Volume 219, number 2, 321–325 FEB 04921 July 1987

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

Received 15 May 1987

Previous studies on the ability of CF_1 fragments to reconstitute photophosphorylation in CF_1-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 CF_0 [(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 CF_1. Starting from ammonium sulfate-precipitated CF_1, we found that a low content of bound ADP (1 mol ADP/mol CF_1) seemed to stabilize the β–δ 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 $\beta_3\delta$ and $CF_1(-\delta)$ (both containing one ADP) or, after washing with alcohol/glycerol mixtures, β (nucleotide-free) and $CF_1/CF_1(-\epsilon)$. On the other hand, with a further 2 ADP and 2 ATP bound to CF_1 (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 CF_1-depleted thylakoids, reconstituted a high rate of photophosphorylation.

CF_1; Nucleotide content; Phosphorylation; Photosynthesis; H^+-ATPase

1. INTRODUCTION

The H^+-ATP synthase of thylakoids consists of the membrane-embedded proton channel CF_0 and the extrinsic catalytic part CF_1. After isolation (e.g. by incubation of thylakoids in EDTA-containing buffer) CF_1 catalyses ATP hydrolysis. CF_1 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 CF_0CF_1 [6–9], we reexamined the purification of various fragments of CF_1. Our previous procedures for subunit depletion and fragmentation involved anion-exchange chromatography of purified CF_1 in the presence of the anionic surfactant Mega 9. This yielded $\beta_3\delta$ and $CF_1(-\delta)$. Washing CF_1 that was bound to an anion-exchange column by alcohol/glycerol mixtures followed by elution with Tris-HCl/NaCl yielded the β-subunit

Correspondence address: S. Engelbrecht, Abt. Biophysik, Fachbereich Biologie/Chemie, Universität Osnabrück, Barbarastr. 11, D-4500 Osnabrück, FRG

Abbreviations: CF_0CF_1, chloroplast ATP synthase; CF_1, chloroplast ATPase; CF_0, chloroplast ATP synthase, proton-conducting part; $CF_1(-\delta)$, CF_1 lacking the δ-subunit; $CF_1(-\epsilon)$, CF_1 lacking the ϵ-subunit; $CF_1(-\delta\epsilon)$, CF_1 lacking the δ- and ϵ-subunits; Mega 9, *N*-(D-gluco-2,3,4,5,6-pentahydroxylhexyl)-*N*-methylnonanamide

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50 © 1987 Federation of European Biochemical Societies

and $CF_1/CF_1(-\epsilon)$ [8]. Upon reconstitution into CF_1-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 CF_1 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 CF_0. Such a function, however, was only inferred from experiments with δ-containing CF_1 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 $\alpha\beta\gamma$ complex, thus facilitating the detachment of δ.

2. MATERIALS AND METHODS

CF_1 was isolated and purified as in [10]. CF_1 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 CF_1 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 CF_1 (CF_1AS) were: ATP, 0.09 ± 0.09; and ADP, 1.15 ± 0.20 mol/mol CF_1AS ($n = 6$). 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 (Bobingen, FRG), the luciferin/luciferase ATP assay kit from LKB, pyruvate kinase and phospho*enol*pyruvate 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 CF_1 (CF_1AS) [8]. It contained approx. 1 mol bound ADP/mol CF_1. Anion-exchange chromatography in the presence of the surfactant Mega 9 ('TSK Mega 9') yielded a $\beta\delta$ complex (probably $\beta_3\delta$ [8]) and $CF_1(-\delta)$. If CF_1 was reduced by 100 mM dithiothreitol (DTT) before loading it onto the column, $CF_1(-\delta\epsilon)$ was obtained instead of $CF_1(-\delta)$. The ϵ-subunit was lost. Washing CF_1 on the column with ethanol/glycerol, followed by elution with Tris-HCl/NaCl ('TSK Glyc/EtOH') yielded the ϵ-subunit (in the wash), the β-subunit and a mixture of CF_1 and $CF_1(-\epsilon)$. This procedure was developed by Richter et al. [13]. If CF_1 was converted into the 2 ATP/1 ADP containing form by successive treatment with Mg-ATP, pyrophosphate and phosphate [11] prior to chromatography, neither $\beta_3\delta$ nor β was obtained. In the presence of Mega 9 during chromatography (TSK Mega 9) $CF_1(-\delta)$ was still obtained, but the δ-subunit was lost. Saturating amounts of ATP converted CF_1, containing one ADP per molecule (i.e. CF_1AS), 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 CF_1 (without removal of excess nucleotide by gel filtration) to the two chromatographic procedures resulted either in the appearance of δ and $CF_1(-\delta)$ (TSK Mega 9) [and δ and $CF_1(-\delta\epsilon)$ if CF_1 was reduced prior to chromatography; TSK Mega 9, DTT] or, after the ethanol/glycerol wash procedure (TSK/Glyc/EtOH), in ϵ and the $CF_1/CF_1(-\epsilon)$ mixture. The various CF_1 species were freed of excess nucleotide by anion-exchange chromatography (nucleotides eluted earlier than CF_1 from the column) and their ATP and ADP contents were 1 and 3, respectively. Whereas the isolated β-subunit was free from bound nucleotide, $\beta_3\delta$ 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

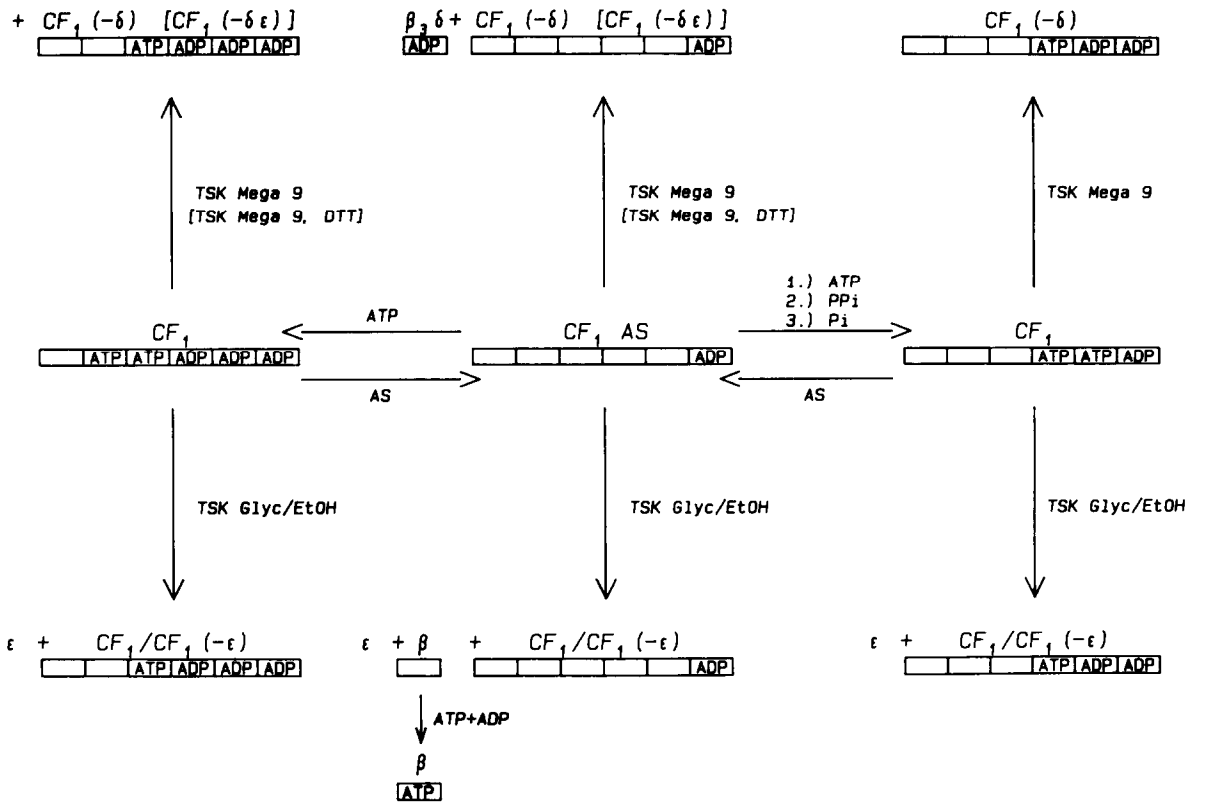

Fig.1. Summary of the fragmentation procedure of CF_1 in dependence of bound nucleotides. TSK Mega 9 denotes anion-exchange chromatography on Merck Fractogel TSK DEAE 650 (S) with 25 mM Tris-HCl, 0.5 mM EDTA, 20 mM Mega 9, pH 7.8, and increasing NaCl; column size 0.5 × 2 cm; flow rate 2 ml/min, 10 bar. 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 [8]. AS denotes ammonium sulfate and CF_1AS 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 β 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 δ 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 $CF_1(-\epsilon)$. 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

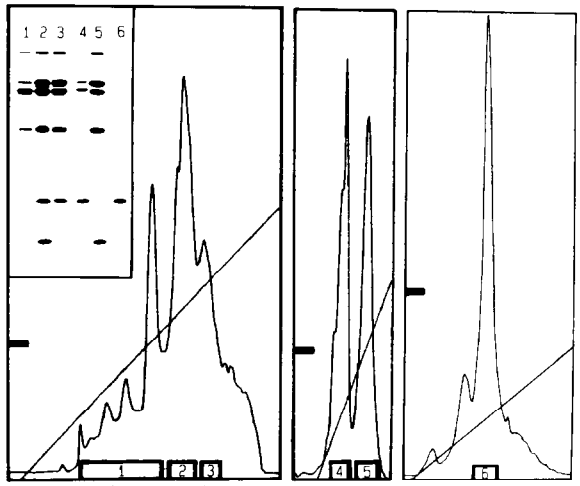

Fig.2. Summary of elution profiles as obtained during preparation of subunit δ from CF_1. Left part, chromatography on a 20 ml Fractogel TSK DEAE 650 (S) column eluted with 25 mM Mes/NaOH, pH 6.0, and increasing amounts of NaCl, flow rate 10 ml/min, 8 bar. Center, chromatography on a 2 ml column of Fractogel TSK DEAE 650 (S), developed with an NaCl gradient in 25 mM Tris-HCl, 0.5 mM EDTA, 20 mM Mega 9, pH 7.8; 2 ml/min, 10 bar. Right part, rechromatography of peak 4 under the same conditions after removal of salt and ATP by repeated ultrafiltration on Amicon YM 10. (Inset) Silver-stained SDS electrophoretic separations of the indicated pools. 6 μg CF_1 and 2 μg δ were separated in a 12.5% gel, migration from top (−) to bottom (+). The bars on the left side of each chromatogram indicate absorption (280 nm) = 0.2. The ascending lines indicate the NaCl gradient, left part 0–400 mM, center and right part 0–300 mM. The absorption profiles are not corrected for delay of the gradient through the column.

TSK DEAE 650 (S) column in 10-mg aliquots or in one batch on the 20 ml HR 16/10 column in the presence of 20 mM Mega 9 yielded two peaks, the first of which (4) was composed of ATP, δ and traces of other CF_1 subunits, mainly β. The high absorption at 280 nm was due to ATP which was eluted at about the same salt concentration. In some runs δ was eluted in an additional peak right before the ATP/δ peak. The second peak (5) in the center part of fig.2 was $CF_1(-\delta)$. The eluted δ fractions (4) were pooled, dialysed by ultrafiltration on an Amicon YM 10 membrane and rechromatographed. This yielded the pure δ-subunit (fig.2, right part, peak 6). The first minor peaks contained lower-M_r degradation products. 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 δ (peak 6). High yields were obtained only with freshly prepared columns. 100–200 μl 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 δ-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 δ reconstituted photophosphorylation. Spinach thylakoids with ATP-synthesis rates of 1400 μmol ATP/h per mg Chl before extraction (measured in the presence of 50 μM phenazine methosulfate) and 680 μmol/h per mg Chl after EDTA treatment (100 μM, 10 min) had activities of 980 μmol ATP/h per mg Chl after reconstitution with 50 μg purified δ-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_1AS readily disintegrated into $\beta_3\delta$ and $CF_1(-\delta)$ [8] or, after reduction, into $CF_1(-\delta,\epsilon)$. By another procedure β, ϵ, and a mixture of CF_1 and $CF_1(-\epsilon)$ were obtained. As outlined earlier [8], the $\beta_3\delta$ stoichiometry of the $\beta\delta$ fragment was assigned by comparative SDS gel electrophoresis and rocket immunodiffusion. It is worth mentioning that $\beta_3\delta$ did not reveal a 3-fold symmetry similar to CF_1 (electron-microscopic investigations by Lunsdorf, H., personal communication). Since both $\beta_3\delta$ and CF_1 from which $\beta_3\delta$ was derived contained 1 mol ADP/mol, we attribute the binding site of the 1 mol ADP/mol CF_1AS to the $\beta_3\delta$ portion of the enzyme. The isolated β-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 $\beta\delta$ complex contained ADP, whereas the isolated β-subunit preferentially bound ATP.

Treatment of CF_1 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 CF_1 with 3 nucleotides that resisted fragmentation although it lost its δ-subunit after the TSK Mega 9 procedure.

CF_1 with five (or more) nucleotide-binding sites occupied yielded subunit δ and $CF_1(-\delta)$. The nucleotides seemed to act as a clamp that kept together the $\alpha_3\beta_3\gamma$ moiety. On the other hand, nucleotide depletion might have induced a conformational change in the $(\alpha\beta)_3$ hexagonal chair that facilitated the detachment of $\beta_3\delta$ or of β. The total yield of β indicated separation of only one β out of three in CF_1; 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 CF_1 depletion. This corroborated our previous conclusion that subunit δ may act as a plug to the open proton channel CF_0 [6–9]. As nucleotide depletion stabilized $\beta\delta$ interactions (indicated by fragmentation into $\beta_3\delta$) and high nucleotide occupation stabilized the $(\alpha\beta)_3\gamma$ complex (indicated by detachment of δ) it is tempting to speculate that subunit δ does not act only as a passive plug to CF_0. 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 CF_0 to the active site(s) in CF_1. 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.

ACKNOWLEDGEMENTS

This work was supported by a grant from the DFG (SFB 171/B3). We wish to thank Holger Lill for computer help and to Karin Schürmann for valuable technical assistance.

REFERENCES

[1] Vignais, P.V. and Satre, M. (1984) Mol. Cell. Biochem. 60, 33–71.
[2] Strotmann, H. and Bickel-Sandkötter, S. (1984) Annu. Rev. Plant Physiol. 35, 97–120.
[3] Galmiche, J.M., Girault, G. and Lemaire, C. (1985) Photochem. Photobiol. 41, 707–713.
[4] Godinot, C. and DiPietro, A. (1986) Biochimie 68, 367–374.
[5] Haraux, F. (1986) Biochimie 68, 435–449.
[6] Junge, W., Hong, Y.Q., Qian, L.P. and Viale, A. (1984) Proc. Natl. Acad. Sci. USA 81, 3078–3082.
[7] Lill, H., Engelbrecht, S., Schönknecht, G. and Junge, W. (1986) Eur. J. Biochem. 160, 627–634.
[8] Engelbrecht, S., Lill, H. and Junge, W. (1986) Eur. J. Biochem. 160, 635–643.
[9] Lill, H., Engelbrecht, S. and Junge, W. (1987) in: Progress in Photosynthesis Research (Biggins, J. ed.) vol.III, pp.141–144, Nijhoff, Dordrecht.
[10] Apley, E., Wagner, R. and Engelbrecht, S. (1985) Anal. Biochem. 150, 145–154.
[11] Kironde, F.A.S. and Cross, R.L. (1986) J. Biol. Chem. 261, 12544–12549.
[12] Spielmann, H., Jacob-Müller, U. and Schulz, P. (1981) Anal. Biochem. 113, 172–178.
[13] Richter, M.L., Patrie, W.J. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7371–7373.
[14] Wèber, J., Lücken, U. and Schäfer, G. (1985) Eur. J. Biochem. 148, 41–47.
[15] Carlier, M.F. and Hammes, G.G. (1979) Biochemistry 18, 3446–3451.
[16] Girault, G., Galmiche, J.M., Lemaire, C. and Stulzaft, O. (1982) Eur. J. Biochem. 128, 405–411.
[17] Shoshan, V., Shavit, N. and Chipman, D.M. (1978) Biochim. Biophys. Acta 504, 108–122.
[18] Andreo, C.S., Patrie, W.J. and McCarty, R.E. (1982) J. Biol. Chem. 257, 9968–9975.
[19] Patrie, W.J. and McCarty, R.E. (1984) J. Biol. Chem. 259, 11121–11128.
[20] Berzborn, R.J., Roos, P. and Bonnekamp, G. (1984) in: Advances in Photosynthesis Research (Sybesma, C. ed.) vol.II, pp.587–590, Nijhoff/Junk, Den Haag.