Purified subunit δ of chloroplast coupling factor CF₁ reconstitutes photophosphorylation in partially CF₁-depleted membranes

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The ATP synthase of chloroplasts consists of the proton channel, CF₀, and the catalytic part, CF₁, which carries nucleotide-binding sites on subunits α and β. The still poorly understood interaction between CF₀ and the catalytic sites on CF₁ is mediated by the smaller subunits γ, δ, and ε of CF₁. We investigated the ability of purified δ to block proton leakage through CF₀ channels after their exposure by removal of the CF₁ counterpart.

Thylakoids were partially depleted of CF₁ by EDTA treatment. This increased their proton permeability and thereby reduced the rate of photophosphorylation. Subunit δ was isolated and purified by FPLC [Engelbrecht, S. and Junge, W. (1987) FEBS Lett. 219, 321–325]. Addition of δ to EDTA-treated thylakoids reconstituted high rates of photophosphorylation. Since δ does not interact with nucleotides by itself, the reconstitution was due to a reduction of the proton leakage through open CF₀ channels. The molar ratio of purified δ over exposed CF₀, which started to elicit this effect, was 3:1. However, if δ was added together with purified CF₁ lacking δ, in a 1:1 molar ratio, the relative amount over exposed CF₀ was as low as 0.06. This corroborated our previous conclusion [Lill, H., Engelbrecht, S., Schönknecht, G. and Junge, W. (1986) Eur. J. Biochem. 160, 627–634] that only a very small fraction of exposed CF₀ was actually proton-conducting but with a very high unit conductance. CF₁ including δ was apparently reconstituted preferentially to open CF₀ channels.

Although the ability of δ to control proton conduction through CF₀ was evident, it remains to be established whether δ acts as a gated proton valve or as a conformational transducer in the integral CF₀CF₁ ATPase.

The ATP synthase in thylakoid membranes (CF₀CF₁) couples proton flux to ATP synthesis. It belongs to a class of ATP synthases which also occur in the inner mitochondrial membrane (MF₀MF₁) and in micro-organisms, e.g. Escherichia coli (EF₀EF₁) and thermophilic bacteria (TF₀TF₁). For recent reviews see [1–5]. The chloroplast ATP synthase consists of nine different subunits [6, 7]. Four of them probably form the membrane-embedded proton channel CF₀. Their relative stoichiometry and their quaternary structure are still under debate. The other five subunits form the extrinsic catalytic part, CF₁, comprising α (56–58 kDa), β (53–56 kDa), γ (37–39 kDa), δ (19–21 kDa), and ε (15 kDa). Their stoichiometry is αβγδε. Electron-microscopic investigations of isolated F₁ showed an alternating arrangement of the α and β subunits in a nearly hexagonal conformation (‘cyclohexane chair’) [8, 9]. A center mass, probably composed of subunits γ, δ, and ε is slightly shifted out of the plane. The γ subunit seems to be closer to the membrane and α more distant [8, 10].

The α and β subunits and/or their interfaces contain nucleotide-binding sites [2, 11], ADP–ATP conversion occurs on the β subunit [12]. γ seems to organize the (αβ)₃ complex. Without this subunit neither the αβ hexagon nor ATPase activity is observed [13]. If two cysteine residues within the γ subunit are oxidized, the catalytic activity of CF₁ is decreased. The mitochondrial and bacterial enzymes, on the other hand, are not redox-regulated in this way [14–16]. Additional functions of subunit γ within the proton pathway (e.g. a proton gate) are possible but not yet proven [17]. Subunit ε seems to interact with γ [10] but its role is under debate. While subunit ε seemed to be required for reconstitution of photophosphorylation in NaBr-treated thylakoids [18], in EDTA-treated thylakoids added CF₁(−ε), i.e. CF₁ lacking the ε subunit, reconstituted photophosphorylation as effectively as CF₁ [19].

It has been proposed that subunit δ is essential for the binding of F₁ to F₀, both in chloroplasts and E. coli [20–22]. It has also been proposed that it mediates proton flow through the integral F₀F₁ [17, 23–25], while acting as a stopcock to the open proton channel CF₀ [19, 26–28]. The latter proposal was based on reconstitution experiments using CF₁ fragments with different contents of subunit δ. The ability of δ-containing fragments to reconstitute photophosphorylation, however, did not prove that δ alone can stop or reduce proton conduction through CF₀. This prompted us to investigate the potential of the isolated δ subunit [29] to reconstitute photophosphorylation.
MATERIALS AND METHODS

CF1, CF1(-δ) [19, 30, 31] and subunit δ [29] were isolated and purified as previously described. Partially CF1-depleted thylakoids were prepared as follows. Spinach thylakoids were obtained by standard procedure [32], except for the omission of Mg2+ in the final suspending medium. Thylakoids were suspended at 0.4 mg chlorophyll/ml in a medium with 1 mM EDTA, 1 mM Tricine/NaOH, pH 7.8 and incubated for 20 min on ice in the dark. The suspension was centrifuged for 15 min at 20000 x g. The supernatant (‘extract’), kept and the pellet (‘EDTA-vesicles’) was resuspended in 0.4 M sorbitol, 10 mM Tricine/NaOH, pH 7.8. This was repeated once with smaller volume. The EDTA-vesicle preparation followed the protocol by Shoshan and Shavit [33]. Our former procedure [19] was abandoned because the reconstitutive activity of ‘Shavit vesicles’ was higher. Rocket immunoelectrophoresis revealed an extraction degree of 10–20% of total CF1 and contents of about 0.1 mg CF1/ml extract.

Photophosphorylation in EDTA-vesicles was reduced to about 50% of the rate in control thylakoids. Higher rates were reconstituted by addition of CF1, CF1(-δ) or by δ alone. This was carried out in 25 mM Tris/HCl, pH 7.8 and with 0–50 μg of the respective protein in a total volume of 300 μl. The mixture was cooled on ice, then EDTA-vesicles equivalent to 10 μg chlorophyll were added. After 2 min of incubation 8 mM MgCl2 was added and the incubation was continued for about 10 min on ice and in the dark. For coreconstitution of CF1(-δ) and δ these proteins were first preincubated for 2–10 min before the addition of EDTA-vesicles. After further incubation for 10 min the reagents for photophosphorylation were added and the mixture was illuminated for 1 min. Photophosphorylation was stopped with trichloroacetic acid and the ATP yield was measured by the luciferin/luciferase assay [19]. ATPase assay, protein determinations, SDS gel electrophoresis and silver staining were as described elsewhere [19].

The FPLC set-up was from Pharmacia; Fractogel TSK DEAE 650 (S) from Merck was used for purification of CF1, CF1(-δ) and δ; ultrafiltration membranes (YM 10), for concentration and dialysis of δ, were from Amicon; the ATP assay kit was from LKB and all other chemicals were purchased either from Merck or from Sigma. N-(3-glucosyloxy-2,3,4,5,6-pentahydroxylhexyl)-N-methylammonium (Mega 9) came from Oxyl GmbH (Bobingen, FRG).

The relative stoichiometries of δ, CF1 and CF0 were calculated based on masses of 410 kDa for CF1, 21 kDa for subunit δ and on a ratio of 0.4 mg total CF1/mg chlorophyll [2].

RESULTS

The rate of photophosphorylation in spinach thylakoids and in EDTA-treated thylakoids before and after addition of CF1, subunit δ and the EDTA-extract is given in Table 1. After removal of 10–20% of total CF1 from the membrane (determined via rocket immunoelectrophoresis) the rate of photophosphorylation dropped to about 50% of the control. It recovered upon addition of isolated CF1, the EDTA extract and also by addition of subunit δ. Reconstitution of photophosphorylation was observed only in the presence of phenazine methosulfate as mediator of cyclic electron transport, but not with methyl viologen as acceptor of the linear electron transport chain. As isolated δ did not show any ATPase activity and as it is accepted that it does not interact with nucleotides, its reconstitutinal activity had to be ascribed to its function as a stopcock to open CF0 channels. Therefore, in the following the term ‘reconstitution’ was used to indicate that the addition of δ partially restored the lost phosphorylation activity in EDTA-vesicles, but without implying that δ restored the catalytic activity of one particular CF1 molecule (see Discussion).

In Table 1 comparatively high amounts of δ (i.e. about 1220 mol δ/mol exposed CF0) were used for reconstitution. It was worth looking closely at whether or not a contamination by CF1 in the δ preparation was responsible for the effect. We reconstituted photophosphorylation by adding δ, CF1, and CF1(-δ) separately. Fig. 1 shows the results. As before, δ alone reconstituted photophosphorylation (solid diamonds), CF1 alone (open circles) and CF1(-δ) alone (open squares) also were effective, although to a lower extent. If contamination of the δ preparation by CF1 was responsible for the reconstitutive activity of δ, this contamination had to be in the order of some micrograms of CF1. Inspection of the silver-stained SDS gels of the δ preparations (Fig. 4) showed that this clearly was not the case.

The experiment documented in Fig. 1 did not exclude, however, a synergistic action of subunit δ with very small amounts of CF1(-δ). Fig. 2 shows the rate of photophosphorylation when isolated δ (solid diamonds), CF1(-δ) (open squares) and both together (solid squares) were added to EDTA-vesicles. With one exception (see below) δ and CF1(-δ) were added in a 1:1 molar ratio. In this particular experiment, and in contrast to Fig. 1, the efficiency of CF1(-δ) was higher than that of δ. But it was striking that the most pronounced effect was obtained after addition of both, δ and CF1(-δ). The effect was higher (solid squares) if δ and CF1(-δ) were preincubated briefly before the addition of EDTA-vesicles than if membranes were incubated with one component before addition of the other one (crosses at 10 μg protein in Fig. 2). The photophosphorylation rate of the starting material, EDTA-vesicles without added protein, was always the same (see the asterisk). The arrow at 50 μg protein shows the effect of the addition of 3 mol δ/mol CF1(-δ) (all other data points of the coreconstitution were obtained

<table>
<thead>
<tr>
<th>Sample</th>
<th>ATP synthesis</th>
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<tbody>
<tr>
<td></td>
<td>PMS</td>
</tr>
<tr>
<td>Thylakoids</td>
<td>1399</td>
</tr>
<tr>
<td>EDTA vesicles</td>
<td>680</td>
</tr>
<tr>
<td>EDTA vesicles after addition of</td>
<td></td>
</tr>
<tr>
<td>30 μg CF1</td>
<td>872</td>
</tr>
<tr>
<td>30 μg CF1 (contained within EDTA extract)</td>
<td>868</td>
</tr>
<tr>
<td>50 μg subunit δ</td>
<td>980</td>
</tr>
</tbody>
</table>

Table 1. Reconstitution of photophosphorylation in EDTA-treated spinach thylakoids by addition of isolated CF1, the EDTA extract and purified subunit δ.

Details of the experimental procedure are described in Materials and Methods. Phenazine methosulfate (PMS) and methylviologen (MV) served as mediators of cyclic and linear electron transport. Chl, chlorophyll.
with δ and CF1(-δ) in a 1:1 molar ratio. An excess of δ over CF1(-δ) hardly further enhanced photophosphorylation. Fig. 2 demonstrates that the effectivity of subunit δ in reconstitution of photophosphorylation was greatly enhanced in the presence of CF1(-δ). The addition of only 2.5 ng δ together with 50 ng CF1(-δ) already yielded a sizable effect. The latter implied addition of 0.06 mol 1:1 complex of δ and CF1(-δ) to EDTA-vesicles which contained 1 mol exposed CFo. This suggested that the majority of exposed CFo was inactive and that only a small fraction was actually conducting protons (but see Discussion).

We titrated the stimulation of the reconstitutive activity of CF1 and CF1(-δ) by adding purified subunit δ. The results are shown in Fig. 3. 5 μg CF1 (solid circles) or CF1(-δ) (solid squares) were preincubated with the indicated amount of subunit δ. The effect of both CF1 varieties was higher with added δ than without. It was interesting to note that even CF1 could be further 'pushed' by added δ. At 0.3 μg δ/5 μg CF1 or CF1(-δ) this effect was saturated and the curves merged.

If isolated subunit δ alone was added to EDTA-vesicles it reacted as follows. (a) It was most effective when freshly prepared. (b) If used after storage for one day at -20°C it no longer reconstituted photophosphorylation by itself but kept its reconstitutioactivity in a complex with CF1(-δ). (c) Spinach δ did not reconstitute photophosphorylation in pea EDTA-vesicles although it was partially effective in spinach EDTA-vesicles when added together with pea CF1(-δ). (d) When stored at 4°C and in the presence of 20 mM MEGA 9, purified δ was stable for several days. However, after removal of the tenside it started to break down from the C terminus. 

An example of (b) is shown in Table 2. The data were obtained with an aliquot from the very same sample of δ as in Fig. 1; however, it was kept frozen for some days in between. Note that the order of addition of δ and CF1(-δ) was important: high rates of photophosphorylation were only achieved after preincubation of δ and CF1(-δ) before addition of the CF1-depleted thylakoid membranes.

Fig. 4 shows silver-stained SDS gels of δ preparations along with various preparations of CF1 (lanes 1, 7 and 8) and CF1(-δ) (lane 2). The purest δ preparation in the third lane was used for the experiments in Fig. 2, the δ preparation in the fourth lane was used for the experiments in Table 1, Table 2 and Fig. 3. Lanes 5 and 6 show partially degraded δ
Table 2. Reconstitution of photophosphorylation in EDTA-treated spinach thylakoids by addition of subunit δ, CF₁(−δ) and both together

Details of the experimental procedure are outlined in Materials and Methods section. Phenazine methosulfate served as mediator of cyclic electron transport.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ATP synthesis (μmol h⁻¹ mg Chl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thylakoids</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1496</td>
</tr>
<tr>
<td>EDTA vesicles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>155</td>
</tr>
<tr>
<td>EDTA vesicles after addition of</td>
<td></td>
</tr>
<tr>
<td>10 μg CF₁(−δ)</td>
<td>271</td>
</tr>
<tr>
<td>10 μg δ</td>
<td>167</td>
</tr>
<tr>
<td>10 μg CF₁(−δ) + 0.5 μg δ</td>
<td>376</td>
</tr>
<tr>
<td>1. 10 μg CF₁(−δ)</td>
<td>277</td>
</tr>
<tr>
<td>2. 0.5 μg δ</td>
<td>265</td>
</tr>
<tr>
<td>1. 0.5 μg δ</td>
<td></td>
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<tr>
<td>2. 10 μg CF₁(−δ)</td>
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preparations. The one in lane 6 was used for the experiments in Fig. 1. The degraded δ (δ') was about 1.2 kDa or 10 amino acids shorter than native δ. In reconstitution experiments δ' (fifth lane) was as active as δ (sixth lane). The supposedly degraded δ' was already apparent in the starting material for the purification of δ (crude CF₁, see lane 7). After further purification of CF₁ via FPLC on Fractogel TSK DEAE 650 (S), the band of lower molecular mass showed increased intensity whereas the intensity of the band of higher molecular mass (which corresponds to native δ) was reduced (cf. lane 8).

The validity of this assumption was checked by N-terminal sequencing (performed by Dr J. Hoppe) of the δ and δ'-enriched samples (lanes 5 and 6). These preparations had identical N termi nating cleavage at the carboxy terminus. The sequence found for the first 13 amino acids was in good accordance with the known sequences of E. coli δ [34, 35] and of beef-heart OSCP, the counterpart of δ in mitochondria [36], as shown in the following alignment.

Spinning CF₁ δ
E. coli δ
Beef-heart OSCP

The short sequence given above for spinach CF₁ δ agrees with data from Berzborn et al., who also reported degradation of δ by about 1000 Da [43].

SUMMARY AND DISCUSSION

Isolated subunit δ of CF₁ reconstituted photophosphorylation in partially CF₁-depleted vesicles which were derived from spinach thylakoids by EDTA treatment. This was readily understood in the framework of the chemiosmotic mechanism [44]. Owing to the extraction of CF₁ some of these vesicles had lost their ability for photophosphorylation because they were leaky to protons. This was due to a small fraction of CF₀ which were exposed by CF₁ extraction, typically only 3% of exposed CF₁ (see [27] and [37]). The high proton conductance of leaky vesicles was attributable to the very high unit conductance of the few open channels, which was in the order of 1 pS [37]. Reconstitution was about saturated after addition of 100 mol δ/mol exposed CF₀. The reconstitutational activity of subunit δ was not attributable to a contamination of the δ preparation with CF₁ or CF₁(−δ), as shown very clearly in Fig. 4. Since δ had no catalytic activity by itself and as it does not specifically interact with nucleotides its reconstitutational activity was attributed to its ability to diminish the protonic conductance of open CF₀ channels. Thereby it allowed a sufficiently large proton-motive force to re-establish in previously leaky vesicles which, in turn, reactivated the photophosphorylating capacity of those CF₀CF₁ molecules which had been dormant in such leaky vesicles.

The stopcock action of δ on open CF₀ was not perfect, however, as suggested by the following. Reconstitution of photophosphorylation was obtained only with phenazine methosulfate as mediator of cyclic electron transport but not in the presence of methyl viologen and with linear electron transport. It is well known that phenazine methosulfate yields much higher rates of proton pumping than methylviologen [45]. Therefore, proton pumping by the cyclic electron transport may overcome higher levels of proton leakage than pumping by the linear electron-transport chain. The residual level of proton leakage, which remained after addition of CF₁(+δ) or of δ, could be explained either way. (a) Rebinding of δ to open CF₀ blocked proton leakage only partially. (b) In addition to a few highly conducting 'open CF₀', EDTA-vesicles

Fig. 4. SDS electrophoresis of various preparations of subunit δ (lanes 3–6), of CF₁ (lanes 1, 7, 8) and of CF₁ (−δ) (lane 2). Migration was from top (−) to bottom (+) in a 12.5% Laemmli gel, silver stain. Approximately 2 μg δ and 5 μg CF₁ were run.
contained fewer conducting CF0 fragments which were unable to bind \( \delta \) or \( \text{CF}_1(\pm \delta) \).

The evidence for the ability of \( \delta \) to reduce the proton leakage through open CF0 corroborated previous more indirect evidence [19, 26–28]. In one line of previous experiments the proton conductance of thylakoid membranes was studied as function of \( \text{CF}_1 \) depletion. It was always greater when subunit \( \delta \) was recovered on exposed CF0 than when it was not found in the extract [26–28]. This was interpreted to indicate that some \( \delta \) remained on exposed CF0 to form \( \text{CF}_0(\pm \delta) \), which was relatively proton-tight. By the inverse approach, \( \delta \)-lacking or \( \delta \)-containing \( \text{CF}_1 \) fragments were added back to partially CF0-depleted vesicles. \( \delta \)-containing fragments were more efficient in blocking the proton channel [19]. By the new procedure for the purification of \( \delta \) [29] the ability of this subunit to act as a stopcock to CF0 now was established.

When a 1:1 mixture of subunit \( \delta \) plus \( \text{CF}_1(-\delta) \) was added to partially \( \text{CF}_1 \)-depleted vesicles photophosphorylation was reconstituted at a surprisingly low stoichiometric proportion of this mixture to exposed CF0, namely 1 mol [\( \delta + \text{CF}_1(-\delta) \)] per 17 mol exposed CF0. A straightforward explanation can be offered in terms of the above work on the single-channel conductance of exposed CF0. Since only a few percent of exposed CF0 were actually proton-conducting [27, 37] it was sufficient to block these few channels. If the binding affinity of \( \text{CF}_1(-\delta)+\delta \) for the few open CF0(\( \delta \)) was higher than the affinity to bind to \( \text{CF}_0(\pm \delta) \) and to the majority of degraded channels, this would indeed explain the observed high effectiveness.

The degradative loss of about ten amino acids at the C terminus had no effect on the reconstitutinal activity of subunit \( \delta \), while the folding had. Storage at \(-20^\circ\text{C}\) abolished the function of \( \delta \) alone. However, the synergistic function with \( \text{CF}_1(-\delta) \) was retained, perhaps by refolding in the course of the reassocation of \( \delta \) with \( \text{CF}_1(-\delta) \). Along the same lines we found that the order of addition of subunit \( \delta \) and \( \text{CF}_1(-\delta) \) to the depleted membranes was important. When \( \delta \) was preincubated with \( \text{CF}_1(-\delta) \) before addition of both to \( \text{CF}_1(-\delta) \)-depleted membranes, the reconstituted photophosphorylation rate was higher than when \( \delta \) was added first to the membranes followed by \( \text{CF}_1(-\delta) \).

How do the presented results relate to published work? Statements about the role of \( \delta \) may be divided into two groups. (a) \( \delta \) is necessary or essential for docking \( \text{F}_1 \) to \( \text{F}_0 \) [20–22]. This view has been challenged because \( \text{CF}_1(-\delta) \) rebinds to \( \text{CF}_1(-\delta) \)-depleted membranes specifically [23, 24]. With the evidence for complementary rebinding of \( \text{CF}_1(+\delta) \) to \( \text{CF}_0(-\delta) \) and, perhaps, of \( \text{CF}_1(-\delta) \) to \( \text{CF}_0(+\delta) \) presented here and in [19], these seemingly contradictory statements appear compatible with each other. (b) \( \delta \) can reduce proton conduction through open CF0, without any other \( \text{F}_1 \) subunit present [19, 26–28]. For purified \( \delta \) this was only established in this article. That the described reconstitutive effect of subunit \( \delta \) was not detected in previous work [25, 38, 39] may be attributable to the different plant species used or to the rapid loss of the activity upon storage of purified subunit \( \delta \) as demonstrated in this article.

It is tempting to speculate about the role of subunit \( \delta \) in the integral \( \text{CF}_0\text{CF}_1 \) complex. \( \delta \) can bind to both parts, to exposed CF0 and to \( \text{CF}_1 \). We have shown elsewhere that the interaction between \( \delta \) and the \( \alpha(\delta)_1\beta \) moiety was weakened in the presence of nucleotides [29]. By inversion of this cause/effect relationship it is conceivable, that subunit \( \delta \) is involved in the energy-requiring extrusion [40] of spontaneously formed ATP [41], and that it does so by some unknown interaction with protons and CF0. So far, however, our results do not allow us to discriminate between the two possible actions of \( \delta \): conformational switch, as above, or gated proton conductor from CF0 into \( \text{CF}_1 \) [42].

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