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# Added subunit $\beta$ of $CF_1$ as well as $\gamma/\delta/\epsilon$ restore photophosphorylation in partially $CF_1$-depleted thylakoids

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We investigated the ability of subunits $\beta$, $\gamma$, $\delta$, and $\epsilon$ of $CF_1$, the $F_1$-ATPase of chloroplasts, to interact with exposed $CF_0$ in EDTA-treated, partially $CF_1$-depleted thylakoid membranes. We measured the ability of subunits $\beta$, $\gamma$, $\delta$, and $\epsilon$ to stimulate the rate of photophosphorylation under continuous light and, for subunit $\beta$, also the ability to diminish the proton leakage through exposed $CF_0$ by deceleration of the decay of electrochromic absorption transients under flashing light. The greatest effect was caused by subunit $\beta$, followed by $\gamma/\delta/\epsilon$. Pairwise combinations of $\gamma$, $\delta$, and $\epsilon$ or each of these subunits alone were only marginally effective. Subunit $\gamma$ from the thermophilic bacterium *PS 3* in combination with chloroplast $\delta$ and $\epsilon$ was as effective as chloroplast $\gamma$. The finding that the small $CF_1$ subunits in concert and the $\beta$ subunit by itself specifically interacted with the exposed proton channel $CF_0$, qualifies the previous concept of subunit $\delta$ acting particularly as a plug to the open $CF_0$ channel. The interactions between the channel and the catalytic portion of the enzyme seem to involve most of the small, and at least $\beta$ of the large subunits.

## 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 subunits $\alpha$ (56 kDa), $\beta$ (54 kDa), $\gamma$ (36 kDa), $\delta$ (21 kDa), and $\epsilon$ (15 kDa) with nucleotide binding sites on $\alpha$ and $\beta$ [2,5].

We have shown earlier that purified subunit $\delta$ can reconstitute cyclic photophosphorylation in partially $CF_1$-depleted thylakoids. This was attributed to plugging open $CF_0$ channels and thereby reactivation of residual $CF_0CF_1$ [9–11]. It was disturbing that the reconstitutional activity of subunit $\delta$ was very variable, from active to completely inactive, depending on the preparation and storage of the purified protein [10]. However, all preparations of $\delta$ invariably enhanced the reconstitutional activity of $CF_1$ lacking subunit $\delta$ ($CF_1(-\delta)$) [10]. Obviously, the presence of the other $CF_1$ subunits shifted subunit $\delta$ into a reconstitutionally active state. Evidence for chaperon-like activities of mitochondrial $\alpha$ [12] and chloroplast $\alpha$ [13] has been presented recently.

In order to localize the source of this shift more precisely, subunits $\beta$, $\gamma$, $\delta$, and $\epsilon$ were purified in small amounts from chloroplast coupling factor 1 ($CF_1$) ($\alpha$ was not accessible by these protocols). The purified subunits and combinations thereof were added to EDTA-treated, partially $CF_1$-depleted thylakoids. Rates of phenazine-methosulfate-mediated cyclic photophosphorylation were measured. Two samples yielded significantly accelerated photophosphorylation rates: the $\gamma/\delta/\epsilon$ combination and, much to our surprise, subunit $\beta$.

## Materials and Methods

$CF_1$ was purified from EDTA extracts of spinach thylakoids by anion-exchange chromatography as in Refs. 14,15. Subunit $\delta$ was prepared from purified (ATP-saturated) $CF_1$ by anion-exchange chromatography in the presence of the non-ionic tenside *N*-(D-gluco-2,3,4,5,6-pentahydroxylhexyl)-*N*-methylnonanamide (Mega 9) [9]. Further purification of $\delta$ was achieved either by rechromatography or by hydrophobic interaction chromatography as outlined in Ref. 16.

Subunits $\beta$, $\gamma$, and $\epsilon$ were prepared from purified, dithiothreitol (100 mM) reduced $CF_1(-\delta)$ by anion-ex-

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Abbreviations: $CF_0CF_1$, chloroplast $F_0F_1$-ATPase; $CF_0$, proton channel (membrane-embedded); $CF_1$, ATPase (soluble part).

change chromatography in the absence of nucleotides after washing column-bound $CF_1$ with a mixture of ethanol and glycerol. Subunit $\epsilon$ together with an unidentified 14 kDa protein (probably the small subunit of ribulose bisphosphate carboxylase/oxygenase) eluted in the ethanol/glycerol wash [17]. Subunit $\beta$ eluted at about 180 mM NaCl [9] and was contaminated with subunit $\alpha$. Subunit $\gamma$, along with several impurities consisting mainly of $CF_1$ subunits was obtained after finishing the NaCl gradient elution by washing the column with buffer containing 1 M NaCl and 22 mM Mega 9.

The eluates were further purified as follows: The $\epsilon$/14 kDa mixture dissolved in 20% (v/v) ethanol, 30% (v/v) glycerol, 50 mM Tris-HCl (pH 7.8) was subjected to cation-exchange chromatography ($5 \times 50$ mm column, Fractogel TSK CM-650(S), 25 mM Mes/NaOH $\pm$ 1 M NaCl (pH 6.0) 2 ml/min) after ultrafiltration/dilution in order to remove the organic solvent. This chromatography yielded pure 14 kDa protein and, in another peak, an approximately 50/50 mixture of $\epsilon$ and the 14 kDa protein. Attempts to further purify $\epsilon$ failed. Therefore the approx. 50/50 mixture was used as such.

Fractions containing subunit $\beta$ were pooled, concentrated (which resulted in precipitation of some protein), centrifuged and stored in aliquots in the presence of 1 mM ATP and 25 mM Tris-HCl (pH 7.8) at $-196$°C. This resulted in homogeneous $\beta$ without electrophoretic evidence for remaining $\alpha$. The purity was further corroborated by amino-terminal sequencing.

Fractions containing subunit $\gamma$ were pooled, concentrated and subjected to gel-filtration (column $26 \times 600$ mm, Pharmacia Superdex 200, 25 mM Tris-HCl, 22 mM Mega 9, 1 mM EDTA, 200 mM NaCl (pH 7.8) 0.2 ml/min). A peak with a shoulder eluting at approx. 205 ml contained subunit $\delta$, followed by $\delta$ together with $\gamma$ and finally, in the shoulder, pure $\gamma$.

In another approach, most of the individual spinach $CF_1$ subunits were prepared by repeated gel-filtration in the presence of sodium dodecylsulfate (SDS). 30 mg $CF_1$ ($-\delta$) (10 mg/ml) were gel-filtered via HiLoad 26/60 Superdex 200 (Pharmacia) (column $26 \times 600$ mm, 50 mM Tris-HCl, 0.5% (w/v) SDS (pH 7.8) 0.1 ml/min). The sample did not contain added nucleotides; it was brought to 0.5% (w/v) SDS, but not boiled. Chromatography yielded a major peak at 150–160 ml with two minor peaks preceding (no baseline separation) followed by another small peak at 197 ml. The first peak contained, in this order, high molecular mass impurities, $\alpha(\beta)$, $\alpha\beta$, $\alpha\beta\gamma$, $(\alpha\beta)\gamma$. Brackets indicate substoichiometric amounts of protein as judged from the staining intensity in SDS electrophoresis. The second peak contained a mixture of $\epsilon$ and a 14 kDa protein. Fractions containing subunit $\gamma$ were pooled, concentrated, boiled and rechromatographed. This yielded $\alpha/\beta$ (135 ml) followed by electrophoretically pure $\beta$ (140 ml) and $\gamma$ (149 ml). Rechromatography of the pooled, concentrated $\epsilon$/14 kDa peak resolved the two proteins in two peaks ($\epsilon$: 183 ml, 14 kDa; 193 ml) containing electrophoretically pure peptides.

These samples were concentrated and stored frozen at $-196$°C. Before reconstitution the proteins were renatured by the following protocol. 200 $\mu$l aliquots (containing approx. 50 $\mu$g protein) were diluted with a slight excess of KCl over SDS, which resulted in precipitation of most of the SDS. After removal of the precipitate by centrifugation, urea was added to give a final concentration of 8 M. After a 15 min incubation at room temperature, samples were diluted 10-fold, gel-filtered via NAP-5 columns (Pharmacia, 25 mM Tris-HCl (pH 7.8) and immediately reconstituted.

Preparations of EDTA vesicles and reconstitutions were carried out as before [16], incubation of EDTA-treated thylakoid membranes (10 $\mu$g chlorophyll) was prolonged to 1 h on ice. 10 $\mu$M $P^1,P^5$-di(adenosine-5′)pentaphosphate was included as (competitive) inhibitor of adenylate kinase activity. Photophosphorylation was initiated by illumination with saturating white light (1 min, room temperature) in the presence of 50 $\mu$M phenazine methosulfate [15]. Quenching and determination of ATP by the LKB/Pharmacia (now Colora) luciferin/luciferase assay were as described [15]. Prior to reconstitution, samples were checked for endogenous ATP. If necessary, ATP was removed by gel-filtration/ultrafiltration.

SDS electrophoresis was carried out with commercially available gels in the Pharmacia PhastSystem [18] according to instructions of the manufacturer. Staining was with silver nitrate/tungstosilicic acid ($H_4[Si(W_3O_{10})_4]_{aq}$)/formaldehyde as described [19,20]. An additional 'conditioning' step was performed after fixation/washing of the gel in order to further increase the sensitivity: incubation for 8 min at room temperature in 0.4 M sodium acetate, 30% (v/v) methanol, 0.1% (w/v) sodium thiosulfate (pH 6.0) [21]. Electrochromic absorption changes were measured as described [22]. Amino-terminal sequencing was carried out with an Applied Biosystems 473A protein sequencer (standard protocol) in the University of Osnabrück protein sequencing facility (UNOPS). Subunit $\gamma$ prepared from the thermophilic bacterium *PS 3* ($TF_1$-$\gamma$) was a gift kindly donated by Dr. Y. Kagawa, Japan. All chemicals were of the highest grade available. Ultrafiltration membranes (YM 10) were from Amicon, Mega 9 from Oxyl Chemie.

## Results

Fig. 1 shows a silver-stained SDS gel with the purified $CF_1$ subunits, $TF_1$-$\gamma$ and $CF_1$ as molecular stan-

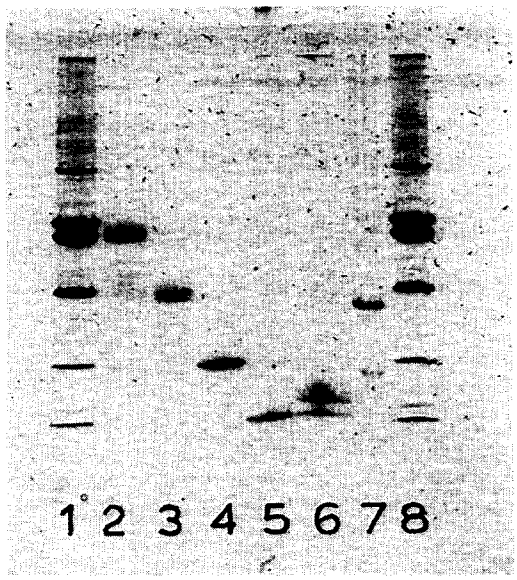


Fig. 1. SDS-electrophoresis of $CF_1$ (lanes 1 and 8, 180 ng each), $CF_1$-$\beta$ (lane 2, 50 ng), $CF_1$-$\gamma$ (lane 3, 30 ng), $CF_1$-$\delta$ (lane 4, 30 ng), 14 kDa protein (lane 5, 20 ng), $CF_1$-$\epsilon$/14 kDa protein (lane 6, 30 ng), $TF_1$-$\gamma$ (lane 7, 30 ng). The samples were separated in an 8–25% PhastGel (Pharmacia) and stained with silver nitrate in the presence of tungstosilicic acid.

dard. It is evident, that subunits $\beta$ (lane 2), $\delta$ (lane 4), 14 kDa protein (lane 5), and $TF_1$-$\gamma$ (lane 7) were homogeneous. $CF_1$ (lanes 1 and 8) contained a high-molecular-mass impurity at about 100 kDa, the smallest peptide was not $\epsilon$ (which stains poorly with silver), but a 14 kDa protein, cf. lane 5. This peptide probably represented the small ribulose bisphosphate carboxylase/oxygenase subunit. Repeated attempts to sequence this protein failed, despite considerable amounts subjected to Edman degradation. The small ribulose bisphosphate carboxylase/oxygenase subunit is known to be blocked at the amino terminus [23]. Subunit $\gamma$ showed a double band; this has been observed before and probably reflects formation/breakage of disulfide bridges. Subunit $\epsilon$ was contaminated by a 14 kDa protein (cf. Materials and Methods). As the pure 14 kDa protein was reconstitutionally inactive (data not shown), the mixture of $\epsilon$/14 kDa was used as such for the reconstitutions. All samples were derived in low amounts from non-denaturing chromatography. Subunits prepared by denaturing chromatography in SDS electrophoresis were indistinguishable from the samples shown, except that $\gamma$ ran as a single band and $\epsilon$ was free from the 14 kDa contamination.

The identity of subunits $\beta$, $\gamma$, and $\epsilon$ was corroborated by amino-terminal sequencing. This gave the following sequences (literature data in brackets):

spinach $CF_1$-$\beta$: R I N P T X S D P X V (MRINPTTSDPGV...[24])
spinach $CF_1$-$\gamma$: A N L R E L R D R I (ANLRELRDRI...[25])
spinach $CF_1$-$\delta$: V D S T A S X Y A S A L A [10] (VDSTASRYASALA...[26])
spinach $CF_1$-$\epsilon$: X L N L X V L X P (MTLNLCVLTP...[24])

Table 1 shows the results of two experiments where mainly subunit $\beta$ was reconstituted. Clearly, $\beta$ reconstituted photophosphorylation in a concentration-dependent manner, although not to the extent of $CF_1$, which represents about 60% of the protein in the extract (the freshly prepared extract serves as a convenient control of the maximal achievable reconstitution [16,27]). Reconstitution of $\beta$ together with $\delta$ in substantial amount enhanced the photophosphorylation rate slightly above noise level, even more enhancement was obtained in the presence of $\gamma$, i.e., with subunits $\beta$, $\gamma$, and $\delta$ (as compared to the effect of comparable amounts of $\beta$ alone). Gel-filtration experiments indicated no interaction in solution between $\beta$ and $\delta$ (data not shown) or between $\gamma$ and $\delta$ (cf. Materials and Methods). Experiments 1 and 2 were conducted on two consecutive days with the same batch of market spinach but freshly prepared thylakoids and vesicles. Note the different activities of the EDTA vesicles, indicating different degrees of depletion (despite identical protocols), and the different effectivity of the extract, resulting in Expt. 2 in higher activity of the extract-reconstituted vesicles as compared to the thylakoid control. $\beta$ also enhanced the photophosphorylation rate in NaBr-treated thylakoids (data not shown).

The reconstitutional activity of subunit $\beta$ was also evident from measurements of the electrochromic absorption changes (Fig. 2). In these experiments, a total of 40 $\mu$g chlorophyll were used with either 40 $\mu$g protein from the extract, or 100 $\mu$g $\beta$. At this excess,

TABLE I

*Phenazine-methosulfate-mediated photophosphorylation in spinach thylakoids and EDTA vesicles in the absence and presence of the indicated amounts of sample*

10 $\mu$g chlorophyll (Chl) were used per experiment. ATP = $\mu$mol ATP synthesized per h per mg Chl. Extract = supernatant obtained after EDTA treatment and centrifugation of thylakoids. All subunits were isolated under nondenaturing conditions from spinach $CF_1$.

| | Expt. 1 | | Expt. 2 | |
|---|---|---|---|---|
| | amount ($\mu$g) | ATP | amount ($\mu$g) | ATP |
| Thylakoids | | 1641 | | 1740 |
| EDTA vesicles | | 278 | | 1251 |
| + extract | 26 | 711 | 23 | 1938 |
| + $\beta$ | 1 | 284 | 1 | 1228 |
| | 5 | 303 | 5 | 1520 |
| | 10 | 356 | 10 | 1483 |
| | 20 | 496 | 20 | 1510 |
| | | | 50 | 1666 |
| + $\beta$ + $\delta$ | 10 + 1 | 356 | 20 + 1 | 1428 |
| | 10 + 2 | 390 | 20 + 2 | 1472 |
| | | | 20 + 4 | 1563 |
| + $\beta$ + $\gamma/\delta$ | | | 20 + 3 | 1642 |

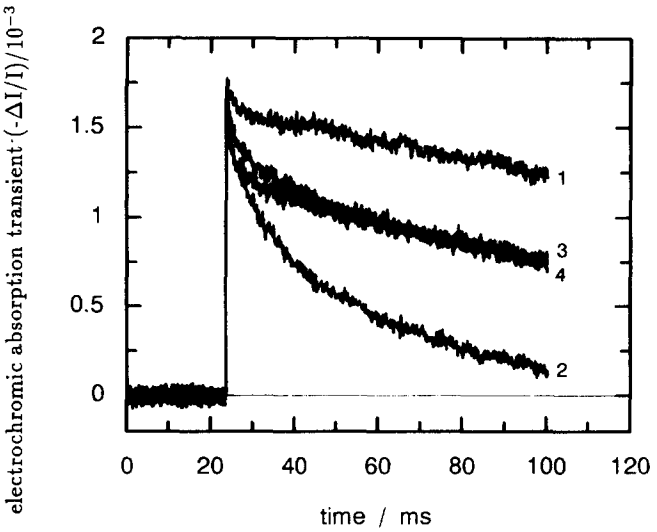


Fig. 2. Flash-induced voltage transients in thylakoids (trace 1), EDTA vesicles (trace 2) and EDTA vesicles reconstituted with 40 $\mu$g protein of the EDTA extract (trace 3) or reconstituted with 100 $\mu$g subunit $\beta$ from $CF_1$ (trace 4). 40 $\mu$g chlorophyll were used per experiment. Electrochromic absorption changes were monitored at 522 nm as described [22].

added $\beta$ relieved the accelerated decay of the transmembrane voltage almost as effectively as added $CF_1$.

Table II shows the results of reconstitution experiments with the three small subunits of $CF_1$ and $TF_1$-$\gamma$. Whereas the single small subunits and combinations of two subunits were either not or only slightly active, the $\gamma/\delta/\epsilon$ sample clearly improved the phosphorylation rate, irrespective of the source of $\gamma$, $CF_1$ or $TF_1$. Note that in this experiment, the $\gamma/\delta/\epsilon$ samples reconstituted the EDTA vesicles to a higher degree than the extract. The samples were mixed and briefly incubated before addition of EDTA-treated membranes. It is not known, whether the three small subunits interacted in solution.

Table III shows results with $\gamma/\delta/\epsilon$ and a $\alpha/\beta/\gamma$ sample which was obtained in anion-exchange chromatography (instead of $(\alpha)\beta$, cf. Materials and Methods) after washing column-bound ATP-saturated (1 mM) $CF_1(-\delta)$ with ethanol/glycerol. The staining intensity in SDS gel-electrophoresis of the $\alpha/\beta/\gamma$ sample indicated equimolar amounts of all three subunits (this is no proof of an $\alpha\beta\gamma$ assembly in solution, though). Again, each small subunit alone exerted no

TABLE II

*Phenazine-methosulfate-mediated photophosphorylation in spinach thylakoids and EDTA vesicles in the absence and presence of the indicated amounts of sample*

10 $\mu$g chlorophyll (Chl) were used per experiment. ATP = $\mu$mol ATP synthesized per h per mg Chl. Extract = supernatant obtained after EDTA treatment and centrifugation of thylakoids. Subunits, if not indicated, were isolated under nondenaturing conditions from spinach $CF_1$. The sample named $\epsilon$ consisted of approx. equal amounts of $\epsilon$ and a 14 kDa protein. Samples given as, e.g., $\gamma/\delta/\epsilon$, indicate that a mixture of the proteins at the indicated amounts, after a brief preincubation without EDTA vesicles, was incubated for 1 h on ice in the presence of membranes.

| | Amount ($\mu$g) | ATP |
|---|---|---|
| Thylakoids | | 1524 |
| EDTA vesicles | | 662 |
| + extract | 20 | 809 |
| + $\gamma$ | 2 | 728 |
| + $TF_1$-$\gamma$ | 2 | 655 |
| + $\delta$ | 2 | 670 |
| + $\epsilon$ | 1 | 684 |
| + $\gamma/\delta$ | 2+2 | 736 |
| + $TF_1$-$\gamma/\delta$ | 2+2 | 736 |
| + $\gamma/\epsilon$ | 2+1 | 721 |
| + $TF_1$-$\gamma/\epsilon$ | 2+1 | 666 |
| + $\delta/\epsilon$ | 2+1 | 719 |
| + $\gamma/\delta+\epsilon$ | 2+2+1 | 861 |
| + $TF_1$-$\gamma/\delta/\epsilon$ | 2+2+1 | 861 |

TABLE III

*Phenazine-methosulfate-mediated photophosphorylation in spinach thylakoids and EDTA vesicles in the absence and presence of the indicated amounts of sample*

All subunits were isolated under nondenaturing conditions from spinach $CF_1$. Other conditions and explanations as for Table II.

| | Amount ($\mu$g) | ATP |
|---|---|---|
| Thylakoids | | 908 |
| EDTA vesicles | | 261 |
| + extract | 48 | 789 |
| + $\gamma$ | 5 | 273 |
| + $\delta$ | 2 | 274 |
| + $\epsilon$ | 1 | 268 |
| + $\gamma/\delta/\epsilon$ | 5+2+1 | 402 |
| + $\alpha/\beta/\gamma$ | 20 | 474 |
| + $\alpha/\beta/\gamma+\delta$ | 20+2 | 702 |

TABLE IV

*Phenazine-methosulfate-mediated photophosphorylation in spinach thylakoids and EDTA vesicles in the absence and presence of the indicated amounts of sample*

All subunits were isolated under denaturing conditions from spinach $CF_1$ and renatured as described under Materials and Methods just before reconstitution. Other conditions and explanations as for Table II.

| | Amount ($\mu$g) | ATP |
|---|---|---|
| Thylakoids | | 640 |
| EDTA vesicles | | 344 |
| + extract | 47 | 589 |
| + $\beta$ | 1 | 341 |
| + $\gamma$ | 0.5 | 350 |
| + $\delta$ | 1 | 369 |
| + $\epsilon$ | 0.5 | 350 |
| + $\gamma/\delta$ | 0.5+1 | 383 |
| + $\gamma/\epsilon$ | 0.5+0.5 | 332 |
| + $\delta/\epsilon$ | 1+0.5 | 365 |
| + $\gamma/\delta/\epsilon$ | 0.5+1+0.5 | 393 |
| + $\beta/\gamma/\delta/\epsilon$ | 1+0.5+1+0.5 | 430 |

effect, in contrast to the $\gamma/\delta/\epsilon$ combination. However, the $\alpha/\beta/\gamma$ sample was even more effective and in the presence of $\delta$ it rivalled the reconstitutional activity of the control (EDTA extract containing about 25 $\mu$g $CF_1$).

Table IV shows results obtained with renatured subunits $\beta$, $\gamma$, and $\epsilon$ originally prepared under denaturing conditions. The amounts of subunits used in this experiment were smaller than in the other experiments because renaturation required greater dilution. Still, the observed changes in photophosphorylation rates, although small, corroborated earlier data sets, especially for the sample containing all four purified $CF_1$ subunits.

## Discussion

$CF_1$-depleted thylakoids show diminished rates of ATP synthesis under continuous light. Structural reconstitution of higher rates can be achieved by adding subunits $\beta$ and $\gamma\delta\epsilon$ (this work) or $\delta$ [9–11] to EDTA-treated thylakoid membranes. Structural reconstitution of ATP synthesis is an indirect effect due to the deceleration or even inhibition of proton flow through open $CF_0$ proton channels. The reduction of the protonic leak conductance allows the generation of a protonmotive force which can drive ATP synthesis by those $CF_0CF_1$ molecules which have remained intact on the membrane despite EDTA treatment. Incremental increases in photophosphorylation rates with increased numbers of different subunits and the failure of certain combinations/preparations of subunits to stimulate photophosphorylation rates would seem to suggest that the observed effects were not due to 'catching' of the added, purified subunits by '$CF_1$-torsos', which had remained on the membrane. In addition, previous reconstitution work has indicated, that EDTA-treatment leaves only five-subunit-$CF_1$ or subunit $\delta$ on $CF_0$ [11,15,28]. Therefore we consider it unlikely that the observed effects were due to complementation and 'repair' of remaining incomplete $CF_1$.

How do these data relate to earlier observations? We have reported that subunit $\delta$ from $CF_1$, under certain conditions, can plug the open proton channel $CF_0$ [10,11,28]. In view of the ability of $\delta$ alone to reconstitute photophosphorylation, the finding that $\gamma/\delta/\epsilon$ were reconstitutionally active was not surprising. In the present experiments, however, a (stored) preparation of purified $\delta$ was used that, when added without $\gamma$ and $\epsilon$, was unable to reconstitute photophosphorylation (cf. Ref. 10). The synergistic effect of $\gamma$ and $\epsilon$ on $\delta$ might indicate an 'educative' effect of the former on $\delta$, as previously observed for the combination of $CF_1(-\delta)$ and $\delta$ (cf. Introduction).

The ability of the $\gamma/\delta/\epsilon$ mixture to reconstitute phosphorylation has been previously reported for thermophilic bacterium *PS 3* [29,30]. We found, that $TF_1$-$\gamma$ together with chloroplast $\delta$ and $\epsilon$ reconstituted photophosphorylation in EDTA vesicles from chloroplasts just as efficiently as chloroplast $\gamma$. This is another example of successful hybrid reconstitution studies that have demonstrated that the secondary and tertiary structure of the smaller subunits of F-type ATPases from a wide range of organisms is more important than their primary structure. *E. coli* $\delta$ could replace chloroplast $\delta$ in reconstitution of photophosphorylation [31], $CF_0$-I could replace $EF_0$-*b*, allowing for growth of the respective mutant strains of *E. coli* similar to the wild type [32]. There are also examples where such replacements do not work, however: the mitochondrial counterpart of chloroplast $\delta$ cannot substitute for chloroplast $\delta$ [28] despite similar secondary structure [33]; chloroplast $\beta$ is unable to functionally replace *E. coli* $\beta$ [34] despite a highly conserved amino-acid sequence [24] (but see below). It remains to be seen to what extent $TF_1$-$\gamma$ can functionally replace chloroplast $\gamma$.

What came as a surprise, then, was the reconstitutive activity of subunit $\beta$. Is this activity relevant? To date, only subunit $\alpha$ was thought to interact directly with $F_0$ subunits [35], although $\beta$ also could be crosslinked to subunit *b* in *E. coli* $F_0$ [36]. The catalytic sites probably reside on the $\beta$ subunits (reviewed in Ref. 2). There is circumstantial evidence that the release of spontaneously formed and bound ATP is the free-energy-requiring step [3]. It has been amply discussed that this could be brought about by long range conformational changes between $F_0$ and $F_1$ [3,37]. This would seem to require at least two different contact regions between $CF_0$ and $CF_1$, one of the bearing part and one of the moving shaft. From this work $\beta$ and $\gamma/\delta/\epsilon$ possibly via $\delta$ seem to directly contact $CF_0$ and both groups likewise can reduce proton conduction. In view of the sequence similarity between $\alpha$ and $\beta$ [2], it is possible that the observed effect of subunit $\beta$ reflects this similarity and that, in intact coupling factors, subunit $\alpha$ plays this role. The contact sites have still to be tracked down.

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## References

1 Schneider, E. and Altendorf, K. (1987) Microbiol Rev. 51, 477–497.

2 Senior, A.E. (1988) Physiol. Rev. 68, 177–231.
3 Boyer, P.D. (1989) FASEB J. 3, 2164–2178.
4 Futai, M., Noumi, T. and Maeda, M. (1989) Annu. Rev. Biochem. 58, 111–136.
5 Junge, W. (1989) Ann. NY Acad. Sci. 574, 268–286.
6 Senior, A.E. (1990) Annu. Rev. Biophys. Biophys. Chem. 19, 7–41.
7 Dimroth, P. (1987) Microbiol. Rev. 51, 320–340.
8 Dimroth, P. (1990) Phil. Trans. R. Soc. London [B] 326, 465–477.
9 Engelbrecht, S. and Junge, W. (1987) FEBS Lett. 219, 321–325.
10 Engelbrecht, S. and Junge, W. (1988) Eur. J. Biochem. 172, 213–218.
11 Lill, H., Engelbrecht, S. and Junge, W. (1988) J. Biol. Chem. 263, 14518–14522.
12 Luis, A.M., Alconada, A. and Cuezva, J.M. (1990) J. Biol. Chem. 265, 7713–7716.
13 Avni, A., Avital, S. and Gromet-Elhanan, Z. (1991) J. Biol. Chem. 266, 7317–7320.
14 Apley, E., Wagner, R. and Engelbrecht, S. (1985) Anal. Biochem. 150, 145–154.
15 Engelbrecht, S., Lill, H. and Junge, W. (1986) Eur. J. Biochem. 160, 635–643.
16 Engelbrecht, S., Althoff, G. and Junge, W. (1990) Eur. J. Biochem. 189, 193–197.
17 Richter, M.L., Patrie, W.J. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7371–7373.
18 Olsson, I., Axiö-Fredriksson, U.B., Degerman, M. and Olsson, B. (1988) Electrophoresis 9, 16–22.
19 Krause, I. and Elbertzhagen, H. (1987) in Elektrophoreseforum 1987 (Radola, B.J., ed.), pp. 382–384, TU München.
20 Willoughby, E.W. and Lambert, A. (1983) Anal. Biochem. 130, 353–358.
21 Heukeshoven, J. and Dernick, R. (1988) Electrophoresis 9, 28–32.
22 Lill, H., Althoff, G. and Junge, W. (1987) J. Membrane Biol. 98, 69–78.
23 Martin, P.G. (1979) Aust. J. Plant Physiol. 6, 401–408.
24 Zurawski, C., Bottomley, W. and Whitfeld, P.R. (1982) Proc. Natl. Acad. Sci. USA 79, 6260–6264.
25 Miki, J., Maeda, M., Mukohata, Y. and Futai, M. (1988) FEBS Lett. 232, 221–226.
26 Hermans, J., Rother, C., Bichler, J., Steppuhn, J. and Herrmann, R.G. (1988) Plant Mol. Biol. 10, 323–330.
27 Shoshan, V. and Shavit, N. (1973) Eur. J. Biochem. 37, 355–360.
28 Engelbrecht, S. and Junge, W. (1990) Biochim. Biophys. Acta 1015, 379–390.
29 Yoshida, M., Okamoto, H., Sone, N., Hirata, H. and Kagawa, Y. (1977) Proc. Natl. Acad. Sci. USA 74, 936–940.
30 Kagawa, Y. (1978) Biochim. Biophys. Acta 505, 45–93.
31 Engelbrecht, S., Deckers-Hebestreit, G., Altendorf, K. and Junge, W. (1989) Eur. J. Biochem. 181, 485–491.
32 Schmidt, G., Rodgers, A.J.W., Howitt, S.M., Munn, A.L., Hudson, G.S., Holten, T.A., Whitfeld, P.R., Bottomley, W., Gibson, F. and Cox, G.B. (1990) Biochim. Biophys. Acta 1015, 195–199.
33 Engelbrecht, S., Reed, J., Penin, F., Gautheron, D.C. and Junge, W. (1991) Z. Naturforsch. 46c, 759–764.
34 Munn, A.L., Whitfeld, P.R., Bottomley, W., Hudson, G.S., Jans, D.A., Gibson, F. and Cox, G.B. (1991) Biochim. Biophys. Acta 1060, 82–88.
35 Gao, Z. and Bäuerlein, E. (1987) FEBS Lett. 223, 366–370.
36 Aris, J.P. and Simoni, R.D. (1983) J. Biol. Chem. 258, 14599–14609.
37 Penefsky, H.S. (1987) Biochem. Soc. Trans. 15, 97–99.