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Cross-reconstitution of the F_0F_1-ATP synthases of chloroplasts and *Escherichia coli* with special emphasis on subunit δ

Siegfried ENGELBRECHT[1], Gabriele DECKERS-HEBESTREIT[2], Karlheinz ALTENDORF[2] and Wolfgang JUNGE[1]

Departments of [1] Biophysics and [2] Microbiology, University of Osnabrück

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F_0F_1-ATP synthases catalyse ATP formation from ADP and P_i by using the free energy supplied by the transmembrane electrochemical potential of the proton. The δ subunit of F_1 plays an important role at the interface between the channel portion F_0 and the catalytic portion F_1. In chloroplasts it can plug the protonic conductance of CF_0 and in *Escherichia coli* it is required for binding of EF_1 to EF_0.

We wanted to know whether or not δ of one species was effective between F_0 and F_1 of the other species and vice versa. To this end the respective coupling membrane (thylakoids, everted vesicles from *E. coli*) was (partially) depleted of F_1 and purified F_1, $F_1(-\delta)$, and δ were added in various combinations to the F_1-depleted membranes. The efficiency or reconstitution was measured in thylakoids via the rate of phenazinemethosulfate-mediated cyclic photophosphorylation and in *E. coli* everted vesicles via the degree of 9-amino-6-chloro-2-methoxyacridine fluorescence quenching.

Addition of CF_1 to partially CF_1-depleted thylakoid vesicles restored photophosphorylation to the highest extent. $CF_1(-\delta)$+chloroplast δ, EF_1, $EF_1(-\delta)$+*E. coli* δ were also effective but to lesser extent. $CF_1(-\delta)$+*E. coli* δ and $EF_1(-\delta)$+chloroplast δ restored photophosphorylation to a small but still significant extent. With F_1-depleted everted vesicles prepared by repeated EDTA treatment of *E. coli* membranes, addition of CF_1, $CF_1(-\delta)$+chloroplast δ and $CF_1(-\delta)$+*E. coli* δ gave approximately half the extent of 9-amino-6-chloro-2-methoxyacridine fluorescence quenching as compared to EF_1 or $EF_1(-\delta)$+*E. coli* δ by energization of the vesicles with NADH, while $EF_1(-\delta)$+chloroplast δ was ineffective. All 'mixed' combinations were probably reconstitutively active only by plugging the protonic leak through the exposed F_0 (structural reconstitution) rather than by catalytic activity. Nevertheless, the cross-reconstitution is stunning in view of the weak sequence similarity between chloroplast δ and *E. coli* δ. It favors a role of δ as a conformational transducer rather than as a proton conductor between F_0 and F_1.

ATP synthesis in thylakoids, mitochondria, and in various microorganisms is mediated by F_0F_1-ATP synthases. These form a class of closely related enzymes consisting of a membrane-embedded F_0 portion, an extrinsic F_1 part and possibly a connecting stalk. Such a stalk was observed already in 1964 in electron microscopic studies of mitochondrial inner membranes by Fernandez-Moran et al. [1] and recently the concept of a stalk has been 'revived' by Gogol et al. [2]. (For recent reviews on F_0F_1 see [3–7]). The F_0 portion functions as a proton channel with extremely high conductance [8], the water-soluble F_1 contains the substrate binding sites (upon detachment from the membrane F_1 catalyses ATP hydrolysis) and the stalk is supposed to serve as the connecting link between F_0 and F_1. F_1 is a heterooligomer with an $\alpha_3\beta_3\gamma\delta\varepsilon$ structure [9–12].

ATP synthesis is thought to occur spontaneously, the energy-requiring step being product release. Three different active sites may by connected allosterically by negative cooperativity: Binding of ADP and P_i to one site causes ATP formation in a second site and ATP liberation in a third one. The concepts of spontaneous ATP formation, grossly enhanced ATP liberation by multisite catalysis, and possibly by functional rotation of active sites in time (the binding change mechanism) have been introduced and recently summarized by Penefsky [13] and Boyer [14].

In this context, it is interesting to study the function of subunit δ. δ is one of the smaller subunits of CF_1 and EF_1. Its molecular mass is around 20 kDa, the primary structures of both the *Escherichia coli* δ and the chloroplast δ are known [15–17] and are only slightly similar [17]. δ of both origins, *E. coli* and spinach thylakoids, appears to be an elongated, rather flexible molecule [18–20]. Originally it was thought that in both *E. coli* and thylakoid membranes δ was the connecting link between F_1 and F_0, being necessary for the association of these two protein complexes [18, 21–23]. A similar conclusion was reached for *Rhodospirillum rubrum* ATP synthase [24]. Only for EF_0EF_1 has this conclusion survived the test of time; for CF_0CF_1 it was clearly demonstrated that δ is not required for rebinding of CF_1 to CF_0 [25]. By various approaches it was shown that chloroplast δ controls proton flow: when isolated and added back to partially CF_1-depleted membranes, it can keep the proton pore through CF_0 closed [26–33]. Thus at least chloroplast δ fulfills a

Correspondence to S. Engelbrecht, Abteilung für Biophysik der Universität Osnabrück, Barbarastraße 11, D-4500 Osnabrück, Federal Republic of Germany

Abbreviations. EF_0, *E. coli* coupling factor 0 (proton channel); EF_1, *E. coli* coupling factor 1 (integral ATPase); $EF_1(-\delta)$, EF_1 lacking the δ subunit; $EF_1(-\varepsilon)$, EF_1 lacking the ε subunit; CF_0, CF_1 etc., the respective terms for the enzyme from chloroplasts; Mega 9, *N*-(D-*gluco*-2,3,4,5,6-pentahydroxyl)-*N*-methylnonanamide.

Enzyme. ATPase (EC 3.6.1.34).

functional and not merely structural role [31, 32]. The structural role of *E. coli* δ in the association of EF_1 with EF_0 does not exclude an additional functional role though, and this has been suggested [34, 35].

It was interesting to compare *E. coli* δ and chloroplast δ directly in mixed reconstitution experiments, adding $CF_1(-\delta)$ + *E. coli* δ to CF_0 and $EF_1(-\delta)$ + chloroplast δ to EF_0. In view of the constraints that are posed by the necessity of precise matching of the interfaces between F_0, δ, and F_1 and considering the weak sequence similarity between the two δ subunits, the finding that *E. coli* δ in the presence of $CF_1(-\delta)$ restored photophosphorylation to a small but significant extent was rather surprising. We interpreted this result as an indication of conformational coupling between proton flow and ATP synthesis, with subunit δ playing the same role both in CF_0CF_1 and EF_0EF_1.

MATERIALS AND METHODS

Spinach thylakoid vesicles that were partially depleted in CF_1('EDTA vesicles') were prepared according to the procedure of Shoshan and Shavit [36], spinach CF_1, $CF_1(-\delta)$ and chloroplast δ according to published procedures [28, 30, 32, 37]. F_1-depleted everted membrane vesicles were prepared from *E. coli* strain ML 308-225 as described by Vogel and Steinhart [38]. EF_1 from strains ML 308-225 or KY 7485 was prepared according to Steffens et al. [39]. Bacterial growth was performed as published [39, 40]. The FPLC purification of $EF_1(-\delta)$ and *E. coli* δ was performed with self-prepared columns [28] of Fractogel TSK DEAE 650(S) and Fractogel TSK Butyl 650(S). Reconstitution of photophosphorylation along with ATP determination was carried out as described [28, 32]. Reconstitution of F_1-depleted everted vesicles from *E. coli* with EF_1 or CF_1 was performed as outlined in [38]. The energization of reconstituted membrane vesicles with NADH (0.2 mM) or ATP (0.5 mM) was determined by 9-amino-6-chloro-2-methoxyacridine fluorescence quenching as described in [41]. Preincubations of $F_1(-\delta)+\delta$ were carried out at room temperature in 25 mM P_i, pH 7.8 for 3–10 min.

Protein determinations were carried out after Sedmak and Grossberg [42], SDS electrophoresis with the Pharmacia PhastSystem [43] with silver/silicotungstic acid staining essentially according to Krause and Elbertzhagen [44] (see also [32, 45]). Rocket immunoelectrophoresis was carried out as in [28, 32].

Mega 9 was obtained from Oxyl Chemie (Bobingen, FRG); the fractogel resins came from Merck, the ATP-monitoring reagent from Pharmacia LKB, Wallac, Finland and the ultrafiltration membranes from Amicon.

Adenosine(5′) pentaphospho(5′) adenosine and tentoxin were purchased from Sigma.

RESULTS

Preparation of E. coli δ *and* $EF_1(-\delta)$

The experiments necessitated the preparation of $EF_1(-\delta)$ and *E. coli* δ. We found that EF_1 isolated from the ATP-synthase-overproducing strain KY 7485 in contrast to strain K12 [46] contained some δ, which rendered this preparation unsuitable as a source of $EF_1(-\delta)$. On the other hand, the amounts of δ were too low for efficient preparation of this subunit from KY 7485. Initial attempts to purify all EF_1 subunits separately followed by reconstitution of $EF_1(-\delta)$ did not look promising. The Smith and Sternweis procedure [47] for preparation of *E. coli* δ in several experiments gave only pure ε but never pure δ and naturally no $EF_1(-\delta)$. Therefore, we focussed on preparation of $EF_1(-\delta)$ and *E. coli* δ by application of the protocol for preparation of $CF_1(-\delta)$ and chloroplast δ [30, 32].

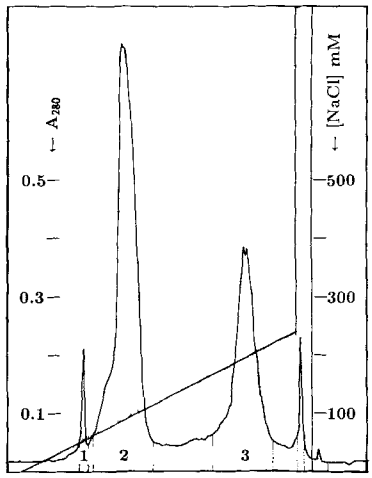

Fig. 1. *Elution profile of anion-exchange chromatography of 10 mg EF_1 on fractogel TSK DEAE 650(S).* The sample was pretreated as detailed in Results. Elution was by a linear gradient (0–300 mM within 150 ml) of NaCl in 25 mM Tris/HCl, 22 mM Mega 9, 1 mM EDTA, pH 7.8 at 5 ml/min. The absorption at 280 nm was detected and the salt gradient as set by the controller is shown as a straight line (the chromatogram is not corrected for the delay between setting of the salt gradient and elution from the column). Note that the second peak contains ADP and ATP, which cause the absorption increase. The run was ended by a high-salt wash, seen on the right edge of the profile as a sharp increase of the salt gradient. The peak that was eluted contained no EF_1-related subunits

After some adjustments the following protocol was adopted, which allowed for the preparation of about 100 µg *E. coli* δ from about 30 mg EF_1 within 8 h (starting from purified EF_1). Freshly prepared EF_1 was dissolved at about 1 mg/ml in 25 mM Tris/HCl, 22 mM Mega 9, 0.25 mM K-ADP, 0.25 mM Na-ATP, 0.5 mM $MgCl_2$, 20% MeOH, pH 7.8 and kept for 1 h on ice. The material was applied in batches not exceeding 15 mg to a 10-ml column packed with fractogel TSK DEAE 650(S) (referred to as anion-exchange column) and eluted with a linear gradient of NaCl in 25 mM Tris/HCl, 22 mM Mega 9, 1 mM EDTA, pH 7.8. A first sharp peak around 50 mM NaCl contained subunits α and ε. A second broad peak contained the nucleotides and subunits α, δ, ε, and, depending on the starting material, small amounts of impurities. A mixture of $EF_1(-\delta)$ and $EF_1(-\delta,\varepsilon)$ eluted in a third peak around 200 mM NaCl. This material precipitated shortly after elution from the column. In order to avoid this, after elution of the δ-containing peak, the column was immediately reequilibrated and eluted with NaCl in 25 mM Tris/HCl, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.8. This completely prevented precipitation of $EF_1(-\delta)/EF_1(-\delta,\varepsilon)$. The first peak and the last peak were combined and 0.1 mM di-isopropylphosphofluoridate was added to the combined $\alpha/\varepsilon/EF_1(-\delta)/EF_1(-\delta,\varepsilon)$ material and the $\alpha/\delta/\varepsilon$ peak. A typical chromatogram is shown in Fig. 1.

A gel filtration of the recombined first and third peak on Superose 12 (Pharmacia) yielded $EF_1(-\delta)$ in the void volume and pure α subunit, thus demonstrating rebinding of ε to $EF_1(-\delta,\varepsilon)$ (cf. Fig. 2). Alternatively, the recombined peak frac-

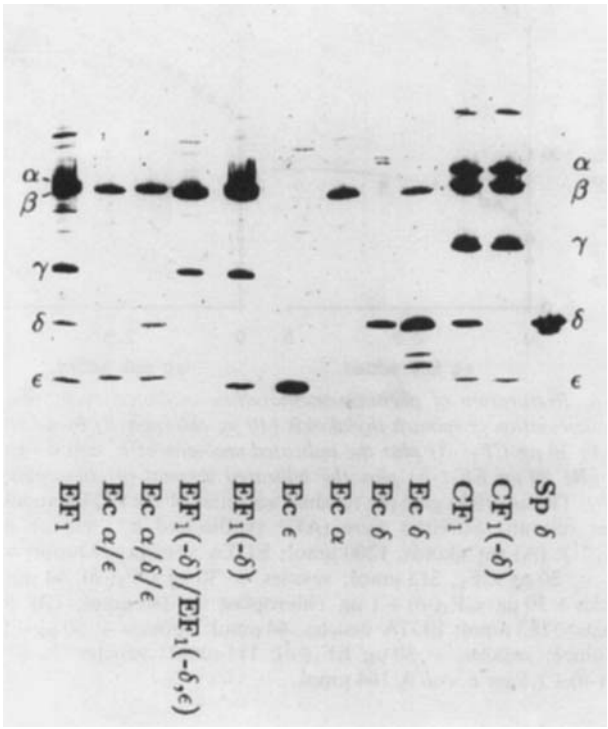

Fig. 2. *SDS electrophoresis of samples obtained during preparation of $EF_1(-\delta)$ and* E. coli *δ and samples that were used for the reconstitution experiments.* Phastgels (10–15%) [43] were silver-stained [32, 44]. The samples are as indicated, ≈ 150 ng EF_1, $EF_1(-\delta)$, CF_1, $CF_1(-\delta)$, and ≈ 30 ng of all other samples were run. Note that the α and β subunits of EF_1 were not separated by the system. The α subunit contained in various samples was identified by doubling the separation time, which resulted in clear distinction of *E. coli* α and β. The two samples of *E. coli* δ represent the achievable purity of the preparation under favorable and unfavorable conditions (cf. Results)

tions were pressure-dialysed via Amicon YM 10 membranes in order to remove detergent and salt. The *E. coli* δ contained in the second peak remained to be further purified. This was achieved by hydrophobic interaction chromatography on fractogel TSK Butyl 650(S). The detergent still present in the second peak was removed by rechromatography on the anion-exchange column. The peak fractions were diluted threefold with 25 mM Tris/HCl, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.8 (buffer A), applied to the column and eluted with 25 mM Tris/HCl, 250 mM $(NH_4)_2SO_4$, pH 7.8. The material was applied immediately to a 1-ml TSK Butyl 650(S) column, the nucleotides and ε passed through, α was washed off the column by lowering the $(NH_4)_2SO_4$ concentration to 25 mM in buffer A and *E. coli* δ was eluted by lowering the $(NH_4)_2SO_4$ concentration to zero (data not shown). The δ-containing peak was pressure-dialysed via an Amicon YM 10 membrane in order to remove traces of $(NH_4)_2SO_4$ that otherwise interfered with the reconstitution experiments. All chromatographies were carried out at room temperature. The eluted material was stored on ice until SDS electrophoretic characterization and after pressue dialysis/concentration in liquid nitrogen.

Taken together, $EF_1(-\delta)$ and *E. coli* δ were prepared by two chromatographies: anion exchange in the presence of detergent and hydrophobic interaction chromatography. The anion-exchange chromatography yielded in three peaks α/ε, $\alpha/\delta/\varepsilon$ and $EF_1(-\delta)/EF_1(-\delta,\varepsilon)$. Recombination of the α/ε and

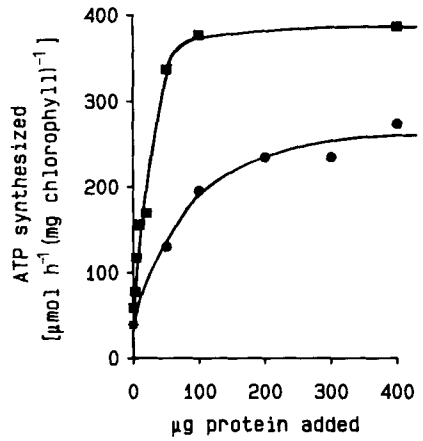

Fig. 3. *Restoration of phenazinemethosulfate-mediated cyclic photophosphorylation in spinach thylakoids (10 μg chlorophyll) by addition of CF_1 (■) and EF_1 (●).* The activity of the thylakoids was 2072 μmol ATP synthesized h^{-1} (mg chlorophyll)$^{-1}$

$EF_1(-\delta)/EF_1(-\delta,\varepsilon)$ peaks gave $EF_1(-\delta)$ and α, which could be separated by gel filtration. δ could be purified out of the second peak by hydrophobic interaction chromatography. In view of the effectiveness of chloroplast δ in the presence of $CF_1(-\delta)$, it was of critical importance to use two different sets of columns and ultrafiltration cells for the preparation of *E. coli* and chloroplast proteins, respectively.

The procedure worked best with EF_1 freshly prepared from strain ML 308-225. The major problem was contamination of *E. coli* δ with α: in some cases contaminating α could not be removed completely, even after incubation with 50 mM dithiothreitol in order to prevent disulfide linkage between α and δ [48]. In these cases the Smith and Sternweis procedure [47] yielded the same α-contaminated δ (in these latter EF_1 preparations, the first peak from the anion-exchange column was about half the size of the third peak so that the 'success' of the *E. coli* δ preparation may be judged at this point already). Fig. 2 shows an SDS electrophoresis of the samples that were used in the reconstitution experiments. Western blot analysis of EF_1, CF_1, chloroplast δ, and *E. coli* δ with antisera prepared against the five subunits of EF_1, against CF_1, and against chloroplast δ showed only cross-reactivity between the two β subunits (G. Deckers-Hebestreit, unpublished results).

Restoration of photophosphorylation in spinach EDTA vesicles

It was our aim to determine whether or not *E. coli* δ could fulfill the role of chloroplast δ in CF_0CF_1 and vice versa. In view of the fact that δ is probably located at the interface between F_0 and F_1 it was interesting to see whether EF_1 would restore photophosphorylation in EDTA-treated thylakoids ('EDTA vesicles') and if so whether the reconstitution was functional, i.e. ATP synthesis by CF_0EF_1, or structural, i.e. plugging of protonic leaks through open CF_0 in a proton-leaky vesicle by EF_1 which indirectly reactivated the catalytic activity of the remaining CF_0CF_1 molecules in the now proton-tight vesicle.

To this end EDTA vesicles were incubated with increasing amounts of EF_1 and, for comparison, CF_1. From Fig. 3 it is evident that EF_1 could restore photophosphorylation although not to the same extent as CF_1. With respect to their binding affinities, EF_1 and CF_1 were roughly comparable (curiously the restoration of photophosphorylation caused by EF_1 was independent of the Mg^{2+} concentration). The

Table 1. *Comparison between rates of phenazinemethosulfate-mediated cyclic photophosphorylation in spinach thylakoids and EDTA-treated thylakoids (before and after addition of CF_1 or EF_1) in the absence and presence of 5 μM tentoxin*
ATP synthesis rates are given in μmol ATP synthesized h^{-1} (mg chlorophyll)$^{-1}$. Values are not corrected for intrinsic ATP (≈ 20 μmol/mg chlorophyll). nd = not determined

Sample	Rate of ATP synthesis (± tentoxin) in expt					
	1		2		3	
	−	+	−	+	−	+
	μmol · h^{-1} · mg^{-1}					
Thylakoids	1120	113	1790	173	1920	nd
EDTA vesicles	46	28	42	22	34	24
+ CF_1 (50 μg)	351	61	450	55	474	60
+ EF_1 (400 μg)	130	68	127	64	190	90

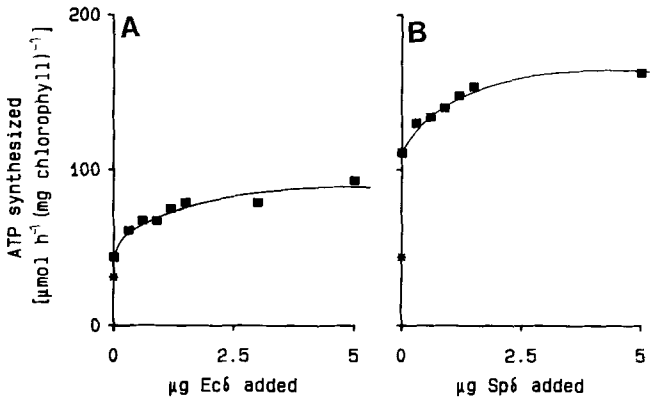

Fig. 4. *Restoration of phenazinemethosulfate-mediated cyclic photophosphorylation in spinach thylakoids (10 μg chlorophyll) by addition of (A) 30 μg $CF_1(-\delta)$ plus the indicated amounts of* E. coli *δ (Ecδ) and (B) 30 μg $EF_1(-\delta)$ plus the indicated amount of chloroplast δ (Spδ)*. The asterisks give the residual activities of the EDTA vesicles. Other relevant activities were (ATP synthesized h^{-1} mg chlorophyll^{-1}): (A) thylakoids, 1200 μmol; EDTA vesicles, 31 μmol; vesicles + 30 μg CF_1, 318 μmol; vesicles + 30 μg $CF_1(-\delta)$, 44 μmol; vesicles + 30 μg $CF_1(-\delta)$ + 1 μg chloroplast δ, 143 μmol; (B) thylakoids, 1183 μmol; EDTA vesicles, 44 μmol; vesicles + 50 μg EF_1, 160 μmol; vesicles + 30 μg $EF_1(-\delta)$, 111 μmol; vesicles + 30 μg $EF_1(-\delta)$ + 1.5 μg *E. coli* δ, 164 μmol

difference in effectiveness could be explained either by the existence of a subpopulation of CF_0 that reacted properly with EF_1, or by suboptimal plugging of leaks. If the CF_0EF_1 complex was functionally active in ATP synthesis, this action should become evident after selective inhibition of CF_0CF_1. We used tentoxin as a specific inhibitor of CF_1 [49]. Table 1 shows the result of three experiments where EDTA vesicles were reconstituted with saturating amounts of CF_1 and EF_1 in the absence and presence of tentoxin. Tentoxin did not prevent a slight restoration of photophosphorylation by addition of both EF_1 and CF_1 to EDTA-treated thylakoids. This was interpreted as an indication of the presence of a small amount of CF_0CF_1 that could not be inhibited by tentoxin and that, upon addition of either CF_1 or EF_1, regained activity. We concluded that EF_1 acted only by plugging leaks whereas the added CF_1, at least in part, was also functionally active. Similar experiments were carried out by Bar-Zvi et al. [50] working with TF_1 from the thermophilic bacterium PS 3 and thylakoid membranes and CF_1 from lettuce chloroplasts. They also concluded that TF_1 acted exclusively by plugging leaks (structural reconstitution), whereas the added CF_1 was active in both structural and functional reconstitution (cf. Table 1 in [50]). In contrast, cross-reconstitution studies with EF_1 and TF_0 and vice versa showed that the F_1 and F_0 parts of *E. coli* and PS 3 were also functionally compatible [41].

Next, we investigated the restoration of photophosphorylation by $CF_1(-\delta)$ + *E. coli* δ and by $EF_1(-\delta)$ + chloroplast δ. Fig. 4 shows the results of experiments where 30 μg $CF_1(-\delta)$ or $EF_1(-\delta)$ was added to EDTA vesicles (equivalent to 10 μg chlorophyll) together with increasing amounts of either spinach or *E. coli* δ. In both cases photophosphorylation was restored to a greater extent after addition of both $F_1(-\delta)$ and δ as compared to the effects of $F_1(-\delta)$ alone. Saturation of the effects at a ratio of about 1.5 μg δ/30 μg $F_1(-\delta)$ corresponded to a molar ratio of 1:1 [18, 32].

The restoration of photophosphorylation was small as compared to the effect of added CF_1, but it was reproducible and clearly above background. Table 2 summarizes the data of six experiments with different preparations of $F_1(-\delta)$ and δ. Not unexpectedly, restoration of photophosphorylation was highest with CF_1, $CF_1(-\delta)$ and EF_1 were comparably effective, while $EF_1(-\delta)$ in most cases was half as effective as EF_1. Addition of chloroplast δ to $CF_1(-\delta)$ greatly improved the degree of restoration of photophosphorylation, although not to the extent of CF_1. In most cases $EF_1(-\delta)$ + *E. coli* δ were as effective as EF_1. $CF_1(-\delta)$ + *E. coli* δ worked in five out of six cases. *E. coli* δ did not enhance the restoration of photophosphorylation to the same degree as chloroplast δ whereas chloroplast δ together with $EF_1(-\delta)$ was indistinguishable from $EF_1(-\delta)$ + *E. coli* δ. Chloroplast δ or *E. coli* δ alone did not restore photophosphorylation and *E. coli* α and *E. coli* ε consistently did not improve the extent of restoration of photophosphorylation by added $CF_1(-\delta)$ or $EF_1(-\delta)$ (data not shown).

Restoration of membrane energization in everted vesicles from E. coli

In an inverse approach the effects of EF_1, $EF_1(-\delta)$, and *E. coli* δ on the leakiness to protons of F_1-depleted everted *E. coli* vesicles were compared to those of CF_1, $CF_1(-\delta)$, and chloroplast δ. To this end F_1-depleted everted vesicles were incubated with increasing amounts of EF_1 and CF_1, respectively (Fig. 5). Energization of reconstituted vesicles with NADH measured by 9-amino-6-chloro-2-methoxyacridine fluorescence quenching showed that CF_1 was effective in plugging protonic leaks through open EF_0 although not to the same extent as EF_1 (see also below). However, the reconstitution of CF_1 was structural as demonstrated by energization of reconstituted vesicles with ATP. In addition, the affinity of CF_1 to EF_0 was somewhat lower (15 μg vs 6 μg) and the degree of restoration of 9-amino-6-chloro-2-methoxyacridine fluorescence quenching was about 60% of the effect of EF_1.

Next, we studied the reconstitution of F_1-depleted vesicles with $CF_1(-\delta)$ + *E. coli* δ or $EF_1(-\delta)$ + chloroplast δ. The results of these experiments are summarized in Table 3. Whereas $CF_1(-\delta)$ + *E. coli* δ were effective in plugging protonic leaks to the same degree as CF_1 or $CF_1(-\delta)$ + chloroplast δ, although very low levels of *E. coli* δ were necessary for reconstitution, chloroplast δ with $EF_1(-\delta)$ did not work. Therefore, $EF_1(-\delta)$ + *E. coli* δ and $CF_1(-\delta)$ + chloroplast δ were used as a control showing that the restoration of 9-amino-6-chloro-2-

Table 2. *Comparison of photophosphorylation data of spinach thylakoids and EDTA-treated thylakoid vesicles before and after addition of the indicated proteins*

Phenazinemethosulfate-mediated cyclic photophosphorylation is given as rate of ATP synthesis/mass chlorophyll; remaining CF_1 after EDTA treatment of thylakoids is given as a percentage of control, determined by rocket immunoelectrophoresis [28, 32]. The restored photophosphorylation by CF_1, $CF_1(-\delta)$, EF_1, and $EF_1(-\delta)$ is given as the increase in photophosphorylation, the improved restoration of photophosphorylation by addition of subunit δ to $F_1(-\delta)$ is given as the increase in photophosphorylation rates obtained with $F_1(-\delta)$ in the presence of δ as compared to $F_1(-\delta)$ alone. 10 µg chlorophyll was incubated with the following amounts of protein: CF_1, 50 µg; $CF_1(-\delta)$, 30 µg; $CF_1(-\delta)$ + chloroplast δ (Spδ), 30 + 1.5 µg; $CF_1(-\delta)$ + *E. coli* δ (Ecδ), 30 + 5 µg; EF_1, 200 µg; $EF_1(-\delta)$, 30 µg; $EF_1(-\delta)$ + *E. coli* δ, 30 + 1.5 µg; $EF_1(-\delta)$ + chloroplast δ, 30 + 5 µg. Details of the reconstitution procedure are given under Materials and Methods

Photophosphorylation	Sample	Value in experiment					
		1	2	3	4	5	6
		µmol · h^{-1} · (mg chlorophyll)$^{-1}$					
Original	thylakoids	1200	1250	1183	1920	1416	1971
	EDTA vesicles	31	44	44	34	82	69
	(remaining CF_1, %)	(41)	(44)	(44)	(44)	(53)	(nd)
Restored	+ CF_1	339	415	nd	434	946	445
	+ $CF_1(-\delta)$	16	75	59	86	97	111
Improved restoration	+ $CF_1(-\delta)$ + Spδ	96	205	218	226	477	171
	+ $CF_1(-\delta)$ + Ecδ	48	39	60	14	209	0
Restored	+ EF_1	55	80	87	164	385	154
	+ $EF_1(-\delta)$	0	36	48	34	97	72
Improved restoration	+ $EF_1(-\delta)$ + Ecδ	14	38	72	32	30	39
	+ $EF_1(-\delta)$ + Spδ	13	48	58	49	89	65

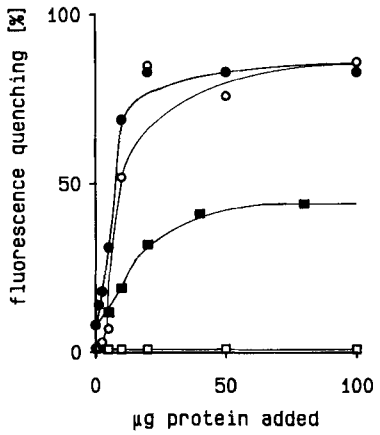

Fig. 5. *Reconstitution of F_1-depleted everted vesicles of* E. coli *with EF_1 (○, ●) or CF_1 (□, ■) measured by 9-amino-6-chloro-2-methoxyacridine fluorescence quenching.* (●, ■) Energization with NADH; (○, □) energization with ATP. For further details cf. Materials and Methods

methoxyacridine fluorescence quenching was similar as compared to the effects of EF_1 and CF_1, respectively. As with thylakoid vesicles, neither *E. coli* δ nor chloroplast δ nor *E. coli* α or ε alone gave any detectable fluorescence quenching and the magnitude of quenching by $EF_1(-\delta)$ was not enlarged by *E. coli* α or ε (data not shown).

Everted vesicles from *E. coli* can be energized either by respiration-driven (NADH) or by ATP-hydrolysis-driven proton translocation, thus allowing for easy discrimination between structural or functional reconstitution. Membrane energization of vesicles reconstituted with $EF_1(-\delta)$ + *E. coli* δ and of $CF_1(-\delta)$ + *E. coli* δ in comparison to EF_1 revealed that both $EF_1(-\delta)$ + *E. coli* δ and EF_1 were functionally active whereas $CF_1(-\delta)$ + *E. coli* δ was only structurally active (data not shown). Thus it was demonstrated (a) that $EF_1(-\delta)$ + *E. coli* δ was equivalent to EF_1 and (b) that subunit δ alone is not sufficient for coupling ATP hydrolysis to proton translocation.

DISCUSSION

Our previous work on chloroplast CF_0CF_1 has demonstrated the ability of purified subunit δ to restore phenazinemethosulfate-mediated cyclic photophosphorylation in partially CF_1-depleted vesicles from spinach thylakoids [31]. This action was due to the fact that δ plugged the protonic leak conductance of a small fraction of 'exposed' CF_0 which were in a high conductance form [8, 27, 33] (see below). This role of δ was detected by three different experimental approaches.

a) When freshly prepared chloroplast δ was added to EDTA-treated spinach thylakoids, it restored photophosphorylation. This ability was rapidly lost upon storage of δ and the loss of activity seemed to involve conformational transitions of isolated δ [31]. Most of the chloroplast δ that was used in this work had been stored at −20° C or −196° C and did not restore photophosphorylation by itself. In gel filtration the protein eluted at an apparent molecular mass of 49 kDa. This may be explained by the highly asymmetric shape of the molecule. It may be regarded also as an indication of dimerization of chloroplast δ and this might prevent rebinding of δ alone to CF_0 (S. Engelbrecht, unpublished observations).

b) When isolated δ was added together with either $CF_1(-\delta)$ or $EF_1(-\delta)$ it improved the restoration of photophosphorylation. This was independent of the age and cold storage of δ [31, 32] (and this article). Thus, it seemed as if the unfavourable condition of stored δ could be overcome by its association with other subunits of F_1.

Table 3. *Resealing of* E. coli F_1*-depleted everted vesicles by addition of the indicated proteins, measured via 9-amino-6-chloro-2-methoxyacridine fluorescence quenching*
Spδ = chloroplast δ, Ecδ = *E. coli* δ. The relatively high quenching by $EF_1(-\delta)$ in experiment 1 probably was due to a contamination by EF_1. The fluorescence quench obtained with rechromatographed $EF_1(-\delta)$ yielded the data given under experiment 2. Energization of the vesicles was achieved with 0.2 mM NADH. nd, not determined. For further details cf. Materials and Methods

Sample	Expt 1		Expt 2	
	mass	quench	mass	quench
	µg	%	µg	%
EF_0 vesicles	100	7	100	11
+ EF_1	10	77	20	80
+ $EF_1(-\delta)$	10	47	20	21
+ $EF_1(-\delta)$ + Ecδ	10 + 0.125	51		nd
	10 + 0.25	68	20 + 0.25	34
	10 + 0.5	80	20 + 0.5	39
	10 + 1	83	20 + 1	72
		nd	20 + 2	85
+ $EF_1(-\delta)$ + Spδ	10 + 2	42		nd
	10 + 20	42	20 + 20	18
+ CF_1	400	49	200	49
+ $CF_1(-\delta)$	400	18	200	15
+ $CF_1(-\delta)$ + Spδ		nd	200 + 1.25	35
	400 + 2.5	32	200 + 2.5	42
	400 + 5	38	200 + 5	50
	400 + 10	45	200 + 10	49
	400 + 20	47		nd
+ $CF_1(-\delta)$ + Ecδ	400 + 0.5	31	200 + 0.5	20
	400 + 1	36	200 + 1	23
	400 + 2	41	200 + 2	33
	400 + 10	50	200 + 4	39
	400 + 20	48		nd

c) Chloroplast δ, if left on CF_0 under certain conditions of EDTA treatment of thylakoids, kept the proton pore through CF_0 closed [26, 29, 33]. This led us to differentiate between 'exposed CF_0', which had lost the CF_1 counterpart through EDTA treatment, but which was mostly not active in proton conduction, and 'open CF_0', which was active [8, 27, 28].

In cross-reconstitution experiments with CF_1-depleted thylakoids both $CF_1(-\delta)$ + *E. coli* δ and $EF_1(-\delta)$ + chloroplast δ restored photophosphorylation to small extent, presumably by plugging protonic leaks through CF_0. $EF_1(-\delta)$ + chloroplast δ did not plug leaks through EF_0 in F_1-depleted everted vesicles from *E. coli*. CF_1 or $CF_1(-\delta)$ + chloroplast δ closed proton leaks through EF_0, although higher amounts were required. If chloroplast δ was substituted by *E. coli* δ the protein amount necessary for observable effects was sharply decreased to about the amount needed with the 'pure' *E. coli* system, i.e. the *E. coli* δ seemed to increase the binding affinity even of $CF_1(-\delta)$ to EF_0. On the other hand $CF_1(-\delta)$ was necessary to 'enable' *E. coli* δ to plug open EF_0. Obviously, subunit δ controls proton flow in both EF_0EF_1 and CF_0CF_1. *E. coli* δ in addition increases the binding affinity of $EF_1(-\delta)$ to EF_0 and likewise of $CF_1(-\delta)$ to EF_0.

The finding that proton pores through EF_0 could be plugged to the extent found in this experiment was surprising in view of recent results that were obtained with CF_0 [8, 27]. In EDTA-treated thylakoids most of the CF_1-depleted CF_0 is converted into a low-conductance form which is sufficient to dissipate the proton motive force built up by methylviologen-mediated linear electron transport. This form constitutes the majority of exposed CF_0. It is sensitive to *N,N'*-dicyclohexylcarbodiimide but cannot be plugged by CF_1. The proton efflux mediated by this 'low-conductance CF_0' can be overcome only by proton pumping at very high rates as mediated through cyclic electron transport with phenazinemethosulfate as cofactor [51]. As the majority of ‚low-conductance CF_0' cannot be plugged by added CF_1, photophosphorylation never can be restored to the rates of the control. Instead, by plugging the minority of CF_0 channels in the high-conductance form, a few rebound CF_1 molecules enable the remaining CF_1 to regain catalytic activity.

Everted vesicles derived from *E. coli* differ from this in one important aspect: the degree of 9-amino-6-chloro-2-methoxyacridine fluorescence quenching is nearly the same in controls and reconstituted everted vesicles. Proton efflux can be inhibited to about the same extent by both EF_1 and *N,N'*-dicyclohexylcarbodiimide. This implies that all exposed EF_0 can react with EF_1, not only a small fraction as with thylakoids.

Conclusive experimental evidence for the presence of δ or (in mitochondria) oligomycin-sensitivity-conferring protein in a connecting stalk is still missing. A comparison of the sequences of spinach and *E. coli* δ reveals that the small number of strictly conserved residues comprises 21 uncharged amino acids, six negatively charged, and four positively charged residues which are located roughly near the N and C termini and the center part of the polypeptide chain. A proton pathway leading through subunit δ up into the $(\alpha\beta)_3\gamma$ complex cannot be definitively excluded as yet. With the relatively low sequence similarity, we find a conformational energy-transducing role of subunit δ more appealing, though.

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