Eur. J. Biochem. *160*, 635–643 (1986)

Reconstitution of CF_1-depleted thylakoid membranes with complete and fragmented chloroplast ATPase

The role of the δ subunit for proton conduction through CF_0

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(Received June 2, 1986) – EJB 86 0543

Chloroplast ATPase (CF_1) was isolated from spinach, pea and maize thylakoids by EDTA extraction followed by anion-exchange chromatography. CF_1 was purified and resolved by HPLC into integral CF_1, and CF_1 lacking the δ & ε subunits: $CF_1(-\delta)$ and $CF_1(-\varepsilon)$. Washing Mono-Q-bound CF_1 with alcohol-containing buffers followed by elution without alcohol produced the β subunit and in separate peaks $CF_1(-\delta)$ and $CF_1(-\varepsilon)$. Elution from Mono Q in the presence of tenside yielded a $\beta\delta$ fragment, $CF_1(-\delta)$ and $CF_1(-\delta\varepsilon)$.

Chloroplasts were CF_1-depleted by EDTA extraction. Reconstitution of photophosphorylation in these 'EDTA vesicles' was obtained by addition of CF_1 and its fragments. CF_1, $CF_1(-\delta)$ and $CF_1(-\delta\varepsilon)$ were active with cross-reactivity between spinach, pea and maize. δ-containing CF_1 always reconstituted higher activities than δ-deficient CF_1. The $\beta\delta$ fragment and dicyclohexylcarbodiimide (DCCD)-inhibited CF_1 also were reconstitutively active while β and DCCD-inhibited $CF_1(-\delta)$ were not.

These results support the notion that subunit δ can function as a stopcock to the CF_0 proton channel as proposed by Junge, W., Hong, Y. Q., Qian, L. P. and Viale, A. [(1984) *Proc. Natl Acad. Sci. USA 81*, 3078–3082].

Energy-transducing membranes make use of the free energy stored in a transmembrane protonmotive force [1]. The structure of the enzymes which couple proton flux to ATP synthesis has been highly conserved during evolution. Proton-ATP synthases consist of two parts, a proton channel F_0, which is embedded in the membrane, and the catalytic part F_1, which is water-soluble and extrinsic to the membrane. For a comprehensive literature survey on F_1 refer to [2].

The ATPase from chloroplasts (CF_1) is a hetero-oligomeric protein of total mass around 410 kDa [3]. Five different subunits with molecular masses ranging from 58 kDa to 15 kDa and named $\alpha,\beta,\gamma,\delta,\varepsilon$ in order of decreasing mass occur in a stoichiometry of 3:3:1:1:1 in CF_1 according to some authors [2, 4]. Recently the stoichiometric number of δ subunits in intact CF_0CF_1 has been proposed to be three rather than one [5].

For an understanding of the role of subunits within the complex, isolation of subunits and reassembly into active enzyme has been attempted. Isolation and reconstitution of F_1 subunits from *E. coli*, from thermophilic bacteria, and from *R. rubrum* has been reported [6–9].

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Abbreviations. SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; CF_1, chloroplast ATPase; CF_0, chloroplast ATP synthase, proton-conducting part; $CF_1(-\delta)$, CF_1 lacking the δ subunit; $CF_1(-\varepsilon)$, CF_1 lacking the ε subunit; $CF_1(-\delta\varepsilon)$, CF_1 lacking the δ and ε subunits; DCCD, dicyclohexylcarbodiimide; Mega 9, *N*-(D-*gluco*-2,3,4,5,6-pentahydroxylhexyl)-*N*-methylnonanamide.

Various approaches have been used in order to isolate and reconstitute the subunits or subunit-deficient CF_1 [10–15]. In three publications by McCarty's group [11–13] the role of δ and ε subunits in photophosphorylation was investigated. The data were conflicting. First it was concluded that the δ subunit is not required for binding of CF_1 to CF_0 but for blocking proton conductance through CF_0 [11]. Then, by introducing a method for removal of the ε subunit from complete CF_1, the same findings were reported for the ε subunit [12]. Finally, it was stated that neither δ nor ε is required for rebinding of CF_1 to CF_0, that δ is not absolutely required for ATP synthesis but that ε-deficient CF_1 does not reconstitute ATP synthesis or proton uptake [13] thus suggesting an essential role of the ε subunit in these processes.

We reinvestigated this issue. We obtained five-subunit CF_1, $CF_1(-\delta)$, and $CF_1(-\varepsilon)$ by HPLC at pH 6. Essentially pure β subunit, $CF_1(-\delta)$ and $CF_1(-\varepsilon)$ were also obtained by HPLC in way similar to that reported by Finel et al. [15] but by the alcohol-wash procedure as introduced by Richter et al. [12]. $CF_1(-\delta)$ and $CF_1(-\delta\varepsilon)$ could be produced by eluting the HPLC column with tenside (surfactant)-containing buffers. This additionally yielded a $\beta\delta$ fragment.

As starting material for reconstitution we used EDTA-extracted chloroplasts. The preparation and the catalytic and proton-conducting properties of these 'EDTA vesicles' are described in the accompanying paper [13a].

MATERIALS AND METHODS

Preparation of CF_1 from market spinach was carried out by EDTA extraction and Whatman DEAE-cellulose (DE-52)

chromatography essentially as described previously [10, 16]. Pooled fractions from the anion-exchange column were centrifuged for 30 min at 28000 × *g* and concentrated by ultrafiltration on YM 10 membranes. Protein in the concentrated solution was precipitated by ammonium sulfate (50% saturation) and the precipitate was kept at 4°C for up to 4 months.

Prior to use, aliquots of the precipitated protein (1 – 5 mg) were pelleted by centrifugation at approximately 20000 × *g*, 5 min. The pellet was dissolved in buffer A of the following HPLC step (as specified in Results). The volume was brought to 2.5 ml with the same buffer and this volume was gel-filtered through a pre-equilibrated disposable PD 10 column (Pharmacia Chemicals, approx. volume 9 ml, Sephadex G-10). The effluent (2.5 ml) was discarded, the column was developed with 3.5 ml buffer and these 3.5 ml effluent were collected and used further. In general, this procedure yielded more than 90% of the applied protein and less than 10% of the initial salt as indicated by protein assay and measurements of electric conductivity.

Protein assays were done according to Sedmak and Grossberg [17].

ATPase was measured in the presence of methanol [18 – 20]. Phosphate was determined according to Taussky and Shorr [21].

SDS gel electrophoresis was carried out according to the Laemmli procedure [22], including some modifications [23].

Staining was performed essentially according to the ammoniacal silver stain procedure of Wray [24]. Further details of experimental procedure are described in the Appendix.

HPLC experiments were performed with a Pharmacia fast protein liquid chromatography set-up using automatic sample injection (500 µl) and spectrophotometric detection at 280 nm. The column was a Pharmacia ready-for-use HR 5/5 Mono Q (5 × 50 mm) or a Pharmacia HR 16/10 column (16 × 100 mm) packed with Merck Fractogel TSK DEAE 650 (S) in the following manner. The gel was decanted twice, taken up in H_2O, heated close to 100°C, treated in an ultrasonic bath for about 5 min and decanted twice, then the slurry was poured into the column which was mounted into the HPLC system and run with water at 10 ml/min for 1 – 2 min. After the top of the gel had settled, more slurry was added in the same way until the desired bed height of about 10 cm had been reached. The flow was then reversed a few times and the column run at 15 ml/min for 10 – 15 min. After that, it was equilibrated with starting buffer (buffer A) or stored in 25% (v/v) EtOH until use.

CF_1-extracted chloroplasts ('EDTA vesicles') were prepared from spinach or pea thylakoids by a 2-min or 10-min incubation at 10 µM chlorophyll, 1 mM NaCl, 100 µM EDTA, 1 mM Tricine/NaOH pH 8.0 at room temperature. Extraction was stopped by bringing the NaCl concentration to 30 mM. Vesicles were sedimented by centrifugation (20 min 30000 × *g*) and resuspended in a medium containing 0.1 M sorbitol, 10 mM NaCl, 10 mM Tricine/NaOH pH 7.8. The procedure resembled the one used by Berzborn [25]. Further details of the preparation and properties of these EDTA vesicles are described in the accompanying publication. Chlorophyll determinations were performed by the method of Arnon [26]. The NaBr-extraction procedure [11] did not result in 90% extracted chloroplasts in our hand. This possibly was due to different starting material.

Reconstitution was optimized and checked with respect to $MgCl_2$ concentration, time, temperature, excess of reconstituted protein over chlorophyll, volume and the presence or absence of reducing compounds. With the exception of the ratio protein/chlorophyll, these parameters did not exert drastic effects. Conditions were chosen as follows: chloroplasts or extracted chloroplasts at 10 µg chlorophyll were diluted with the same medium they were suspended in, so as to achieve the same volume in all samples to be reconstituted (max. final volume 300 µl). Protein was added at concentrations between 0.2 mg/ml and 1.5 mg/ml 20 mM Tris/HCl pH 7.8. Saturating amounts of CF_1 usually were 3 – 5 µg/µg chlorophyll. After addition of $MgCl_2$ to give a concentration of 8 mM, the mixture was incubated for approximately 10 min on ice in the dark. When necessary, protein samples were gel-filtered via PD 10 in order to remove salts, ATP or tenside.

ATP synthesis was induced by strong continuous illumination and with phenazinemethosulfate as cofactor for cyclic electron transport according to standard procedures [11].

ATP was measured via the LKB luciferin/luciferase assay [27]. Test mixtures contained either 250 µl 0.1 M Tris/acetate, 2 mM EDTA, pH 7.8, 50 µl LKB ATP-monitoring reagent and 10 µl sample or half of these amounts. Each sample was calibrated by addition of 100 pmol ATP. Samples were diluted so as not to exceed 300 mV photomultiplier output voltage (LKB luminometer 1250) immediately after mixing. Light output was fairly constant under these conditions for 1 min.

Rabbit immunization was carried out with EDTA-extracted CF_1, purified on Whatman DEAE-cellulose DE-52 and Merck Fractogel TSK DEAE 650 (S). After control sera from three rabbits had been obtained, the animals were immunized subcutaneously at four places on their backs with a total of 0.5 mg CF_1/500 µl H_2O, 500 µl complete Freund's adjuvant. After four weeks the procedure was repeated, but complete adjuvant was substituted for by incomplete Freund's adjuvant. Thereafter the animals were boosted with 250 µg CF_1 biweekly and antisera were drawn between two boosts. Anti-(δ subunit) serum was a gift from Nathan Nelson (then Haifa, Israel).

Rocket immunoelectrophoresis was carried out essentially as described by Laurell [28]. The buffer system used for pouring the plates and in the electrode chambers was modified to 75 mM Tris/boric acid, 340 µM $CaCl_2$, 0.05% (w/v) NaN_3, pH 8.6. Agarose solution was 1% (w/v), plates were 84 × 94 mm. Electrophoresis was performed at 20°C, 16 h, 2.5 V/cm. 100 µl antiserum against CF_1 under these conditions gave approximately 20 mm migration distance/µg CF_1. Staining, destaining and drying of the plates was as outlined in the LKB multiphor 2117 manual [29].

Western blots were run as in [30] with slight modifications. The blot buffer was 192 mM glycine, 125 mM Tris/HCl, 20% (v/v) methanol, pH 8.6. Blotting was for 1 h at 4°C, 1 A. Coating with bovine serum albumin was with a medium consisting of 50 mM Tris/HCl, 150 mM NaCl, 3% (w/v) bovine serum albumin, pH 7.2, 1 h at 37°C. Antisera were added in a dilution of 1:500 and incubation was carried out overnight at 4°C. The wash buffer consisted of 50 mM Tris/HCl, 150 mM NaCl, pH 7.2. Visualization of antigen-bound antibodies was achieved either with fluorescein-labeled or with peroxidase-conjugated goat anti-(rabbit-IgG) antibodies according to protocols provided by the supplier (Sigma Chemicals).

Reagents were of the highest grade available. DEAE-cellulose (DE-52) was from Whatman, Fractogel TSK DEAE 650 (S) and all nucleotides from Merck. YM 10 and Centricon ultrafiltration membranes were from Amicon. Acrylamide (4 × crystallized), other chemicals for electrophoresis and agarose (standard EEO) were from Serva (Heidelberg, FRG).

Table 1. *Comparison of purification data and molecular masses of CF_1 from spinach, peas and maize.* Spinach was bought from the market, peas were from 10-day-old pea seedlings, cut 4 cm above ground, and maize leaves were collected from the local area. Apparent subunit composition (predominant forms) and masses were taken from SDS electrophoresis. For calculation, the molecular mass of spinach β subunit was taken to be 53874 Da and the mass of spinach ε as 14702 [33]. The specific activity is given as μmol ATP hydrolysed min^{-1} mg $protein^{-1}$

Species	Yield from 1 kg leaves	Specific activity	Apparent subunit composition	Apparent molecular mass				
				α	β	γ	δ	ε
	mg	μmol min^{-1} mg^{-1}		kDa				
Spinach	168	8	$CF_1/CF_1(-\delta)$	58	54	38	21	15
Pea	28	22	$CF_1(-\delta)$	54	52	38	(21)	15
Maize	90	1.1	$CF_1(-\delta\varepsilon)$	58	54	40		

Luciferin/luciferase was from LKB, Freund's adjuvant from Difco, nitrocellulose from Sartorius and the labeled goat anti-(rabbit-IgG) antibodies from Sigma. Mega 8 and Mega 9 came from Oxyl GmbH (Bobingen, FRG).

RESULTS

Yield, subunit composition and specific activity of isolated CF_1

CF_1 was extracted in the presence of EDTA (0.1 mg chlorophyll/ml, 0.75 mM EDTA) and the extract was concentrated and chromatographed on Whatman DE-52 cellulose as described previously [10, 16, 32].

If carried out at 4°C elution from the DEAE-cellulose column produced two functional enzymes, ferredoxin:$NADP^+$ oxidoreductase (see Fig. 1 in [16]) and CF_1. CF_1-containing fractions were identified via the Mg^{2+}-ATPase activity in the presence of MeOH. Irrespective of the species, CF_1 from spinach, pea and maize thylakoids usually was eluted at approximately 15–30 mS/cm.

The yield of active CF_1 was found to depend mainly on the quality of the starting leaf material. Table 1 compares yields, specific activities, subunit composition and molecular masses of spinach, pea and maize CF_1 after DE-52 chromatography. A variable degree of extractibility with the three species was observed, spinach yielding the highest and peas the lowest amount of CF_1 (if similar losses during the preparation are assumed). This view was fully substantiated by quantitative immunoelectrophoresis that indicated up to 70% extraction with spinach thylakoids and 20% extraction with pea thylakoids. This result appears to indicate a tighter binding of CF_1 to CF_0 in peas than in spinach.

Specific activities (Mg^{2+}-ATPase) also differed substantially. With spinach the activities of the best preparations after Mono Q purification did not exceed 10–12 U/mg. Pea CF_1 after such treatment sometimes had activities higher than 45 U/mg, maize CF_1 activities remained essentially unchanged. High specific activities in the case of pea CF_1 might reflect activation via proteolytic cleavage though, with different response to the Mg^{2+}/MeOH assay after that cleavage.

According to SDS electrophoresis, after DE-52 cellulose chromatography the spinach preparation consisted of at least 50% five-subunit CF_1, whereas the predominant forms with peas and maize were $CF_1(-\delta)$ and $CF_1(-\delta\varepsilon)$ respectively. This would seem to suggest different binding affinities of the δ subunits to CF_0.

Apparent molecular masses (by sodium dodecyl sulfate electrophoresis) were roughly comparable, the most pronounced differences being the smaller masses of pea α and pea β subunits and a higher mass of maize γ as compared to the respective subunits from the other species. For reconstitution, pea and maize CF_1 preparations after DE-52 chromatography were used without further purification. Spinach CF_1 was purified by HPLC.

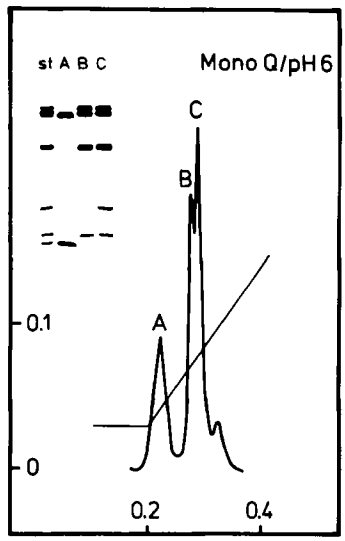

Fig. 1. *Purification of spinach CF_1 from DEAE-cellulose by HPLC on Mono Q.* Column: Pharmacia HR 5/5 Mono Q, 1 ml; buffer: A = 50 mM Mes/NaOH pH 6.0, B = A + 1 M NaCl; flow: 1 ml/min = 1.6 MPa; sample: 2 mg crude CF_1. The insert shows a SDS electrophoresis with 12% separating and 5% stacking gel according to Laemmli/Mattick [22, 23], 11 mA, 16 h. Migration was from top (–) to bottom (+). Silver stain was according to Wray et al. [24]. Samples 'st' represent the starting material. Peak A contains ribulose-bisphosphate carboxylase. Numbers on the abscissa and the inserted line indicate NaCl concentrations (M), numbers on the ordinate indicate absorption at 280 nm. (This and Fig. 2 are not corrected for the lag time between generation of the gradient and elution of the column)

Further purification of CF_1 by HPLC and separation of subunit-deficient CF_1

Fig. 1 shows a typical Mono Q run with spinach CF_1 at pH 6. The first peak at 215 mM NaCl contained ribulose-bisphosphate carboxylase, followed by $CF_1(-\delta)$ at 275 mM NaCl and CF_1 at 290 mM NaCl. The ratio of total CF_1:ribulose bisphosphate carboxylase was 2:1, the ratio CF_1:$CF_1(-\delta)$ was approximately 1:1, specific activity increased by 60%, no difference in activity between CF_1 and

Table 2. *Elution data of CF_1 species separation by HPLC*
The Mono Q column was a commercially available Pharmacia HR 5/5 1 ml column operated at 1 ml/min, 1.8 MPa. The TSK DEAE 650 (S) column was a self-prepared HR 16/10 20-ml column operated at 10 ml/min, 0.4 MPa. The buffers were in both cases A = 50 mM Mes/NaOH pH 6.0 and B = A + 1 M NaCl. Elution was by a linear gradient of buffer B in A

Column	Max. load/run	Elution point ([NaCl])					
		Spinach CF_1			Pea CF_1		
		$CF_1(-\delta)$	CF_1	$CF_1(-\varepsilon)$	$CF_1(-\delta)$	CF_1	$CF_1(-\varepsilon)$
	mg protein	mM					
Mono Q	8	275	290	330	275	320	380
TSK DEAE 650 (S)	100	225	240	280	200	210	250

$CF_1(-\delta)$ was observed (10–12 U/mg). Table 2 gives a summary of elution data for spinach and pea CF_1 on Mono Q and Fractogel TSK DEAE 650 (S). The table shows that with both spinach and pea CF_1 and on both columns the order of elution was $CF_1(-\delta)$, CF_1, $CF_1(-\varepsilon)$, i.e. removal of the δ subunit resulted in a weaker binding and removal of the ε subunit resulted in a tighter binding to the anion-exchangers as compared to five-subunit CF_1. The insensitivity against turbid solutions, high resolution and recovery rates and low costs make self-prepared TSK columns interesting candidates for further applications in HPLC.

Extraction of spinach thylakoids resulted in equal amounts of $CF_1(-\delta)$ and CF_1 whereas extraction of peas mainly yielded $CF_1(-\delta)$. Rechromatography of spinach CF_1 purified on Mono Q/pH 6 did not result in any further appearance of $CF_1(-\delta)$. This showed that the occurrence of CF_1 and $CF_1(-\delta)$ was not introduced during chromatography on Mono Q. Small amounts of $CF_1(-\varepsilon)$ also were present in spinach CF_1 preparations as evident from the minor peak behind peak C in Fig. 1.

Further fragmentation of spinach CF_1

This could be achieved by two different protocols: either by washing first Mono-Q-bound CF_1 with alcohol-containing buffers followed by elution without alcohols or by immediate elution in the presence of tensides. In both cases essentially three peaks around 190 mM, 320 mM and 350 mM salt were obtained. These peaks contained (after the alcohol wash) the β subunit with some contaminants ('βx'), $CF_1(-\delta)$, and $CF_1(-\varepsilon)$ respectively. In the presence of tensides a $\beta\delta$-fragment, $CF_1(-\delta)$ and $CF_1(-\delta\varepsilon)$ were eluted. The various CF_1 species deficient in the δ and/or ε subunit showed ATPase activities in the usual range of 10–12 U/mg. Mg^{2+}-ATPase activity in the peaks containing 'βx' and $\beta\delta$ usually was lower than 0.2 U/mg. Appearance of the first peak (βx or $\beta\delta$) was dependent on the hydrophobicity of the wash buffer or the tenside, the starting material and the absence of ATP/ADP. The protein contained in these peaks will be denominated simply 'βx' and '$\beta_n\delta$'. Experimental evidence for a stoichiometry of approximately 3:1 for the $\beta\delta$ fragment is given further below.

Elution profiles of two typical runs are shown in Fig. 2. Mono-Q-bound, glycerol/ethanol-washed enzyme yielded in the first elution peak mainly the β subunit. The amount and composition of accompanying proteins varied slightly from preparation to preparation. The contaminants consisted mostly of γ subunit and some bands between γ and β subunits. As proteolytic degradation was unlikely at that stage of purity

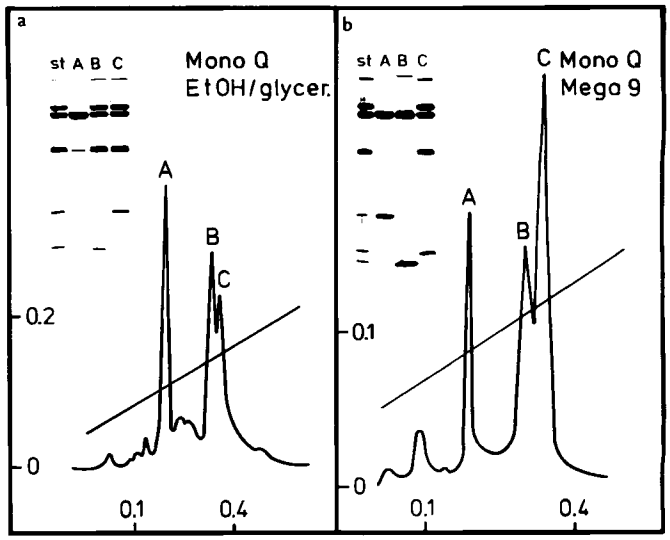

Fig. 2. *HPLC/anion-exchange runs of purified spinach CF_1 at Mono Q.* (a) Elution after washing with glycerol/ethanol. (b) Elution in the presence of Mega 9. Column: HR 5/5 Mono Q, 1 ml. Buffers: (a) A = 20 mM Tris/HCl pH 7.8, B = A + 1 M NaCl, C = A + 30% (v/v) glycerol +20% (v/v) ethanol, D = 25 mM Tris/HCl, 20 mM Mega 9, pH 7.8, E = D + 1 M NaCl; flow: 1 ml/min = 18 bar (buffers A, B, D, E) 0.5 ml/min = 40 bar (buffer C); sample: 2 mg CF_1. Inserts show SDS electrophoretic patterns, conditions as in Fig. 1. Sample 'st' indicates the starting material; peak B (a) contained ribulose-bisphosphate carboxylase. Washing with 30 ml buffer C was before onset of the gradient and is not shown in the elution profile. Numbers on the abscissa and the inserted line indicate NaCl concentration (M), numbers on the ordinate indicate absorption at 280 nm

it is probable that these bands originated from partially degraded α subunits that happened to become enriched in this peak. The α subunit usually was the most sensitive to denaturation. A series of experiments with different wash buffers, containing different amounts of methanol, ethanol, and glycerol, revealed that the ratio of the βx peak to the $CF_1(-\delta)$ and $CF_1(-\varepsilon)$ peaks was dependent on the dielectric constant of and the washing time with that buffer. The relative yield of βx as compared to $CF_1(-\delta)$ and $CF_1(-\varepsilon)$ increased with decreasing dielectric constant of the wash buffer, the total yield of protein decreased with decreasing dielectricity of that buffer. This indicated that the appearance of βx was related to some denaturation of CF_1, leaving the most stable subunit(s) or subunit clusters behind. Another interesting observation concerned the appearance of $CF_1(-\delta)$. This only was obtained with mixtures of CF_1 and $CF_1(-\delta)$ as starting

material. If purified CF_1 was used, besides βx only CF_1 and $CF_1(-\varepsilon)$ were eluted. It is tempting to speculate that $CF_1(-\delta)$ did not lose its ε subunit or at least not as rapidly as five-subunit CF_1. With respect to the order and composition of eluted samples [βx – $CF_1(-\delta)$ – $CF_1(-\varepsilon)$] we obtained results similar to those of Finel et al. [15], but by a different elution protocol. Interestingly, application of the same procedure as that described by Finel et al. [15] did not lead to elution of these proteins. This probably reflects differences between the CF_1 samples that were used: EDTA-extracted versus chloroform-extracted CF_1.

The same fragment $\beta_n\delta$ was obtained with different amounts of the tenside Mega 9 present in the elution buffer or with Mega 8. The relative yield of $\beta_n\delta$ in the first elution peak increased linearly with increasing amounts of Mega 9 (10–20 mm) and (at identical molarities of tenside) also increased by use of the more hydrophobic Mega 9 instead of Mega 8. This indicated that the relative hydrophobicity was important but not the critical micellar concentration (which should be between 10 and 20 mM according to the manufacturer). Precipitation of $\beta_n\delta$, $CF_1(-\delta)$, and $CF_1(-\delta\varepsilon)$ within 2 h after completion of the run with some preparations showed that, as with alcohols, denaturation participated in the process. Gel filtration of the samples on PD 10 immediately after the run removed enough tenside to prevent precipitation and to allow reconstitution. Elution (in the presence of Mega 9) of crude CF_1 (from the DE-52 step) separated the ribulose-bisphosphate carboxylase from $CF_1(-\delta)$ (peak B in Fig. 2b). In these cases resolution into $CF_1(-\delta)$ and $CF_1(-\delta\varepsilon)$ often could not be achieved (cf. Fig. 2b). Elution of $CF_1(-\delta)$ in the presence of Mega 9 did not yield the peak around 190 mM NaCl. This indicated that presence of the δ subunit was essential for appearance of the peak and made it likely that a $\beta_3\delta$ complex was eluted.

Both processes, i.e. the appearance of the βx peak after washing column-bound enzyme with alcohols and the appearance of the $\beta_n\delta$ peak upon elution in the presence of tenside, were strictly dependent on the absence of ATP: even micromolar amounts of ATP (or ADP) in the starting material were sufficient to suppress quantitatively the occurrence of βx or $\beta_n\delta$. In view of the well-known stabilizing effects of ATP on F_1, this again substantiates the notion that denaturing effects participated in and were necessary for the two processes. Despite this, in more than fifty single experiments the essential compounds βx, $\beta_n\delta$ and the various δ/ε-subunit-depleted CF_1 species were obtained, only contaminants especially with βx varied slightly.

Inspection of the SDS-electrophoretic pattern at a lower protein amount than that used in the insert of Fig. 2b (not shown) indicated a 3:1 stoichiometry for the $\beta\delta$ fragment. In order to evaluate the composition and ratio of subunits in this sample more precisely, western blot and (rocket) immunoelectrophoresis were applied. Fig. 3 shows on the left side a western blot of five-subunit spinach CF_1 reacted with anti-CF_1 and with anti-δ antibodies. It was apparent that the α, β and δ subunits were most reactive with the anti-CF_1 serum and that the anti-δ antibodies reacted exclusively with the δ subunit. On the right side of the same figure the results of a rocket immunoelectrophoresis are shown. It is evident that same masses of CF_1 and $\beta_n\delta$ gave approximately the same areas of rockets with the anti-CF_1 serum. With the anti-δ serum slightly larger areas with CF_1 than with $\beta_n\delta$ were obtained. These results showed that the $\beta_n\delta$ sample indeed was composed of β and δ subunits. Numerical evaluation revealed a probable stoichiometry of $\beta_3\delta$. Calculation was based on

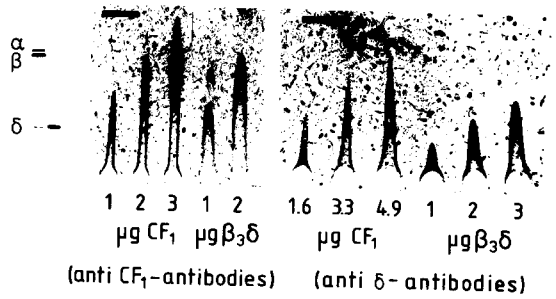

Fig. 3. *Western blot and rocket immunoelectrophoresis.* Left side: western blot with 10 μg CF_1 labeled with anti-CF_1 or anti-δ antibodies, visualization by fluorescein-labeled goat anti-(rabbit-IgG) antibodies. Right side: rocket immunoelectrophoresis with samples and amounts as indicated

the assumption that same areas of anti-CF_1 rockets with same masses of CF_1 and $\beta\delta$ do indicate the same stoichiometry with respect to the β subunit.

In principle it should have been possible to evaluate the stoichiometry of the δ subunit in CF_0CF_1 by the same technique. We obtained immunoelectrophoretic results that were in accordance with a stoichiometry of three δ subunits per CF_0CF_1. The 'rockets' were blasted, however, and therefore this result must be considered with caution. Probably the δ antibodies failed to react properly with CF_0CF_1 from thylakoids because the δ subunits were hidden within the complex, thus confirming an earlier observation by Berzborn et al. [5].

Reconstitution

Fig. 4 shows the reconstitution of cyclic photophosphorylation as a function of the amount of added CF_1 in CF_1-depleted chloroplasts from spinach (top) and peas (bottom). The amount of added CF_1, as given by the abscissa, has to be related to the amount of chlorophyll in each sample (10 μg). CF_1 depletion was performed as given in Materials and Methods and with 2 min incubation with EDTA. CF_1 was obtained from DE-52 chromatography or HPLC. It consisted mainly of integral CF_1 and $CF_1(-\delta)$ for spinach, $CF_1(-\delta)$ for pea and $CF_1(-\delta\varepsilon)$ for maize.

The following is immediately evident from Fig. 4. Under otherwise identical extraction conditions spinach chloroplasts lost photophosphorylating activity more readily than pea chloroplasts. The corresponding loss of CF_1, as measured via immunoelectrophoresis, was 70% (spinach) and 20% (pea). Cross-reconstitution between spinach and pea CF_1 and CF_0 was possible. In CF_1-depleted spinach chloroplasts pea $CF_1(-\delta)$ reconstituted less efficiently than spinach CF_1 and $CF_1(-\delta)$.

This could be interpreted either way. Less specific recognition of pea $CF_1(-\delta)$ by spinach CF_0 or failure of rebound $CF_1(-\delta)$ to plug the proton leak through CF_0.

We checked rebinding by quantitative immunoelectrophoresis and found it to be equal for both species. This argued against less specific recognition of pea $CF_1(-\delta)$ by spinach CF_0 and provided evidence for the δ-plug hypothesis. The apparent lack of discrimination between spinach and pea CF_1 in the lower part of Fig. 4 is discussed further in the Discussion.

The reconstitutional activity of subunit-depleted spinach CF_1 was studied in further detail. In order to obtain the relative efficiencies of the various subunit-depleted CF_1

Table 3. *Reconstitution of CF_1-depleted thylakoids from peas with spinach CF_1, $CF_1(-\varepsilon)$, $CF_1(-\delta)$, and $CF_1(-\delta\varepsilon)$*

Sample	ATP synthesized (single experiment)	Activity range as compared to CF_1 in n experiments	Bound CF_1
	$\mu mol\ h^{-1}\ mg\ Chl^{-1}$	%	mg CF_1/mg Chl
Pea thylakoids (= control)	660		0.35
CF_1-depleted thylakoids	61		0.20
CF_1-depleted thylakoids, reconstituted with			
CF_1	140	100	0.29
$CF_1(-\varepsilon)$	155	85–117 (n = 5)	0.39
$CF_1(-\delta)$	107	27–69 (n = 10)	0.49
$CF_1(-\delta\varepsilon)$	110		n.d.

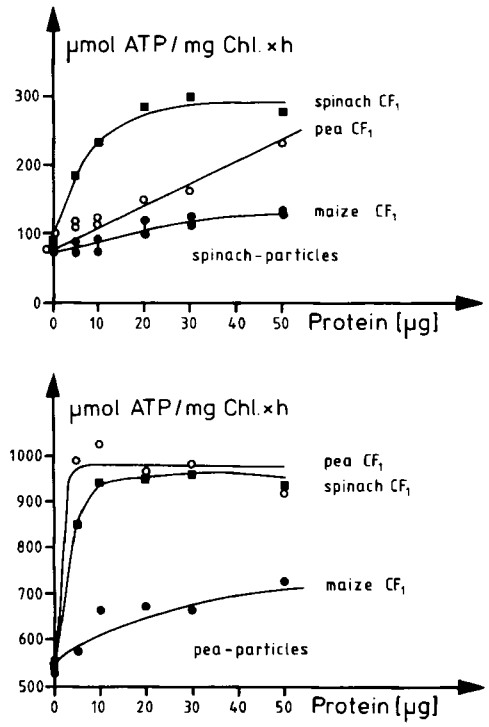

Fig. 4. *Reconstitution of cyclic photophosphorylation in CF_1-depleted chloroplasts as function of the amount of added CF_1.* Spinach chloroplasts synthesized 1064 µmol ATP h^{-1} µg chlorophyll^{-1}, pea chloroplasts 980 µmol h^{-1} mg chlorophyll^{-1}

species they were added at subsaturating amount (15 µg/10 µg chlorophyll). The purity of the species CF_1 (integral), $CF_1(-\delta)$, $CF_1(-\varepsilon)$, and $CF_1(-\delta\varepsilon)$ can be inferred from Figs 1 and 2. Pea chloroplasts underwent 10 min incubation in EDTA, which dropped the rate of photophosphorylation to 10% of control.

Table 3 shows the rebinding of (last column) and the reconstituted activities (second column) by the added CF_1 species. It is evident from the table that δ-subunit-deficient CF_1 was significantly less efficient in reconstitution than δ-subunit-containing CF_1 although neither lack of δ nor ε subunits affected the extent of rebinding. We concluded that the δ subunit was required for plugging the proton-conducting channel CF_0. A similar degree of rebinding of CF_1, $CF_1(-\delta)$ and $CF_1(-\varepsilon)$ to EDTA vesicles does not necessarily imply that the δ or ε subunit(s) were not required for rebinding. Instead it is possible that a complementary rebinding of CF_1 to CF_0 and of $CF_1(-\delta)$ to $CF_0(+\delta)$ occurred.

Reconstitution experiments also were conducted with the $\beta_n\delta$ and the βx fragments, with dicyclohexylcarbodimide(DCCD)-inhibited CF_1 and with DCCD-inhibited $CF_1(-\delta)$. The result is documented in Table 4. $\beta_n\delta$ reconstituted as well as CF_1. DCCD-inhibited CF_1 reconstituted one-half the activity that was reconstituted by active CF_1. On the other hand βx and DCCD-$CF_1(-\delta)$ were reconstitutively inactive. While the experimental results in Fig. 4 and Table 3 were suggestive of a plugging action of subunit δ on the proton channel, we consider the results shown in Table 4 as a proof for such a role.

SUMMARY AND DISCUSSION

CF_1 preparation by EDTA extraction of chloroplasts yielded different amounts and compositions of the extracted CF_1 in dependence of the plant species. While spinach thylakoids were extractable to 70%, pea thylakoids lost only 20% of their total CF_1. The extracted spinach CF_1 was composed of integral CF_1 and of $CF_1(-\delta)$ in approximately equal proportion. Pea CF_1 consisted mainly of $CF_1(-\delta)$. It was conceivable that the CF_1-depleted chloroplasts ('EDTA vesicles') also differed in the nature of the CF_0 portion which remained exposed on the membrane, namely CF_0 and $CF_0(+\delta)$ in spinach and (mainly) $CF_0(+\delta)$ in pea thylakoids.

Application of three different elution protocols in HPLC produced the following varieties of subunit-depleted or fragmented CF_1: reconstitutively active, integral CF_1, $CF_1(-\varepsilon)$, $CF_1(-\delta)$ and $CF_1(-\delta\varepsilon)$; reconstitutively active $\beta_n\delta$ and reconstitutively inactive βx.

Reconstitution of cyclic photophosphorylation in EDTA vesicles from spinach and from pea by rebinding of spinach CF_1/$CF_1(-\delta)$ and pea $CF_1(-\delta)$ yielded an interesting result (Fig. 4). While the spinach CF_1/$CF(-\delta)$ mixture acted similarly in both (spinach and pea) EDTA-extracted vesicles, pea $CF_1(-\delta)$ was much less efficient in the spinach vesicles than in the pea vesicles. This difference in reconstituted ATP-synthetic activity was not due to different degrees of rebinding.

We interpreted the different reconstitutional efficiencies between these species in the light of the double requirements for ATP synthesis, namely the integrity of the enzyme and concomitant availability of a sufficiently large protonmotive force. Enhanced rates of ATP synthesis in CF_1-depleted thylakoids can be induced by two different mechanisms: addition of catalytic capacity either without or with concomitant

Table 4. *Reconstitution of CF_1-depleted pea thylakoids with CF_1, $\beta_n\delta$, βx, $CF_1(-\delta)$, DCCD-CF_1, and DCCD-$CF_1(-\delta)$*
Zero-time values (i.e. samples that were quenched before illumination) were in all cases 20 μmol ATP h^{-1} mg chlorophyll^{-1}. Neither the buffers in which the samples were prepared (after gel filtration against 20 mM Tris/HCl) nor 50 μg ovalbumin, 50 μg bovine serum albumin nor 50 μg ribulose-bisphosphate carboxylase affected ATP synthetic activities of CF_1-depleted chloroplasts significantly. In a total of 20 reconstitution experiments with $\beta_n\delta$, 12 times the reconstituted activity was comparable to that reconstituted by CF_1, 6 times it was significantly lower, and 2 times there was no reconstitution at all. CF_1 and $CF_1(-\delta)$ in 25 mM Tris/HCl, pH 7.8 were incubated 1.5 h at room temperature in the presence of 2 mM DCCD, gel-filtered via PD 10 and concentrated via HPLC on Mono Q. Specific activities after this treatment were lowered by 80%

Sample	ATP synthesized		
	experiment 1	experiment 2	experiment 3
	μmol ATP h^{-1} mg chlorophyll^{-1}		
Pea thylakoids	810	622	637
CF_1-depleted thylakoids	349	229	237
CF_1-depleted thylakoids, reconstituted with			
50 μg CF_1	466	270	380
50 μg $\beta_n\delta$	445		
50 μg βx		237	
50 μg $CF_1(-\delta)$			319
50 μg DCCD-CF_1			309
50 μg DCCD-$CF_1(-\delta)$			226

plugging of proton leaks. The first possibility only can occur with vesicles which still were able to build up the necessary protonmotive force. Upon rebinding of e.g. $CF_1(-\delta)$ to $CF_0(+\delta)$ catalytic capacity is simply added to those vesicles. The second possibility (i.e. plugging proton leaks) results in enhanced ATP synthesis rates only if all open CF_0 per vesicle were plugged since in the accompanying paper it was demonstrated that a single open CF_0 per EDTA vesicle was sufficient to dissipate the protonmotive force within a few milliseconds. After plugging of all open CF_0 per vesicle with CF_1, for example, even coupling factors so far inactive are reactivated because the vesicle regained the ability to build up the necessary protonmotive force. We observed failure of reconstitution with vesicles still containing 10^7 chlorophyll molecules: these vesicles were so large and had lost so many CF_1 per vesicle that complete blocking of all open CF_0 became virtually impossible.

How are the different reconstitutional activities of spinach, pea and maize CF_1 in spinach and pea EDTA-vesicles to be explained? It was plausible to assume that (a) δ acted as a plug to otherwise proton-conducting CF_0 and that (b) integral CF_1 selectively rebound to CF_0 and $CF_1(-\delta)$ selectively rebound to $CF_0(+\delta)$. The spinach $CF_1/CF(-\delta)$ mixture then plugged proton leaks (by rebinding of CF_1) thereby increasing the amount of photophosphorylation vesicles to 30% of the control; further reconstitution of ATP-synthetic activity was not possible because the remaining vesicles had still too many proton-conducting CF_0. The fraction of $CF_1(-\delta)$ which was contained in spinach CF_1 also reconstituted by adding catalytic capacity to $CF_1(-\delta)$-depleted non-leaky vesicles. Pea CF_1, upon addition to spinach vesicles, was reconstitutively less active because it contained the necessary integral CF_1 only in low amount. Maize CF_1 contained even smaller amounts of integral, five-subunit CF_1 and therefore it reconstituted still lower activities than pea CF_1. Taken together this means that spinach thylakoids upon extraction lost CF_1 and $CF_1(-\delta)$ in roughly equal amounts. Spinach EDTA vesicles then were composed of several subpopulations. Vesicles that lost several CF_1 were not reconstitutable at all, therefore a maximum of 30% of the activity of the control was obtained upon reconstitution. The remaining populations contained either predominantly (proton-conducting, open) CF_0 or (still sealed, exposed) $CF_0(+\delta)$. In both cases the leaks first had to be sealed by reconstitution of five-subunit CF_1, then addition of catalytic capacity by rebinding of $CF_1(-\delta)$ to $CF_0(+\delta)$ became detectable.

The major difference between pea and spinach EDTA vesicles was that peas not only lost less CF_1 but also predominantly $CF_1(-\delta)$. Again, first the proton-conducting CF_0 had to be plugged before further addition of catalytic capacity by rebinding of $CF_1(-\delta)$ to $CF_0(+\delta)$ became detectable. However, with peas only a small amount of five-subunit CF_1 was needed in order to fulfill this role, these amounts of CF_1 were contained in both spinach and pea CF_1 preparations, therefore the curves of reconstitution of photophosphorylation were very similar both with pea and spinach CF_1. The foregoing interpretation implied that the δ subunit might participate in rebinding as it was always present in all cases where functional reconstitution was obtained.

The suggested function of subunit δ as a plug to open CF_0 was further substantiated by the experiments presented in Table 3: δ-deficient CF_1 was less reconstitutively active than δ-containing CF_1 although its relative efficiency (as compared to five-subunit CF_1) was surprisingly high (50%). This again was in accordance with the view that $CF_1(-\delta)$ selectively rebound to $CF_0(+\delta)$: as upon rebinding to CF_0 no catalytic activity could have been regained, extensive rebinding of $CF_1(-\delta)$ to the 'wrong' CF_0 is hard to reconcile with the relatively high amount of reconstituted photophosphorylating activity after addition of δ-deficient CF_1 to EDTA vesicles. The observed high efficiency of $CF_1(-\delta)$ reconstitution might be related to the fact that more $CF_0(+\delta)$ was present for rebinding than CF_0.

The most interesting result was the reconstitution of photophosphorylation by catalytically inactive $\beta_n\delta$. We took this as proof for the previously proposed role of subunit δ to act as plug to the open proton channel [11, 36, 37]. $\beta_n\delta$ reconstituted photophosphorylation at high rates in a majority of experiments. Occasional failure of reconstitution (10% of experiments) with $\beta_n\delta$ might have been due to the

requirement of more than one δ subunit per CF_0CF_1 as proposed by Berzborn et al. [5]. DCCD-inhibited CF_1 was reconstitutively active, while DCCD-inhibited $CF_1(-\delta)$ was not. This finding also is on line with the previous suggestion that δ may function as a stopcock to CF_0 proton channels. As the δ subunit alone was not investigated but always accompanied by β, strictly speaking we cannot exclude the possibility that δ and β acted in a concerted manner. It is possible, for example, that the function of δ as a plug to CF_0 depended on a proper orientation achieved by β.

In intact CF_0CF_1 the stopcock would be of no value if it was not relieved to admit controlled proton transport during ATP synthesis. Thus it may be visualized that in energy-transducing chloroplast coupling factors δ not only acts as stopcock but as valve or even as energy transducer (like a piston in a combustion engine).

APPENDIX

Protein assays were done according to Sedmak and Grossberg [17]. Protein concentrations were calculated by assuming $A_{595} = 0$ at 0 µg protein and $A_{595} = 0.57$ at 25 µg protein. The calibration curve is linear between these two points with ovalbumin as standard. If absorbance readings higher than 0.57 were obtained, the measurement was repeated with diluted samples. The assay was compared with the more commonly used Lowry procedure (with ovalbumin or bovine serum albumin as standards) and with the recently introduced Pierce BCA protein assay, which is a derivative of the Lowry assay. Results, however, were very similar and independent of the standard (with purified CF_1 as sample). In general higher amounts of sample protein gave lower protein concentrations. This effect is probably due to adsorption and aggregation. It was compensated for by measuring at least three values at different protein amounts per assay and averaging the results.

ATPase was measured in the presence of methanol [18–20]. The reaction mixture contained 40 mM Tris/HCl, 4 mM ATP, 1.5 mM $MgCl_2$, pH 8.0, 30% (v/v) MeOH, 0.5–5 µg CF_1, in a total volume of 0.5 ml. After 10 min incubation at 37 °C, the reaction was terminated by addition of 0.5 ml 0.5 M trichloroacetic acid; P_i was complexed then by adding 0.5 ml of a solution containing 4 ml 16% (w/v) ammonium heptamolybdate in 5 M H_2SO_4/36 ml H_2O with 2 g $FeSO_4 \cdot 7\,H_2O$ and monitored via absorption at 740 nm [21]. Specific activity was calculated according to $(1000 \times A_{740})/(22.75 \times CF_1$ per assay, µg).

ATP synthesis was measured 'on line' by adding a medium consisting of 0.1 M sorbitol, 10 mM NaCl, 50 mM Tricine/NaOH, 2 mM KH_2PO_4, 5 mM $MgCl_2$, pH 8.0 to the reconstitution mixture up to a volume of 965 µl. After addition of 30 µl 0.1 M potassium-ADP and 5 µl 0.01 M phenazinemethosulfate, the mixture was exposed for 1 min to saturating white light, heat-filtered through water cuvettes. Quenching was then achieved by adding 250 µl 0.5 M trichloroacetic acid followed by 750 µl H_2O. Samples were kept on ice and measured within 60 min.

SDS gel electrophoresis was carried out essentially according to the Laemmli procedure [22], including some modifications [23]. The acrylamide stock solution: acrylamide: *N,N'*-methylenebisacrylamide(Bis) = 40:1, contained 30 g sucrose, 30 g acrylamide and 0.75 g Bis in 100 ml. Separating gel buffer was 1.5 M Tris/HCl, pH 8.8; stacking gel buffer was 0.5 M Tris/HCl, pH 6.8; sample buffer contained in a volume of 24 ml 3.8 ml 10% (w/v) SDS, 3 ml stacking gel buffer, 3 ml glycerol, 0.24 ml 2-mercaptoethanol, 0.6 ml 0.05% (w/v) bromophenol blue, 1.44 g urea, pH 7.2. Sometimes, prior to use, phenylmethylsulfonylfluoride to a final concentration of 1.0 mM was added. Electrode buffer contained in 1 l 3 g Tris, 14.4 g glycine, 10 ml 10% (v/v) SDS, 30 g urea, and was used without any setting of the pH. Separating gel (12%) was usually prepared 5 h before use and contained 11.78 ml H_2O, 8.75 ml separating gel buffer, 0.35 ml 10% (v/v) SDS, 14 ml acrylamide:Bis = 40:1, 0.12 ml freshly prepared 10% (w/v) ammonium persulfate. Polymerization was initiated by adding 9 µl *N,N,N',N'*-tetramethylethylenediamine (Temed) and degassing was achieved by ultrasonication in a bath for 10–20 s. Stacking gel (5%) was cast 1.5 h prior to use and contained 5.6 ml H_2O, 2.5 ml stacking gel buffer, 0.1 ml 10% (w/v) SDS, 1.7 ml acrylamide:Bis = 40:1, 0.1 ml 10% (w/v) ammonium persulfate, 5 µl Temed. Gel dimensions were $13 \times 13 \times 0.15$ cm, stacking gel length approximately 1 cm. Samples were prepared by mixing 100 µl sample buffer with up to 50 µl sample. Total protein content was around 5 µg with CF_1 alone, in cases where several proteins were to be expected, protein amounts were chosen to be higher. Samples in sample buffer were either heated at 100 °C for 2 min or kept for several hours at room temperature. Electrophoresis was performed overnight at initial settings of 35 V, 11 mA, constant current.

Staining was performed essentially according to the ammoniacal silver stain procedure as published by Wray [24]. After completion of the run the gel was washed briefly with deionized water, sometimes stained with 0.05% (w/v) Serva blue G in 50% (v/v) MeOH, 5% (v/v) acetic acid for 2 min, then destained in 25% (v/v) MeOH, 5% (v/v) acetic acid at least for 3 h with at least four changes of destain solution or, if Coomassie staining was performed, until the blue background had disappeared completely. Then the gel was incubated for 3 h in 50% (v/v) MeOH (technical grade, i.e. in the presence of traces of formaldehyde) with at least two changes of the solution. This was followed by two washing steps in H_2O for about 2 min each. Between all changes of solution the gel and the tray were rinsed briefly with deionized water. Silver solution was prepared [A = 0.85 g $AgNO_3$ in 5 ml H_2O; B = 22.2 ml 0.36% w/v NaOH + 1.5 ml conc. NH_3; A added dropwise to B with vigorous stirring, if precipitation of $Ag(OH)_2$ occurred, more NH_2 was added; finally made up to 100 ml with H_2O] and the gel was incubated in the silver solution in the dark for 15–20 min. Afterwards the gel was washed with 2–3 changes of H_2O in 2-min intervals, then 500 ml H_2O containing 5 ml 1% (w/v) citric acid and 0.5 ml 37% formaldehyde were added. Development of the stain occurred within 20 min. When the background started to turn yellow or brown, 100 ml MeOH were added to the mixture, followed by 1 min further incubation, then the gel was washed with deionized H_2O, briefly immersed into a solution containing 200 mM ammonium thiosulfate with a few drops of acetic acid and immediately photographed. Sometimes the gel was washed 2×20 min in H_2O and dried. All incubations from the very beginning were performed under continuous shaking. Gels were touched with vinyl gloves only in order to avoid fingerprints.

We would like to thank Mrs K. Schürmann for skilled and dedicated assistance and Mrs H. Kenneweg for preparation of the figures and photographs. The introduction to rabbit immunization by Dr Radunz (Uni Bielefeld) and suggestions and modifications by G. Deckers-Hebestreit concerning the western blot are greatly appreciated. Rabbit anti-δ antibodies were a kind gift of Prof. N. Nelson,

Israel. Financial support by the *Deutsche Forschungsgemeinschaft* (SFB 171/B2/B3) is gratefully acknowledged.

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