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Over-production, renaturation and reconstitution of δ and ϵ subunits from chloroplast and cyanobacterial F_1

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Abstract

We studied the functioning of chimeric F_0F_1-ATPases by replacing subunits δ and ϵ of spinach CF_1 with their counterparts from *Synechocystis* sp. PCC 6803. The sequence identities between these subunits are 26 and 41%, respectively. For a systematic approach to such studies and later extension to genetically modified subunits recombinant proteins are required. The genes coding for spinach and *Synechocystis* δ and ϵ were cloned into pET3 expression vectors and expressed in *Escherichia coli*. Upon expression at 37°C the recombinant subunits formed inclusion bodies within the host cells except for spinach δ, which was soluble. *Synechocystis* δ and ϵ could be obtained in soluble form upon expression at 20°C. After purification (and refolding of spinach ϵ) both ϵ subunits inhibited the Ca^{2+}-ATPase activity of soluble $CF_1(-\epsilon)$. Subunits δ and ϵ from both species raised the rate of ATP synthesis in partially CF_1-depleted spinach thylakoids when added together with $CF_1(-\delta)$ or $CF_1(-\delta,\epsilon)$. This showed the functionality of recombinant *Synechocystis* and spinach δ and ϵ together with spinach $\alpha_3\beta_3\gamma$. The molar excess of ϵ necessary for saturation was higher for Ca^{2+}-ATPase inhibition than for reconstitution of photophosphorylation thus pointing to a direct interaction between ϵ and both CF_1 and CF_0.

Keywords: ATPase; F_1; Over-expression; Photophosphorylation; Reconstitution; Subunit; (*Synechocystis* sp. PCC 6803)

1. Introduction

F_0F_1-ATPases synthesize ATP at the expense of protonmotive force [1–6] (or sodiummotive force in *Propionigenium modestum* [7,8]). The enzymes consist of the membrane-embedded proton (sodium) channel F_0, and the extrinsic, water-soluble part F_1. CF_1 of chloroplasts consists of five different subunits, α (56 kDa), β (54 kDa), γ (36 kDa), δ (21 kDa), and ϵ (15 kDa) with a stoichiometry of 3:3:1:1:1 and six nucleotide binding sites on α and β. The architecture of *Synechocystis* F_1 is very similar. The major difference between the chloroplast and *Synechocystis* enzyme is the redox regulation of the former, brought about by an additional amino acid stretch within subunit γ, which contains two cysteines.

A considerable number of chimeric, but still active enzymes has been constructed [9–12]. Surprisingly, neither a highly conserved subunit is a guarantee for its interchangeability nor does a low degree of conservation prevent it. Subunit β is the most conserved subunit in F_0F_1-ATP-synthases but spinach β cannot fulfill the role of *E. coli* β in the *E. coli* enzyme (66% identical residues between spinach and *E. coli* β) [12,13]. On the otherhand e.g. subunit δ of spinach chloroplast CF_1 can substitute for its counterpart in *E. coli* F_1 (24% conserved residues) [12].

The "small" subunits γ, δ, and ϵ are located at the interface between the membrane-embedded F_0 and the extrinsic F_1. They are instrumental for the coupling between ion movements through F_0 and ATP release from F_1 [1–6,14]. We have shown earlier that purified subunit δ enhanced the reconstitutional activity of CF_1 lacking subunit δ ($CF_1(-\delta)$) in partially CF_1-depleted thylakoids. This activity was attributed to the plugging

Abbreviations: CF_0CF_1, chloroplast F_0F_1-ATPase; CF_0, proton channel (membrane-embedded); CF_1, ATPase (soluble part); $CF_1(-\delta)$, CF_1 lacking the δ subunit; $CF_1(-\epsilon)$, CF_1 lacking the ϵ subunit; $CF_1(-\delta,\epsilon)$, CF_1 lacking both the δ and ϵ subunits.

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of open CF_0 channels. The reduced proton leak allowed for restoration of the protonmotive force and activated both the reconstituted and remaining CF_0CF_1 [15–17]. A similar effect of ϵ on $CF_1(-\epsilon)$ was reported by Richter et al. [18]. In order to unravel the detailed role of these subunits in further biochemical and spectroscopic experiments we aimed at the production of larger amounts of both wild-type and mutant proteins by over-expression.

2. Materials and methods

2.1. Materials

Enzymes and reagents for molecular biology were obtained from AMS Biotechnology (Bioggio-Lugano, Switzerland), Bethesda Research Laboratories, Boehringer-Mannheim, and New England Biolabs. Chromatographic media were from Merck and Pharmacia Biotech, ultrafiltration membranes (YM 10) from Amicon, electrophoresis equipment from Pharmacia Biotech (PhastSystem). Tentoxin was supplied by Dr. B. Liebermann, Institut für Pharmazie, Neugasse 23, Friedrich-Schiller-Universität Jena, D-07743 Jena. Urea was purchased from ICN Biochemicals, guanidinium hydrochloride from Boehringer-Mannheim and L-arginine from Biomol, Hamburg. Other chemicals were either from Merck or Sigma.

2.2. Plasmids, bacterial strains and molecular genetics

We have recently cloned the genes for the five subunits of spinach CF_1 and of *Synechocystis* sp. PCC 6803 F_1 into pJLA expression vectors [12]. The ten respective inserts were further cloned into pET vectors [19]. These were transformed into expression hosts *E. coli* BL21(DE3) and HMS174(DE3). Upon induction with 0.4 mM isopropyl β-D-thiogalactoside for up to 16 h, the cells produced the desired proteins. Inclusion bodies were isolated by standard methods [20].

At least 50% of spinach δ was expressed in soluble form, whereas spinach ϵ was found in inclusion bodies under all conditions tested. Subunits δ and ϵ from *Synechocystis* precipitated into inclusion bodies at 37°C but remained soluble upon induction and further growth of the cells at 20°C.

2.3. Purification of Synechocystis F_1 and of the recombinant subunits

Synechocystis F_1 was separated from membranes by a low-salt wash with 2 mM Tris-Tricine, 50 mM sucrose (pH 7.5) [21] after removal of phycobilisomes by two washes with 10 mM $Na_2P_4O_7$. The resulting solution was chromatographed on a Fractogel TSK DEAE-650(S) anion exchange column. *Synechocystis* F_1 eluted in a peak around 240 mM salt. The eluate was used for measurements after desalting through Pharmacia PD 10, without further treatment.

Recombinant spinach δ was purified by anion exchange chromatography followed by hydrophobic interaction chromatography (HIC) as described earlier [22]. The eluate from the anion exchange column at 120–180 mM salt was diluted 1.5-fold and 150 mM ammonium sulfate were added. This solution was applied to a HIC column, unbound proteins were discarded and pure spinach δ was eluted from the column by lowering the ammonium sulfate concentration in the elution buffer in one step from 125 mM to zero.

Spinach ϵ inclusion bodies were dissolved in 8 M urea, 50 mM Mes/NaOH (pH 5.5). This solution was chromatographed on a cation exchange column (Fractogel TSK CM-650(S)) and yielded pure ϵ at 90–100 mM NaCl. ϵ could be refolded from this solution by 2-fold dilution resulting in a 4 M urea solution (0.5 mg ϵ/ml), followed by gel filtration (through Pharmacia PD 10 or NAP 5 columns) against 50 mM Mes/NaOH, 500 mM L-arginine [23] (pH 7.5) and finally gel filtration against 50 mM Mes/NaOH, 100 mM NaCl (pH 5.5). Spinach ϵ is a "sticky" protein, possibly because of its positive charge (distribution). This complicates routine procedures like ultrafiltration because the bulk of the protein is lost easily. For many purposes a solution of spinach ϵ in 4 M urea also could be used just by diluting it directly into the assay medium.

Synechocystis δ was prepared both from inclusion bodies and by purification of the soluble protein. Inclusion bodies were dissolved in 6 M guanidinium hydrochloride, 50 mM Tris-HCl (pH 7.8), 5 mg protein/ml. After dilution to 1.3 M guanidinium hydrochloride the solution was diluted 5-fold with water under vigorous stirring and then dialyzed against 25 mM Tris-HCl (pH 7.8). During this step most of the δ subunit precipitated whereas accompanying proteins remained in solution. Precipitated δ was dissolved in 8 M guanidinium hydrochloride (0.5 mg protein/ml), diluted 8-fold to 1 M guanidinium hydrochloride and then gel filtrated through PD10 columns equilibrated with 25 mM Tris-HCl, 500 mM L-arginine (pH 7.8). The eluate was pressure-dialyzed and concentrated by ultrafiltration (Amicon YM 10).

The soluble recombinant *Synechocystis* δ is uncharged at pH 7.8. The soluble cell fraction containing the over-expressed subunit was applied to a "tandem" consisting of a 1 ml Resource Q and a 1 ml Resource S column (Pharmacia Biotech). During this step most of the impurities bound to the two columns, whereas *Synechocystis* δ was not retarded. The resulting solution was concentrated (YM 10) and gel filtrated through Superdex 200 (2.6 × 60 cm, Pharmacia Biotech) equili-

brated with 25 mM Tris-HCl, 100 mM NaCl. δ eluted around $V_e = 214$ ml. Like δ, *Synechocystis* ϵ is uncharged at pH 7.8 and it was purified from the soluble cell fraction by the same procedure.

The primary structure of the proteins [24,25] was confirmed by nucleotide sequencing [26].

Preparation of thylakoids [16,17], EDTA-treated thylakoids ("EDTA vesicles") [16,22], EDTA- and tentoxin-treated thylakoids ("tentoxin vesicles") [22], CF_1 and CF_1 lacking δ and/or ϵ [15,18], reconstitution of cyclic photophosphorylation [16,22,27], ATP, ATPase and phosphate assays [16,17,27] and protein determination [28] were performed according to published procedures.

3. Results and discussion

Fig. 1 shows an SDS electrophoresis with crude and purified recombinant δ and ϵ subunits from spinach and *Synechocystis* F_1 and complete F_1 from both sources. Evidently, the purified recombinant subunits did not differ in their migration behavior from their counterparts as present in intact F_1. Spinach δ is encoded by nuclear DNA and therefore the leader sequence has to be removed prior to expression in *E. coli*. The correct primary structure was confirmed by

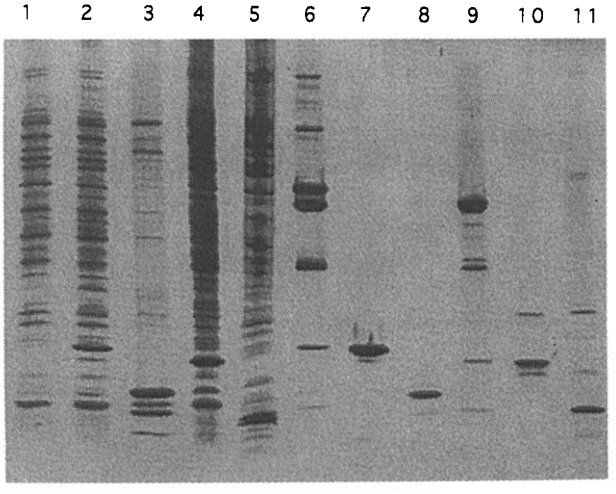

Fig. 1. SDS electrophoresis of crude and purified recombinant δ and ϵ subunits from spinach CF_1 and from *Synechocystis* sp. PCC 6803 F_1. Lane 1, soluble fraction of BL21(DE3) cells; lane 2, soluble fraction of induced BL21(DE3)/pET-spiδ cells containing spinach δ; lane 3, insoluble fraction of induced BL21(DE3)/pET-spiϵ cells containing spinach ϵ; lane 4, soluble fraction of induced BL21(DE3)/pET-cysδ cells containing *Synechocystis* δ; lane 5, soluble fraction of induced BL21(DE3)/pET-cysϵ cells containing *Synechocystis* ϵ; lane 6, purified spinach CF_1; lane 7, purified recombinant spinach δ; lane 8, purified recombinant spinach ϵ; lane 9, purified *Synechocystis* F_1; lane 10, purified recombinant *Synechocystis* δ; lane 11, purified recombinant *Synechocystis* ϵ. 8–25% PhastGel™, migration from top to bottom, silver/silicotungstic acid stain [27].

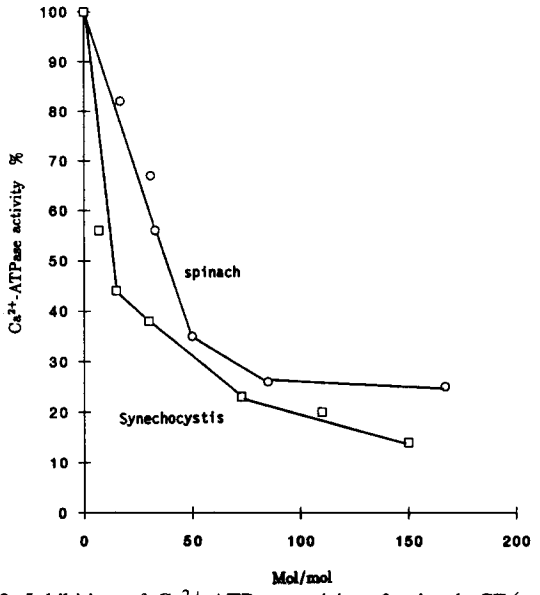

Fig. 2. Inhibition of Ca^{2+}-ATPase activity of spinach $CF_1(-\epsilon)$ by purified recombinant spinach and *Synechocystis* ϵ. 100% of activity correspond to 11 U/mg spinach $CF_1(-\epsilon)$. Mol/mol indicates the ratio $\epsilon/CF_1(-\epsilon)$. Assay conditions: 5 nm $CF_1(-\epsilon)$, 50 mM Tris-HCl (pH 7.8), 5 mM ATP, 5 mM $CaCl_2$, 5 min preincubation with ϵ (in the absence of substrate), 10 min incubation with substrate at 37°C followed by termination of the reaction with trichloroacetic acid and photometric determination of inorganic phosphate after complexation of P_i by molybdate [10].

N-terminal amino acid sequencing:

(M)VDSTA SRYAS ALADV ADVTG TLEAT NSDVE KLIRI FSEEP VYYFF ...

The N-terminal methionine was removed nearly entirely.

The biological activity of F_1 subunit ϵ is easily measurable by its inhibition of Ca^{2+}-ATPase activity [29,30]. Fig. 2 shows the Ca^{2+}-ATPase activity of spinach $CF_1(-\epsilon)$ in the presence of increasing amounts of ϵ. It is evident that subunit ϵ from either species, spinach or *Synechocystis* inhibited the Ca^{2+}-ATPase activity of the spinach enzyme. A rather high molar excess was necessary to achieve significant inhibition. This may reflect a low yield of refolding (spinach ϵ), loss of ϵ due to unspecific binding or a low binding affinity [31]. Although we cannot exclude the first two explanations, we favor the latter. In reconstitution of photophosphorylation ϵ was much more effective on a molar basis (see below).

Table 1 shows the reconstitution of photophosphorylation by adding recombinant δ from spinach and *Synechocystis* plus spinach $CF_1(-\delta)$ to partially CF_1-depleted spinach thylakoids. The improved reconstitutive activity of $CF_1(-\delta)$ in the presence of recombinant δ as compared to the effectivity of $CF_1(-\delta)$ alone demonstrated the structural and functional integrity of the recombinant δ subunits. Not unexpectedly, *Synechocystis* δ was not as effective as spinach δ in core-constitution with spinach $CF_1(-\delta)$.

Table 1
Reconstitution of phenazine-methosulfate-mediated photophosphorylation in EDTA-treated spinach thylakoids by CF_1, $CF_1(-\delta)$, and $CF_1(-\delta)+\delta$

Sample	μg	ATP
Thylakoids		1370
Vesicles		250
Vesicles +		
CF_1	10	720
$CF_1(-\delta)$	10	260
$CF_1(-\delta)$ + spiδ	10+1	620
$CF_1(-\delta)$ + cysδ	10+5	440

"Vesicles" are thylakoids which were partially CF_1-depleted by treatment of a 0.4 mM chlorophyll solution with 1 mM EDTA on ice for 1 min [16,22,27]. ATP indicates μmoles ATP synthesized per h and mg chlorophyll. 10 μg chlorophyll, 50 μM phenazine-methosulfate were used per assay. The given amounts of enzyme and subunits were saturating under these conditions (data not shown). Preincubation of samples and membranes was 1 h on ice in the dark. spi = spinach, cys = *Synechocystis* sp. PCC 6803.

Table 2 shows the results of an experiment which aimed at the reconstitutive activity of both small spinach subunits, δ and ε, in coreconstitution with $CF_1(-\delta,\epsilon)$. Spinach ε in reconstitution of photophosphorylation was much more effective (8:1 mol/mol) than in inhibition of Ca^{2+}-ATPase activity (cf. Table 1). This might be due to a conformational rearrangement which CF_1 undergoes both upon removal from CF_0 and/or depletion of the ε subunit, thereby decreasing its affinity for ε.

Table 3 shows reconstitution of photophosphorylation in EDTA-treated thylakoids from spinach with integral F_1 from *Synechocystis* and spinach. The cyanobacterial enzyme had to be prepared within 1 day by anion exchange chromatography of a low-salt extract of *Synechocystis* membranes. The preparation lost its enzymatic and reconstitutive activity very rapidly. The loss in activity apparently was not caused by proteolytic digestion and it could not be overcome by a variety of additives (data not shown). Therefore *Synechocystis* F_1 could not be further characterized with respect to its exact composition ($F_1/F_1(-\delta)/F_1(-\epsilon)/F_1(-\delta\epsilon)$). *Synechocystis* F_1 was effective in reconstitution, although to a much lesser extent than spinach CF_1.

Table 2
Reconstitution of phenazine-methosulfate-mediated photophosphorylation in EDTA-treated spinach thylakoids by CF_1, $CF_1(-\delta,\epsilon)$ and $CF_1(-\delta,\epsilon)$ + recombinant spinach δ + recombinant spinach ε

Sample	μg	ATP
Thylakoids		843
Vesicles		150
Vesicles +		
CF_1	10	670
$CF_1(-\delta,\epsilon)$	10	155
$CF_1(-\delta,\epsilon)+\delta$	10+1.5	210
$CF_1(-\delta,\epsilon)+\epsilon$	10+3	190
$CF_1(-\delta,\epsilon)+\delta+\epsilon$	10+1+3	440

Preparation of samples, vesicles, reconstitution procedure and measurement of synthesized ATP were as detailed in Materials and methods and in the legend to Table 1.

Table 3
Reconstitution of phenazine-methosulfate-mediated photophosphorylation in EDTA-treated spinach thylakoids by F_1 from spinach ("spi") and from *Synechocystis* ("cys") and recombinant *Synechocystis* δ and ε

Sample	μg	ATP
Thylakoids		1468
Vesicles		305
Vesicles +		
spiCF_1	10	810
cysF_1	10	400
cysF_1 + cys subunits		
cysF_1 + δ	10+2	440
cysF_1 + ε	10+3	430
cysF_1 + δ + ε	10+2+3	500

For experimental details see Table 1 and Materials and methods.

It was interesting to know whether recombinant δ and ε complemented spinach $CF_1(-\delta,\epsilon)$ in reconstitution only indirectly by blocking exposed proton channels CF_0, or whether they formed a fully functional enzyme. We have previously obtained a discrimination between the former, named "structural reconstitution" and the latter, "functional reconstitution", by poisoning remaining CF_1 molecules on the membrane with

Table 4
Reconstitution of phenazine-methosulfate-mediated photophosphorylation in EDTA- and tentoxin-treated spinach thylakoids ("tentoxin vesicles") by spinach CF_1, $CF_1(-\delta,\epsilon)$, and recombinant spinach ("spi") and *Synechocystis* ("cys") δ and ε

Sample	μg	ATP
Thylakoids		996
Tentoxin vesicles		8
Tentoxin vesicles +		
CF_1	20	244
$CF_1(-\delta,\epsilon)$	20	42
spiδ/ε	20/20	12
cysδ/ε	4/4	14
$CF_1(-\delta,\epsilon)$ (20 μg) +		
spiδ	20	44
spiε	20	41
spiδ/spiε	20/20	225
cysδ/cysε	4/4	108
cysδ/spiε	4/20	70
spiδ/cysε	20/4	183
spiδ/spiε	20/20	67 *
cysδ/cysε	4/4	63 *

For experimental details see Table 1 and Materials and methods. Data marked with * were obtained by first mixing $CF_1(-\delta,\epsilon)$ (240 μg) with the respective subunits (60 μg δ and 90 μg ε), followed by ion-exchange chromatography of the mixtures. These samples thus were expected to contain integral CF_1 (containing either spinach δ and ε or *Synechocystis* δ and ε).

tentoxin [22]. Table 4 shows results of a reconstitution experiment carried out with "tentoxin vesicles" i.e., EDTA-treated, partially CF_1-depleted thylakoid membranes, where the remaining CF_1 was inhibited by tentoxin. Increased photophosphorylation rates upon addition of CF_1 in these vesicles indicate functional reconstitution [22].

It is evident that $CF_1(-\delta,\epsilon)$ when complemented by recombinant δ and ϵ subunits was nearly as effective in reconstitution of photophosphorylation as integral CF_1. This proved once again the functional integrity of the recombinant subunits. However, a rather large excess of spinach ϵ was required. In this experiment the 4 M urea solution of ϵ was used (cf. Materials and methods), because the solution of refolded ϵ was to dilute. Urea concentrations up to 500 mM did not affect the ATP synthetic activity of (reconstituted) EDTA vesicles from spinach (data not shown). Spinach ϵ thus was refolded in situ and it was not possible to decide to what extent the large excess necessary for reconstitution was due to largely misfolded protein or whether it reflected low binding affinities.

As an alternative approach, we attempted to first reconstitute integral CF_1 by mixing $CF_1(-\delta,\epsilon)$ with recombinant δ and ϵ in solution, then to isolate the enzyme by anion exchange chromatography, and only

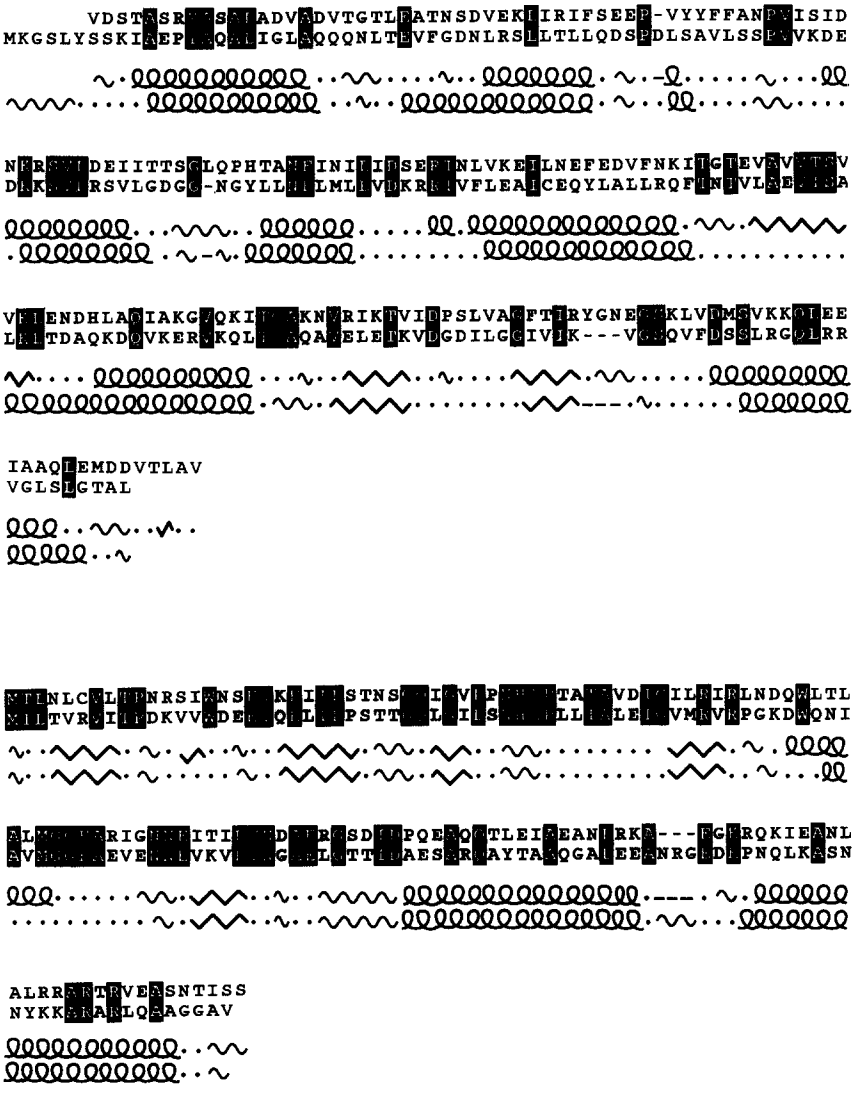

Fig. 3. Amino acid alignment and secondary structure prediction of spinach and *Synechocystis* F_1 δ (upper part) and ϵ (lower part) subunits. The first row always shows the amino acid sequence of spinach δ, the second row the amino acid sequence of *Synechocystis* δ, the third row the secondary structure prediction for spinach δ, and the fourth row the secondary structure prediction for *Synechocystis* δ etc. The alignment was obtained with the program ClustAlV running under the DKFZ (Heidelberg) HUSAR package. Identical residues are indicated by box shading. Helices are indicated as spirals, β-strands as zig-zag lines and loops as wavy lines. The secondary structure prediction was obtained with the program PredictProtein (Internet: Predict-Help@MBL-Heidelberg.DE). This program is reported to have an accuracy of more than 70% [33].

then to reconstitute ATP synthesis in CF_1-depleted vesicles. Even 50 μg of this "$CF_1(-\delta,\epsilon)+\delta+\epsilon$" did not match the effectivity of the components mixed in situ. The smaller extent of reconstitutive activity of these samples in comparison to those where all components were mixed in situ indicated that under these conditions $CF_1(-\delta,\epsilon)$ did not bind the small subunits properly. It is conceivable that CF_0 facilitated binding of ϵ to CF_0CF_1 either indirectly by increasing the binding affinity of $CF_1(-\epsilon)$ for ϵ or directly by providing another binding site for ϵ: a conformational change of CF_1 could accompany removal of the ϵ subunit. This change might be permanent in solubilized $CF_1(-\epsilon)$, but reversed upon binding of $CF_1(-\epsilon)+\epsilon$ to CF_0.

The two recombinant *Synechocystis* subunits δ and ϵ complemented spinach $CF_1(-\delta,\epsilon)$ functionally, despite a rather low percentage of sequence identity. Fig. 3 shows an alignment of the two δ and the two ϵ subunits together with a prediction of their secondary structure. The percentage of identical residues is 26% and 41% for δ and ϵ respectively. In pairwise comparison *Synechocystis* ϵ thus resembles more its spinach counterpart than *Synechocystis* δ resembles spinach δ. The predicted secondary structure assignments, however, are very similar for both subunits. When comparing spinach δ, *E. coli* δ and their mitochondrial counterpart oligomycin-sensitivity-conferring protein (23% identical residues), a very similar content of secondary structure has been experimentally demonstrated by CD spectroscopy [32].

On a molar basis, *Synechocystis* ϵ was more effective in reconstitution of photophosphorylation (together with $CF_1(-\delta,\epsilon)$) than spinach ϵ. In combination with spinach δ it nearly matched the effectivity of spinach $CF_1(-\delta,\epsilon)$ + spinach δ + spinach ϵ. This was not the case if *Synechocystis* δ was used in connection with spinach $CF_1(-\delta,\epsilon)$ + spinach ϵ. Either *Synechocystis* ϵ "fits" better than *Synechocystis* δ (in comparison with their spinach counterparts) or the biological function of δ is more elaborate: it would seem that spinach and *Synechocystis* ϵ are nearly interchangeable, whereas *Synechocystis* δ allows for some slip in coupling. Presently, a differentiation between less binding or impaired function is not possible.

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