Biochimica et Biophysica Acta, 1144 (1993) 278–284

BBABIO 43887

Complementation of *Escherichia coli unc* mutant strains by chloroplast and cyanobacterial F_1-ATPase subunits

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(Received 26 February 1993)

Key words: ATPase, F_1-; Mutant strain; Gene complementation; Mutant strain complementation; Gene expression; (*E. coli*); (*Synechocystis* sp. PCC 6803)

The genes encoding the five subunits of the F_1 portion of the ATPases from both spinach chloroplasts and the cyanobacterium *Synechocystis* sp. PCC 6803 were cloned into expression vectors and expressed in *Escherichia coli*. The recombinant subunits formed inclusion bodies within the cells. Each particular subunit was expressed in the respective *unc* mutant, each unable to grow on non-fermentable carbon sources. The following subunits restored growth under conditions of oxidative phosphorylation: α (both sources, cyanobacterial subunit more than spinach subunit), β (cyanobacterial subunit only), δ (both spinach and *Synechocystis*), and ϵ (both sources), whereas no growth was achieved with the γ subunits from both sources. Despite a high degree of sequence homology the large subunits α and β of spinach and cyanobacterial F_1 were not as effective in the substitution of their *E. coli* counterparts. On the other hand, the two smallest subunits of the *E. coli* ATPase could be more effectively replaced by their cyanobacterial or chloroplast counterparts, although the sequence identity or even similarity is very low. We attribute these findings to the different roles of these subunits in F_1: The large α and β subunits contribute to the catalytic centers of the enzyme, a function rendering them very sensitive to even minor changes. For the smaller δ and ϵ subunits it was sufficient to maintain a certain tertiary structure during evolution, with little emphasis on the conservation of particular amino acids.

Introduction

In photosynthesis and in respiration, the electron-transport chains pump protons and generate a transmembrane protonmotive force. The electrochemical potential difference is used by proton-translocating ATP synthases for the endergonic synthesis of ATP [1]. These enzymes belong to the family of F-ATPases. Bacteria with an energy supply exclusively dependent on fermentation use F-ATPases in the opposite direction, for energization of their plasma membrane by proton pumping at the expense of ATP hydrolysis. This provides the driving force for active uptake of substrates and flagellar rotation. The F-ATPase is a multi-subunit enzyme consisting of two distinct subcomplexes. The membrane sector (F_0) spans the membrane and acts as a proton conductor. The catalytic sector (F_1) binds to F_0, it contains the nucleotide-binding sites and controls proton flux through the whole enzyme (for reviews, see Refs. 2–4).

The F_1 part contains three copies each of the large α and β subunits with molecular masses around 55 kDa. These subunits bind nucleotides and cooperate in the catalytic cycle of the enzyme. The γ subunit comprises a central position within the F_1 complex [5]. The chloroplast subunit contains 36 additional amino acids as compared to *E. coli* γ. This extra stretch is involved in the thiol modulation of the activity of CF_0CF_1 [6]. Cyanobacteria show an intermediate situation: their γ subunit has 27 amino acids more than the *E. coli* counterpart, but it is missing a 7-amino-acid stretch which contains the regulatory cysteines in the chloroplast γ [6]. Subunit δ is involved in the regulation of proton flow [7,8] and indispensable for catalysis [9]. In chloroplasts, ϵ acts as an ATPase inhibitor under non-energized conditions. Apparently, it changes its conformation in dependence of the energization state of the membrane in chloroplasts [10] and its position relative

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Abbreviations: *A*, absorbance; OSCP, oligomycin sensitivity-conferring protein; TIR, translational initiation region.

to the other subunits depending on the occupation state of the six nucleotide binding sites in *Escherichia coli* [11].

Despite the high tertiary and quaternary structural similarity found between F_0F_1 from different sources, significant differences in the primary structure emerged within particular subunits responding to the demands of organisms living under different conditions. By comparing *E. coli* and spinach, homology of different subunits is ranging from 66% identical residues (β subunits) down to less than 25% (δ, ϵ and some F_0 subunits). In order to define regions of interaction between subunits, as well as critical residues, it is interesting to study hybrid enzymes, containing subunits from different organisms. To this end, we cloned all five genes coding for the F_1 subunits both of spinach chloroplasts and the unicellular cyanobacterium, *Synechocystis* sp. PCC 6803, into *E. coli* expression vectors. After transformation of these vectors into the appropriate *E. coli unc* mutants, three out of five spinach subunits and four out of five cyanobacterial subunits complemented the respective *E. coli unc* mutants.

Materials and Methods

Materials

Enzymes and reagents for molecular biology were obtained from Bethesda Research Laboratories, New England Biolabs, Promega Biotech, Perkin Elmer Cetus, Boehringer-Mannheim or Pharmacia LKB. Materials for oligonucleotide synthesis were from Pharmacia, and synthesis of oligonucleotides was carried out on a Pharmacia GeneAssembler. Most other chemicals and antibiotics were from Sigma.

Bacterial strains and plasmids

For the expression of the ATPase genes in *E. coli*, we used pJLA vectors originally constructed and described by McCarthy and co-workers [12]. These vectors contain the λ p_Rp_L promoters in tandem, under the control of the bacteriophages *c*I857 temperature-sensitive repressor. In addition, they contain the TIR region of the *E. coli uncE* gene, which enhances initiation of translation of this gene as compared to the other genes of the operon. pJLA502 provides an ATG start codon within a *Nco*I site, and pJLA503 contains an ATG start codon within a *Nde*I site. Vectors were maintained and proteins were expressed either in *E. coli* DH5α (Bethesda Research Labs) or in *E. coli* pop2136 (Boehringer-Mannheim). Mutant *E. coli* strains used in complementation experiments are described in Table I. DNA used for amplification of the genes of interest was obtained from the following sources: Genes for spinach subunits α β and ϵ were from P.R. Whitfield, Canberra [13,14]. The gene for α was supplied on a 2.5-kb *Sal*I fragment in pBR322, the one for β on an 1.95-kb *Eco*RI fragment in pBR325, and the DNA for ϵ was located on an 11-kb *Bam*HI insert in pBR322. The cDNA coding for the spinach γ-subunit was provided in two overlapping fragments (pSG302/pSG402) by M. Futai, Osaka [15]. We merged the two fragments into pGEM3Z (Bethesda Research Labs) prior to amplification. The gene coding for spinach δ was obtained as a *Kpn*I/*Eco*RI fragment in p6SocD-3 from R.G. Herrmann, Munich [16]. The genes for the *Synechocystis* ATPase have been cloned and sequenced recently by Lill and Nelson [17].

TABLE I

Genotypes of E. coli strains used in this work

Strain	Genotype	Reference
RH304	*uncA*206, *recA*56, *srl*::Tn*10*, *bgl*R, *thi*1, *rel*1, HfrP01	33
RH344	*uncD*244, *recA*56, *srl*::Tn*10*, *bgl*R, *thi*1, *rel*1, HfrP01	33
AT753-26-28	F′, *uncG*270, *thi*1, *arg*H, *metB*1, *lacY*1 or *lacZ*4, *gal*6, *strA*8	34
KF96rA	*uncH* (Gln23 → end), *thy*, *thi*	35
KF148	*uncC*148 (SD$^-$), *thy*, *thi*	28
DH5α	F$^-$, ϕ80d*lacZ*Δ(*lacZYA-argF*)U169, *recA1*, *endA*1, *hsdR*17(r$^-$, m$^+$*K*), *supE*44, λ^-,*gyrA*, *relA*1	36
pop2136	F$^-$, *endA*, *thi*, *hsdk*, *malT*, *malPQ*, λ *c*I857	37

Molecular genetics

All genes were amplified from the original plasmids (see above) by the polymerase chain reaction [18]. Amplification of DNA was carried out using Vent Polymerase (New England Biolabs). Primers were constructed containing the ATG start codon of the genes within a *Nco*I or *Nde*I site which was introduced without changing the downstream coding region. A second primer, also containing an appropriate restriction site, was placed into the noncoding region downstream of the reading frame of the gene of interest. In the two nuclear-encoded spinach genes which contain sequences coding for transit peptides (subunits γ and δ), we placed a start codon directly adjacent to the start site of the mature peptides. The amplification products were precipitated with ammonium acetate [19]. The genes for the F_1 subunits were either cut out directly from the amplification products by means of the restriction sites defined by the primers and cloned into the appropriate expression vector, or, in some cases, amplification products were subcloned into pUC18 or pGEM4Z. Plasmid DNA was isolated by the alkaline lysis method [19] and the coding sequence was cut out and cloned into pJLA (see above), using the newly defined restriction sites. To minimize potential errors introduced by the amplification procedure, we

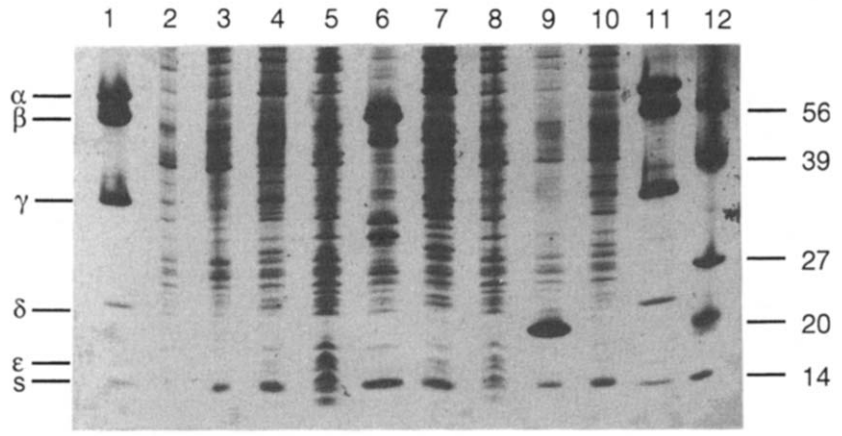

Fig. 1. Fractionation of *E. coli* cells carrying different pJLA vectors. A 10–15% gradient gel was run with the following samples (approx. 300 ng protein per lane): lanes 1 and 11, spinach CF_1; lane 2, total protein of *E. coli* DH5cα cells containing pJLA503 grown at 30°C; lane 3, insoluble fraction of *E. coli* DH5α pJLA503 grown at 42°C, lane 4, as in lane 3, but soluble fraction; lane 5, total protein of *E. coli* DH5α cells containing pJLA503 with the gene coding for the spinach β subunit, grown at 30°C; lane 6, same cells as in lane 5, but grown at 42°C, insoluble fraction; lane 7, same as lane 6, soluble fraction; lane 8, total protein of *E. coli* DH5α cells containing pJLA503 with the gene coding for the *Synechocystis* δ subunit, grown at 30°C; lane 9, same cells as in lane 8, but grown at 42°C, insoluble fraction; lane 10, same as lane 9, soluble fraction; lane 12, molecular mass standards (Boehringer-Mannheim).

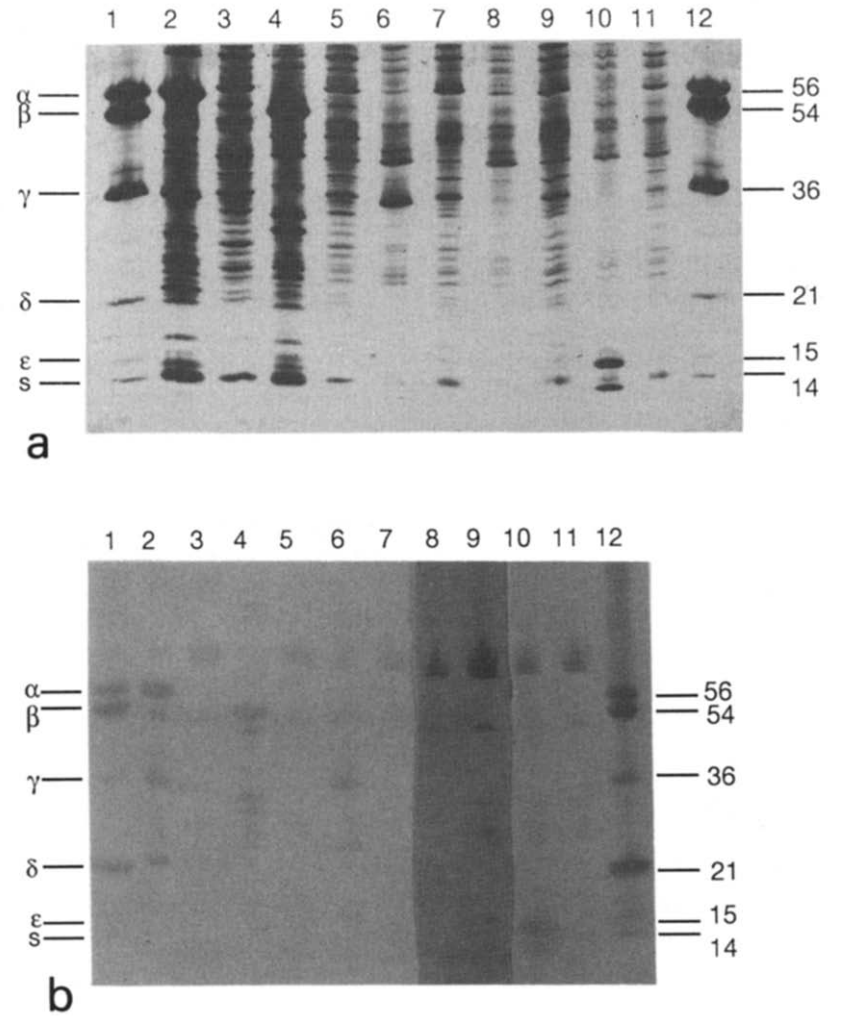

Fig. 2. Expression of spinach F_1 genes in *E. coli*. Always 300 ng protein from insoluble (even numbers) and soluble (odd numbers) fractions from cells grown at 42°C for 16 h before fractionation were loaded per lane. Lanes 1 and 12, spinach CF_1 as a standard (s stands for the small subunit of ribulose-bisphosphate-carboxylase, 14 kDa). The respective genes cloned into pJLA vectors present in the cells were, lanes 2 and 3, α; lanes 4 and 5, β; lanes 6 and 7, γ; lane 8 and 9, δ; lane 10 and 11, ϵ. (a), Silver-stained gel; (b), Western blot. Anti-spinach CF_1 antiserum was used in dilutions of 1:20000 (lanes 1–7), 1:5000 (lanes 8 and 9) or 1:2000 (lanes 10–12). In lanes 8 and 9, the spinach antiserum was supplemented with anti-spinach δ antiserum at 1:1000 dilution.

removed the largest possible parts of the amplified sequences from the expression vectors and replaced them with the respective DNA cut out of the templates used for amplification.

Expression of foreign proteins in E. coli

For expression of proteins from pJLA vectors, cells were grown at 30°C in LB medium containing ampicillin at 50 μg/ml until they reached A 0.5 at 600 nm. The culture was then shifted to 42°C and grown for another 16 h. The cells were pelleted, lysed with lysozyme and Triton X-100 and fractionated into soluble and insoluble material by centrifugation [19]. Aliquots of both fractions were run on 10–15% SDS-PAGE gels (Pharmacia Phast System). The gels were either silver-stained [20] or proteins were transferred onto nitrocellulose for Western blot analysis [21].

Complementation of E. coli unc mutants

Strains carrying mutations which affect different subunits have been generously provided by R.D. Simoni (strains RH304, RH344, AT753-26-28) and M. Futai (strains KF96rA and KF148). Table I lists the genotypes of the strains used throughout this work. The cells were transformed with expression plasmids according to standard procedures [22]. Their growth was tested at various temperatures on solid minimal medium [23], supplemented with 0.5 μg/ml thiamine and 0.4% succinate or 0.4% glucose as the only carbon source. With strain AT753-26-28, the medium was further supplemented with arginine and methionine at 4 μg/ml.

Computer analysis

Alignments and comparisons of sequence data were performed using the GCG software package [24] implemented on a DEC μVAX 3800 under VMS.

Results and Discussion

Fig. 1 shows the expression of the chloroplast F_1-ATPase β subunit and the cyanobacterial δ subunit. Samples were taken from cells grown at 30°C, as well as at 42°C. Recombinant proteins were present exclu-

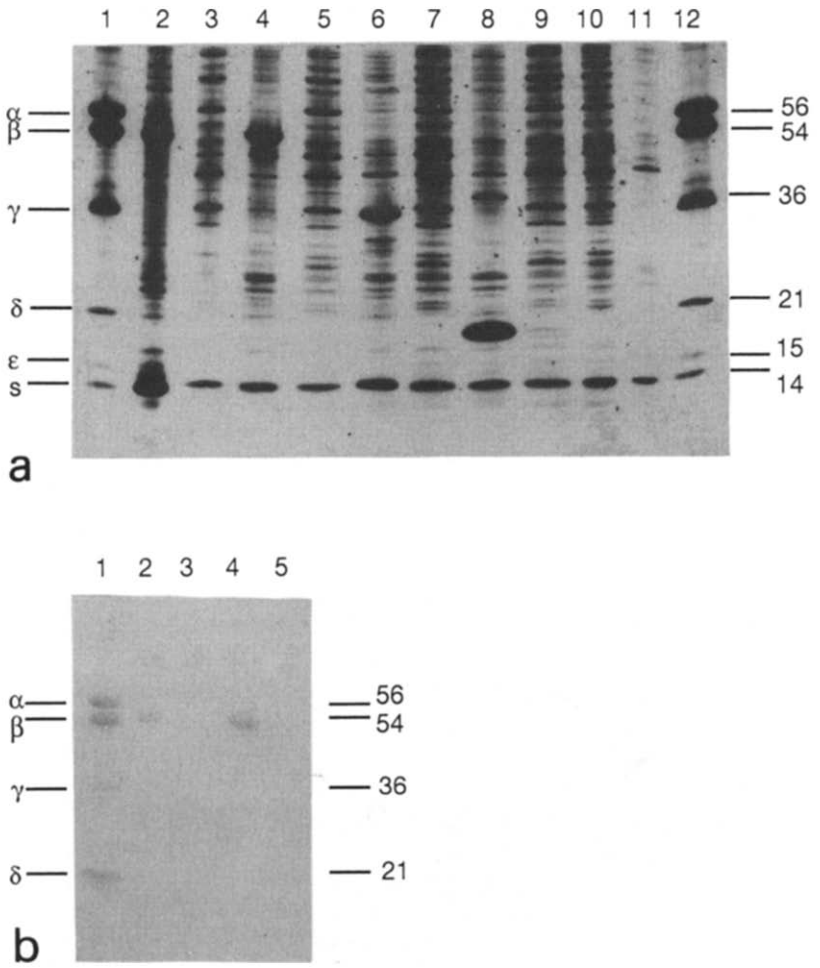

Fig. 3. Expression of *Synechocystis* sp. PCC 6803 F_1 genes in *E. coli*. Loading of the gel, standards and blot as in Fig. 2; anti-spinach CF_1 antiserum was used at 1:500 dilution. *Synechocystis* α and β subunits are not separated in 10–15% Phast gradient gels.

sively in the insoluble fraction of the cells (inclusion bodies). Also included in the figure are negative controls with cells carrying only expression vectors without insert. In Fig. 2a, expression of all five spinach chloroplast F_1 subunits is shown. Samples of the soluble and insoluble fractions of cells carrying the respective pJLA vectors and grown 16 h at 42°C were run on SDS-PAGE and silver stained. Proteins from an identical gel were also electrotransferred onto nitrocellulose, decorated with anti-spinach CF_1 IgG and visualized after reaction with an anti-rabbit antibody conjugated to alkaline phosphatase (Fig. 2b). With the exception of δ, spinach CF_1 subunits could be detected as silver-stained bands (lane 2, α; lane 4, β; lane 6, γ; lane 10, ϵ). All five spinach CF_1 subunits were detected immunologically in Western blots. The expressed proteins were found exclusively in inclusion bodies, with virtually no recombinant peptide detectable in the soluble fractions of the cells. Fig. 3 shows the expression of cyanobacterial F_1 subunits in *E. coli*. Experimental procedures were as in Fig. 2. Protein staining shows all subunits except ϵ present in inclusion bodies in considerable amounts. Due to the lack of species-specific antisera, Western blot analysis was performed with anti-spinach CF_1 antiserum as in Fig. 2b. Only the cyanobacterial α and β subunits were detectable in the insoluble fractions.

For complementation, five different *E. coli unc* mutant strains were used. Cells were transformed with the respective plasmids carrying the spinach or cyanobacterial genes, and controls were transformed with plasmid pAP55, which contains the complete wild-type *E. coli unc* operon [25]. The cells were streaked out on minimal medium containing either succinate or glucose as sole carbon source, and incubated at 30, 37 and 42°C, respectively. Transformations were done in duplicate and all streakouts in triplicate in order to avoid false positive results caused by revertants. Table II summarizes the results: Both α subunits (from spinach and *Synechocystis*) complemented strain RH304, allowing poor but significant growth on minimal medium supplemented with succinate. *Synechocystis* α permitted growth also at 30°C, whereas spinach α was ineffective at this temperature. The spinach β-subunit did not complement at all, whereas significant cell growth occurred upon expression of the cyanobacterial β-subunit at all temperatures tested. The γ subunits from either species did not complement. δ and ϵ subunits showed the most effective complementation of *E. coli*

TABLE II

Growth of E. coli unc mutants carrying various plasmids under different conditions

Cells were grown as described in Materials and Methods. Plasmids named pchlx carry genes for chloroplast subunits, plasmids named pcysx genes for cyanobacterial subunits. Symbols are: −, no growth; +/−, poor but significant growth; +, growth; ++, growth to high yields; +++, growth to high yields at high rates.

Strain / Plasmid	Succinate			Glucose		
	30°C	37°C	42°C	30°C	37°C	42°C
RH304						
pJLA503	−	−	−	++	++	++
pchlα	−	+/−	+/−	++	++	+
pcysα	+	+/−	+/−	+++	++	+
pAP55	++	++	++	+++	+++	+++
RH344						
pJLA503	−	−	−	++	++	++
pchlβ	−	−	−	++	++	+
pcysβ	+/−	+	+/−	++	++	+
pAP55	++	++	++	+++	+++	+++
AT753-26-28						
pJLA503	−	−	−	++	++	++
pchlγ	−	−	−	++	+	+/−
pcysγ	−	−	−	++	+	+
pAP55	++	++	++	+++	+++	+++
KF96rA						
pJLA503	−	−	−	++	++	++
pchlδ	+	+	+/−	++	++	+
pcysδ	++	++	+	++	++	+
pAP55	++	++	++	+++	+++	+++
KF148						
pJLA503	−	−	−	++	++	++
pchlϵ	+/−	+	+/−	++	++	+
pcysϵ	+/−	+	+/−	++	++	+
pAP55	++	++	++	+++	+++	+++

mutants with cyanobacterial δ being more effective than spinach δ and almost as effective as the wild-type subunit. This was of special interest in view of the previous finding that *E. coli* δ was able to partially replace spinach δ in reconstitution experiments with assembled CF_1 lacking δ, but not vice versa [26]. Obviously, spinach δ cannot be introduced into assembled *E. coli* F_1 lacking δ, but can replace its bacterial counterpart when present during biosynthesis and assembly. We could not detect any difference in growth of mutants complemented with the spinach or cyanobacterial ϵ. Interestingly enough, the *E. coli* ϵ-subunit is anionic [27], whereas spinach ϵ is slightly cationic [20]. A multiple sequence alignment of subunit ϵ from spinach, *Synechocystis* and *E. coli* reveals a rather balanced distribution of charges throughout the three sequences, except for a small cluster near the C-terminus (spinach, ...RRARTR...; *Synechocystis*, ...KKARAR...; *E. coli*, ...AQASAE...). The fact that despite these differences in charge, ϵ subunits from both spinach and *Synechocystis* complemented well agrees with the observation that out of the 138 residues of *E. coli* ϵ 46 amino acids could be truncated from the C-terminus without significant loss of EF_1 function [28]. In addition, our data suggest a location of the C-terminal part of subunit ϵ at the surface of F_1.

Since the recombinant subunits were found almost exclusively in inclusion bodies, their ability to complement *unc* mutants points towards a competition between the precipitation of proteins and their assembly into nascent ATP synthases. Therefore, minor differences in complementation efficiency at different temperatures probably reflect the kinetics of folding and assembly, as well as gene dosage effects, rather than the inherent ability of particular subunits to substitute for the *E. coli* wild-type protein (cf., different growth efficiencies with different expressed subunits on glucose plates, second half of Table II). Nevertheless, considering the similarities of the respective subunits as depicted in Table III, some general conclusions may be drawn: First, despite a relatively high fraction of identical residues, the two large subunits α and β were found to be less competent in the complementation experiments than δ and ϵ. Pairwise comparison between β subunits reveals 71% identity between *E. coli* and *Synechocystis*, and 66% between *E. coli* and spinach, respectively. Obviously, there are some additional changes in spinach β, sufficient to render it inactive in *E. coli*. This finding corroborates earlier data that spinach β does not replace its *E. coli* counterpart [29], and it is not surprising, since even single site mutations in *E. coli* β were sufficient to prevent the assembly of the whole complex [2,27].

Best complementations were achieved with δ and ϵ from both sources. These two F_1 subunits have the lowest overall identity scores, 12 and 17%, respectively.

TABLE III

Identical or similar residues in E. coli F_1 subunits as compared to Synechocystis sp. PCC 6803 and spinach F_1

Values have been calculated using the GCG GAP program, using the similarity score matrix provided by the program for the calculation of similarities (shown in parentheses). Overall values have been calculated scoring only residues as identical or similar which where conserved or similar in all three species.

Subunit	*E. coli / Synechocystis*	*E. coli /* Spinach	Spinach/ *Synechocystis*	Overall values
α	54 (72)	54 (71)	72 (84)	46 (65)
β	71 (84)	66 (80)	80 (87)	62 (73)
γ	38 (60)	38 (58)	57 (73)	28 (46)
δ	24 (51)	24 (48)	27 (54)	12 (27)
ϵ	28 (52)	25 (48)	42 (66)	17 (37)

This might reflect the importance of a maintained secondary structure of these subunits with only a few strictly-conserved amino acids. This suggestion is reinforced by the finding that subunit δ from *E. coli* and spinach chloroplasts and OSCP from beef heart mitochondria all possess almost identical fractions of secondary structure elements, despite low conservation of primary structure [30]. A similar situation seems to exist in the F_0-channel portion. Whereas *E. coli b* could be replaced by its chloroplast counterpart, CF_0 *I* [31], subunits thought to be more closely involved in proton translocation, i.e., EF_0 *c* [32] and *a* (Burkovski, A., data not shown), could not be exchanged. Neither one of the two γ subunits was able to complement the respective *E. coli* mutant. We cannot exclude a particular tendency of γ subunits to precipitate and thereby escape assembly into nascent EF_1, but we favour the view that γ plays a central topological role [5] in F_0F_1-ATPases, perhaps with the highest number of contact sites to other subunits and thereby the highest species-specificity.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, SFB 171 B3, B4 and B8. Skilled assistance of Martina Blomeier and Petra Völler is gratefully acknowledged. Critical reading of the manuscript by G. Deckers-Hebestreit is appreciated.

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