicidin	7	5
	5 mM NH_4Cl	12	8
F_1-depleted		35	24
	F_1-extract, 45 μg protein	82	56
	$\alpha\beta\gamma\delta\varepsilon$, 10 μg protein	87	60

Synechocystis thylakoid vesicles have been prepared according to [16]. Depletion of F_1 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 μmol ATP synthesized/h.

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 reconsitute ATP synthesis in F_1-depleted cyanobacterial thylakoid membranes

Table 3
ATPase activity of native *Synechocystis* F_1 and of mixed recombinant subunits

Sample	μmol P_i/(mg protein · min)
F_1	3.20
$\alpha\gamma\delta\varepsilon$	0.03
$\beta\gamma\delta\varepsilon$	0.01
$\alpha\beta$	0.02
$\alpha\beta\gamma$	0.06
$\alpha\beta\gamma\delta\varepsilon$	0.15

Methanol-stimulated ATPase activity was determined as before [17]. 2–10 μg F_1 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 F_1 complexes though, since a correct refolding of only a part of the enzyme interacting with F_0 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* F_1. 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 F_1 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.

Acknowledgement: We are grateful to P. Völler and M. Blomeier for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 171).

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