Fragmentation of chloroplast coupling factor in dependence of bound nucleotides

Preparation of a reconstitutionally active form of subunit δ

S. Engelbrecht and W. Junge

Abt. Biophysik, Fachbereich Biologie/Chemie, Universität Osnabrück, Barbarastr. 11, D-4500 Osnabrück, FRG

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Previous studies on the ability of CF₁ fragments to reconstitute photophosphorylation in CF₁-depleted thylakoids have shown that the degree of reconstitution was correlated with the presence of subunit δ in the fragment. This was taken as evidence that subunit δ was necessary for plugging the active proton channel CF₂₉ [(1986) Eur. J. Biochem. 160, 635-643]. We questioned whether or not δ alone had this ability. In order to obtain δ we investigated the role of bound nucleotides in the stability of CF₁. Starting from ammonium sulfate-precipitated CF₁, we found that a low content of bound ADP (1 mol ADP/mol CF₁) seemed to stabilize the δ-S interaction, while loosening the interaction between α, β and γ. By elution from an anion-exchange column in the presence of the nonionic surfactant Mega 9 we obtained δδδ and CF₁(−δ) (both containing one ADP) or, after washing with alcohol/glycerol mixtures, δ (nucleotide-free) and CF₁/CF₁(−ε). On the other hand, with a further 2 ADP and 2 ATP bound to CF₁ (after incubation with excess ATP) the α-β-γ interaction was stabilized in such a way that subunit δ alone could be isolated from the complex. Subunit δ, when isolated by this procedure and added back to CF₁-depleted thylakoids, reconstituted a high rate of photophosphorylation.

CF₁; Nucleotide content; Phosphorylation; Photosynthesis; H⁺-ATPase

1. INTRODUCTION

The H⁺-ATP synthase of thylakoids consists of the membrane-embedded proton channel CF₀ and the extrinsic catalytic part CF₁. After isolation (e.g. by incubation of thylakoids in EDTA-containing buffer) CF₁ catalyses ATP hydrolysis. CF₁ is composed of five types of subunits, 3 α, 3 β, 1 γ, 1 or more δ and 1 ε. Only the α- and β-subunits contain nucleotide-binding sites (reviews [1–5]).

Motivated by the search for the role of subunit δ in the regulation of proton transport through CF₀CF₁ [6–9], we reexamined the purification of various fragments of CF₁. Our previous procedures for subunit depletion and fragmentation involved anion-exchange chromatography of purified CF₁ in the presence of the anionic surfactant Mega 9. This yielded δδδ and CF₁(−δ). Washing CF₁ that was bound to an anion-exchange column by alcohol/glycerol mixtures followed by elution with Tris-HCl/NaCl yielded the δ-subunit.
and CF₁/CF₁(−ε) [8]. Upon reconstitution into CF₁-depleted thylakoid membranes, δ-containing fragments reconstituted higher rates of photophosphorylation than fragments lacking δ [8]. Measurements of the proton permeability of the thylakoid membrane after extraction of CF₁ revealed that the leakiness for protons was correlated with the appearance of subunit δ in the supernatant [9]. This was taken as further evidence of the ability of δ to block the proton channel CF₀. Such a function, however, was only inferred from experiments with δ-containing CF₁ fragments. In order to obtain subunit δ alone, for both reconstitution and further characterization, we investigated more systematically the role of bound nucleotides in fractionation. We found that bound nucleotides stabilized the αβγ complex, thus facilitating the detachment of δ.

2. MATERIALS AND METHODS

CF₁ was isolated and purified as in [10]. CF₁ and δ were further purified by anion-exchange chromatography on fractogel TSK DEAE 650 (S) columns of either 20 ml (HR 16/10) or 2 ml (HR 5/10) inner volume [8]. Nucleotide extraction and conversion of CF₁ into the 2 ATP/1 ADP containing state were performed as described by Kironde and Cross [11]. The ATP content and the content of ADP after pyruvate kinase-mediated conversion into ATP was determined by the luciferin/luciferase assay as described [8,12]. Typical nucleotide contents for the ammonium sulfate-precipitated CF₁ (CF₁AS) were: ATP, 0.09 ± 0.09; and ADP, 1.15 ± 0.20 mol/mol CF₁AS (n = 6). The values given in fig.1 are rounded off to the nearest integer.

Protein determinations, SDS gel electrophoresis, silver staining and reconstitution experiments were carried out essentially as in [8].

Mega 9 was obtained from Oxyl Chemie (Bohningen, FRG), the luciferin/luciferase ATP assay kit from LKB, pyruvate kinase and phosphoenolpyruvate from Boehringer Mannheim, Fractogel TSK DEAE 650 (S) from Merck, diisopropyl phosphofluoridate from Sigma (Munich) and YM 10 ultrafiltration membranes from Amicon (Witten, FRG); all other chemicals were from either Merck or Sigma (Munich). The FPLC apparatus and columns and PD 10 columns were from Pharmacia.

3. RESULTS

The scheme in fig.1 summarizes the results. The starting material, shown in the middle, was ammonium sulfate-precipitated CF₁ (CF₁AS) [8]. It contained approx. 1 mol bound ADP/mol CF₁.

Anion-exchange chromatography in the presence of the surfactant Mega 9 ('TSK Mega 9') yielded a βδ complex (probably βδ• [8]) and CF₁(−δ). If CF₁ was reduced by 100 mM dithiothreitol (DTT) before loading it onto the column, CF₁(−δε) was obtained instead of CF₁(−δ). The ε-subunit was lost. Washing CF₁ on the column with ethanol/glycerol, followed by elution with Tris-HCl/NaCl ('TSK Glyc/EtOH') yielded the ε-subunit (in the wash), the δ-subunit and a mixture of CF₁ and CF₁(−ε). This procedure was developed by Richter et al. [13]. If CF₁ was converted into the 2 ATP/1 ADP containing form by successive treatment with Mg-ATP, pyrophosphate and phosphate [11] prior to chromatography, neither βδ nor β was obtained. In the presence of Mega 9 during chromatography (TSK Mega 9) CF₁(−δ) was still obtained, but the δ-subunit was lost. Saturating amounts of ATP converted CF₁, containing one ADP per molecule (i.e. CF₁AS), into a form with 2 ATP and 3 ADP (left part of fig.1, center; nucleotide content measured after 3–5 successive gel filtrations in order to remove unbound nucleotide). Subjecting the ATP-saturated CF₁ (without removal of excess nucleotide by gel filtration) to the two chromatographic procedures resulted either in the appearance of δ and CF₁(−δ) (TSK Mega 9) [and δ and CF₁(−δε) if CF₁ was reduced prior to chromatography; TSK Mega 9, DTT] or, after the ethanol/glycerol wash procedure (TSK/Glyc/EtOH), in ε and the CF₁/CF₁(−ε) mixture. The various CF₁ species were freed of excess nucleotide by anion-exchange chromatography (nucleotides eluted earlier than CF₁ from the column) and their ATP and ADP contents were 1 and 3, respectively. Whereas the isolated β-subunit was free from bound nucleotide, βδ contained one ADP. With saturating amounts of Mg-ATP and Mg-ADP the isolated β-subunit bound one ATP (measured after 4 successive gel filtrations). Mg-ATP was bound preferentially. In
Fig. 1. Summary of the fragmentation procedure of CF$_1$ in dependence of bound nucleotides. TSK Mega 9 denotes anion-exchange chromatography on Merck Fractogel TSK DEAE 650 (S) with 25 mM Tris-HCl, 0.5 mM EDTA, 20 mM Mega 9, pH 7.8, and increasing NaCl; column size 0.5 x 2 cm; flow rate 2 ml/min, 10 bar. TSK Glyc/EtOH indicates chromatography on the same column. After loading with CF$_1$, the column was washed with 10 ml of 50 mM Tris-HCl, 30% (v/v) glycerol, 20% (v/v) ethanol, pH 7.8, at 0.5 ml/min, 30–40 bar and then eluted with 25 mM Tris-HCl, pH 7.8, with increasing amounts of NaCl. The same procedures with Mono Q as column are described in [18]. AS denotes ammonium sulfate and CF$_1$AS represents CF$_1$ that was precipitated by 50% saturated ammonium sulfate, centrifuged, and desalted into the appropriate starting buffer by gel filtration through Pharmacia PD 10 columns. This CF$_1$ was subjected to the indicated chromatography either directly or after reduction with 100 mM dithiothreitol (room temperature, 1 h). Conversion into the 2 ATP/1 ADP containing form was by treatment with 10 mM Mg-ATP, 5 mM PP$_i$, and 50 mM P$_i$ (each incubation followed by one or more PD 10 gel filtrations) as detailed by Kironde and Cross [11]. CF$_1$ containing 2 ATP and 3 ADP was prepared by brief incubation with 10 mM ATP and subsequent gel filtration on PD 10/25 mM Tris-HCl, pH 7.8. Usually at least 3–5 gel filtrations had to be carried out in order to remove all unbound nucleotide. The surplus nucleotide was not removed prior to the indicated anion-exchange chromatographies.

the absence of nucleotides $\beta$ was unstable and precipitated within 3 h at room temperature in Tris-HCl, pH 7.8.

The two steps leading to a pure preparation of subunit $\delta$ are documented in detail in fig. 2. The left part of this figure shows a typical chromatogram of a CF$_1$ preparation which was prepurified on Whatman DE 52 cellulose [8,10]. It was further purified on a 20 ml HR 16/10 TSK DEAE 650 (S) column. The DE 52 pool was separated in 4 aliquots of about 70 mg protein each. The first four peaks contained mainly ribulose-bisphosphate carboxylase along with some other proteins. The main peak (2) consisted of CF$_1$, the last peak (3) of CF$_1$($-\varepsilon$). Two preparations of 1 kg spinach leaves each typically yielded about 80–120 mg CF$_1$ at this point. Rechromatography of CF$_1$ (after addition of 0.5–1.0 mM ATP) either on a 2 ml HR 5/10
Traces of CF$_1$ were eluted at higher salt concentrations (not shown). 100 mg CF$_1$ (peak 2) yielded between 1.5 and 5 mg subunit $\delta$ (peak 6). High yields were obtained only with freshly prepared columns. 100–200 $\mu$l of diisopropyl phosphofluoridate were added to the EDTA suspension, DE 52 pool and HR 16/10 TSK pool. This helped to suppress proteolytic degradation of the isolated $\delta$-subunit. In some cases, however, the DE 52 pool showed signs of proteolytic digestion, thus indicating degradative processes in the starting material already.

When isolated by this procedure subunit $\delta$ reconstituted photophosphorylation. Spinach thylakoids with ATP-synthesis rates of 1400 $\mu$mol ATP/h per mg Chl before extraction (measured in the presence of 50 $\mu$M phenazine methosulfate) and 680 $\mu$mol/h per mg Chl after EDTA treatment (100 $\mu$M, 10 min) had activities of 980 $\mu$mol ATP/h per mg Chl after reconstitution with 50 $\mu$g purified $\delta$-subunit. These results will be detailed elsewhere.

4. DISCUSSION

Not unexpectedly, the stability of isolated CF$_1$ against fragmentation was dependent on the nucleotide content of the enzyme. Tentatively, this was discussed in terms of a total of six binding sites. Six binding sites for nucleotides have been established for MF$_1$ [14], the mitochondrial analogue to CF$_1$, while so far only three binding sites are established for CF$_1$ [15,16].

We started from the ammonium sulfate-precipitated form of purified CF$_1$, which, in accordance with published values [15–17], contained only 1 mol ADP per mol CF$_1$. CF$_1$AS readily disintegrated into $\beta$S and CF$_1$($\delta$) [8] or, after reduction, into CF$_1$($\delta$,$\epsilon$). By another procedure $\beta$, $\epsilon$, and a mixture of CF$_1$ and CF$_1$($\epsilon$–$\gamma$) were obtained. As outlined earlier [8], the $\beta$S stoichiometry of the $\beta$S fragment was assigned by comparative SDS gel electrophoresis and rocket immunodiffusion. It is worth mentioning that $\beta$S did not reveal a 3-fold symmetry similar to CF$_1$ (electron-microscopic investigations by Lunsdorf, H., personal communication). Since both $\beta$S and CF$_1$ from which $\beta$S was derived contained 1 mol ADP/mol, we attribute the binding site of the 1 mol ADP/mol CF$_1$AS to the $\beta$S portion of the enzyme. The isolated $\beta$-subunit did not contain...
any ATP or ADP. After incubation with both ADP and ATP simultaneously, it bound 1 mol ATP/mol β. It was somewhat puzzling to observe that the βδ complex contained ADP, whereas the isolated β-subunit preferentially bound ATP.

Treatment of CF₁ with excess Mg-ATP followed by removal of exchangeable nucleotide by several incubations and gel filtrations in the presence of pyrophosphate and phosphate [11] yielded a form of CF₁ with 3 nucleotides that resisted fragmentation although it lost its δ-subunit after the TSK Mega 9 procedure.

CF₁ with five (or more) nucleotide-binding sites occupied yielded subunit δ and CF₁(−δ). The nucleotides seemed to act as a clamp that kept together the αβγ moiety. On the other hand, nucleotide depletion might have induced a conformational change in the (αβ)₃ hexagonal chair that facilitated the detachment of βδ or of β. The total yield of β indicated separation of only one β out of three in CF₁; the rest of the molecule was lost [8]. The yield of β increased with increasing storage time of the starting material as ammonium sulfate precipitate.

The described procedure for preparation of spinach chloroplast δ resembles published methods [18,19]. Differences are: the use of (cheaper) Mega 9 instead of octylglucoside, DEAE Fractogel instead of hydroxyapatite and omitting of nucleotide from the elution buffers. We were unable to obtain subunit δ by the published procedures and application of our protocol to hydroxyapatite columns also did not give the desired result. The reason for this discrepancy is unclear. It was noteworthy that subunit δ, isolated by the described procedure, was functional. It reconstituted photophosphorylation in vesicles which were made proton-leaky by CF₁ depletion. This corroborated our previous conclusion that subunit δ may act as a plug to the open proton channel CF₀ [6–9]. As nucleotide depletion stabilized βδ interactions (indicated by fragmentation into βδδ) and high nucleotide occupation stabilized the (αβ)γ complex (indicated by detachment of δ) it is tempting to speculate that subunit δ does not act only as a passive plug to CF₀. By binding to at least one of the three β-subunits (or three δ being bound to the three β-subunits [20]) it could connect the proton channel CF₀ to the active site(s) in CF₁. It is conceivable that subunit δ serves to conduct protons into the β-subunit during ATP synthesis. Alternatively, δ may act as a conformational transducer that facilitates nucleotide binding and release.

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REFERENCES