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Studies on the heterogeneity of the soluble chloroplast coupling factor 1: the formation of ϵ-deficient isozymes

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Release of the chloroplast coupling factor 1 (CF_1) from thylakoid membranes by chloroform yields a heterogeneous population of isozymes. Following solubilization, subtle changes in the isozyme distribution occur. In particular, the percentage of ϵ-deficient isozymes increases. Five parameters were evaluated for their role in the distribution of isozymes and net enzyme yield. (i) Extraction of spinach thylakoid membranes yields a five-subunit holoenzyme and derivative isozymes whereas *Chlamydomonas reinhardtii* membranes yield isozymes lacking a δ-subunit. (ii) Supplementing extraction buffers with $MgCl_2$ or $CaCl_2$ dramatically suppresses the release of total CF_1, but does not appear to affect the distribution of isozymes. (iii) Loss of CF_1 by non-specific protein denaturation becomes detectable 24–48 h after enzyme solubilization. Analysis of CF_1 samples stored at room temperature for extended periods (up to 2 months) shows that the loss of solubility is uniform for the isozyme complex, rather than selective for a given subunit. (iv) Proteolysis does not significantly affect the distribution of the ϵ-subunit either before or after enzyme solubilization. (v) Dithiothreitol greatly enhances the formation of ϵ-deficient isozymes from ϵ-containing isozymes in both species studied and appears to account for the subtle changes observed following CF_1 solubilization.

Introduction

The chloroplast coupling factor 1 (CF_1) contains the catalytic domain of the proton-translocating ATP synthetase. It is an oligomeric protein which can be readily solubilized from the membrane-embedded proton channel, CF_0. The stoichiometry of non-identical subunits in CF_1 is most likely $\alpha_3\beta_3\gamma_1\delta_{1-2}\epsilon_1$ (in order of descending mass) and the molecular weight of the holoenzyme has been estimated to be approx. 420000 (see Refs. 16, 26 and 27 for reviews).

Conceivably, an oligomeric protein might exist as a population of subset oligomers. Evidence in the literature supports heterogeneity in the composition of CF_1. In attempts to establish the CF_1 subunit stoichiometry of the spinach enzyme, Binder et al. [5] noted that the ϵ and δ subunits did not fit a reproducible whole-number stoichiometry as well as the three larger subunits did. Indeed, they suggested "...that isolated CF_1 should be thought of as a complex, rather than as a single enzyme with one entirely unique composition". Similarly, heterogeneity was observed for the *Chlamydomonas reinhardtii* CF_1 which electrophoresed as a doublet in non-denaturing polyacrylamide gels [20,24].

Stronger evidence for the existence of heterogeneity was obtained through the use of high-resolution chromatographic resins [4,7,8,9]. In these references, CF_1 was first purified via low-resolution techniques, then rechromatographed via high-resolution chromatography to obtain the five subunit holoenzyme, $CF_1(-\epsilon)$, $CF_1(-\delta)$, and $CF_1(-\delta,\epsilon)$ isozymes. However, there is some ambiguity as to whether rechromatography itself generates, rather than resolves, the CF_1 isozymes.

There is also variability regarding the retention of the δ-subunit as an integral and stable part of CF_1 when the enzyme is isolated via the chloroform extraction technique [7,9,11,24,28].

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TLCK, *N*(α)-*p*-Tosyl-L-lysine chloromethyl ketone; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tricine, *N*-tris(hydroxymethyl)methylglycine; DTT, dithiothreitol; Tris, tris(hydroxymethyl)-amino methane; CF_1, chloroplast coupling factor 1; $CF_1(-\delta)$, chloroplast coupling factor 1 lacking the δ subunit; $CF_1(-\epsilon)$, chloroplast coupling factor 1 lacking the ϵ-subunit; $CF_1(-\delta,\epsilon)$, chloroplast coupling factor 1 lacking the δ- and ϵ-subunits; Chl, chlorophyll; Mega 9, *N*-D-gluco-2,3,4,5,6-penta(hydroxylhexyl)-*N*-methylnonanamide.

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We have, therefore, re-examined the question of the heterogeneity of CF_1 isolated via this technique. In this paper, we show that the distribution of the isozymes in the crude CF_1 extract varies following solubilization. This variation is probably not the result of specific proteolysis or selective denaturation of any of the isomeric forms of the enzymes or of any of the constituent subunits. Rather, this variation can be greatly modulated by subtle modifications of the purification technique. In particular, we demonstrate the dramatic effect that DTT has on the stability of ϵ-containing isozymes.

Materials and Methods

Preparation of CF_1. The purification procedure used was a composite of previously described techniques [4,7,9,11,24,28]. Cells were broken (algal with a glass bead beater; spinach in a Waring blender) in a buffer containing 50 mM Tricine-NaOH (pH 8.0) supplemented with 100 μM PMSF. Membrane particles were sedimented by centrifugation at $20000 \times g$ and washed five times at a concentration of 0.1–0.2 mg Chl/ml in 10 mM sodium pyrophosphate (pH 7.8) supplemented with 100 μM PMSF. Washed membranes were resuspended to about 2–3 mg Chl/ml in extraction buffer (10% (v/v) glycerol, 1 mM EDTA, 1 mM ATP, 5 mM DTT, 0.1 mM PMSF, 0.1 mM *p*-aminobenzamidine, 0.1 mM TLCK, 20 mM Tricine-NaOH (pH 7.5)), and then mixed with 1/2 volume of cold chloroform. The phases were separated by centrifugation of the suspension at $4000 \times g$. The CF_1-containing aqueous phase was clarified by further centrifugation at $30000 \times g$ for 20–30 min. Samples were kept ice-cold up through the solubilization step to minimize proteolysis. Clarified crude CF_1 was loaded directly onto a Mono Q HR 5/5 (or 10/10) column 1 h after mixing with chloroform (with the exception of the data presented in Fig. 1). The column was first washed isocratically with low salt buffer. Protein was then eluted with a continuous gradient of low to high salt buffer (120–400 mM NaCl in 10% glycerol, 1 mM DTT, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5). Column size and gradient volumes were selected based on anticipated protein yields (see figure legends for details). In order to prevent cold denaturation, chromatographed samples were stored at room temperature until further assayed.

Proteolysis protection. Sample aliquots were incubated in 1.5 ml microfuge vials with one-tenth volume of 0.15% sodium deoxycholate for 10 min, followed by the addition of one-tenth volume of ice-cold 72% trichloroacetic acid as described in Ref. 19. The samples were centrifuged, the supernatants discarded, and the pellets stored at −80 °C until further prepared for SDS-PAGE.

Assaying the loss of solubility of CF_1 over extended storage times. CF_1 isozymes which had been initially purified as above were stored at room temperature for 7 weeks to allow the putative isozyme redistribution to completely equilibrate. Soluble protein was separated from insoluble (denatured) protein by centrifugation at $13000 \times g$ for 10 min. The pellet was washed once with 50 mM Tricine-NaOH (pH 7.5), then centrifuged as above. 'Total' isozymes were prepared by precipitating the sample (with deoxycholate and ice-cold trichloroacetic acid as in Ref. 19) before centrifugation and dissolving the resultant pellet with SDS-PAGE sample buffer; 'insoluble' isozymes were separated as described above, then dissolved in SDS-PAGE sample buffer; 'soluble' isozymes were prepared by precipitating the centrifugal supernatants as above and dissolving the resultant pellet with SDS-PAGE sample buffer. The dissolved proteins were characterized by SDS-PAGE [12].

Miscellaneous. Immunoblots were performed essentially as described in Ref. 30, except that 3% gelatin was used as a blocking reagent rather than 5% fetal calf serum. Protein [6] and chlorophyll [2] assays were performed according to previously published procedures. All SDS-PAGE analyses used a 4% acrylamide stacking gel and a 15% running gel as described previously [12]. Unstimulated MgATPase assay incubation mixtures contained 4 mM [γ-^{32}P]ATP, 2 mM $MgCl_2$, and 40 mM Tris-HCl (pH 8.0). $\frac{1}{2}$–1 μg of enzyme was assayed at 37 °C for 2 min essentially as described in Ref. 25. The octylglucoside-stimulated MgATPase incubation mixtures additionally contained 40 mM *n*-octylglucoside [21] and were assayed in the same fashion.

Protein profiles of chromatographs were obtained by using a modification of the Bradford assay [6]. 20–100 μl of sample were mixed in microtiter plates with 230–150 μl of Bradford reagent (sample and reagent proportions are listed for each figure), and the absorbance at 590 nm was measured with a BIO-TEK model EL-308 EIA Reader.

Materials. Spinach (Bloomsdale variety) was grown indoors with a 12 h light cycle. Leaves were harvested about 6–8 weeks after germination. *Chlamydomonas reinhardtii* wild-type strain 137+ was grown under continuous light in 14 l cultures under conditions previously described [24]. [γ-^{32}P]ATP was prepared essentially as previously described [15]. [^{32}P]Phosphate was purchased from New England Nuclear. Dithiothreitol was obtained from Boehringer Mannheim Biochemicals. The fast protein liquid chromatograph (FPLC), the Mono Q HR 5/5 and 10/10 anion-exchange columns and the Superose 12 gel-permeation column were purchased from Pharmacia. The ultrafiltration apparatus and the PM-10 ultrafiltration membranes were from Amicon. Polyclonal antibodies directed against the ϵ-subunit of *Chlamydomonas reinhardtii* CF_1 were raised in rabbits and were the generous gift of Dr. Sabeeha Merchant.

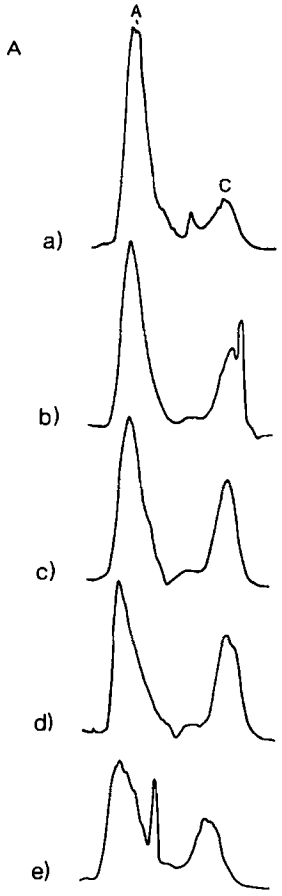
Results

Subtle, time-dependent changes in isozyme distribution

A series of chromatographs were obtained by direct application, at timed intervals, of aliquots taken from the clarified aqueous phase of chloroform-treated *C. reinhardtii* thylakoid membranes onto an FPLC (Mono Q) column. This series is presented in Fig. 1 (panel A). Based on the protein profiles of the column eluate, individual peaks were pooled, re-assayed for total protein, and assayed for both latent (octylglucoside-stimulated) and manifest (already activated) Mg^{2+}-dependent ATPase activity. In addition to a non-ATPase protein peak which eluted in the isocratic phase, extracts of *C. reinhardtii* membranes usually contained only two major protein peaks (labelled A and C in Fig. 1). Both of these peaks had latent ATPase activity (average specific activity was 18 $\mu mol \cdot mg^{-1} \cdot min^{-1}$); however, only peak C had manifest activity (average specific activity was 1.4 $\mu mol \cdot mg^{-1} \cdot min^{-1}$).

Fig. 1 illustrates that the relative proportions of the total protein in the two peaks changed with time. For example, when the aliquot was chromatographed within 1 h of preparation of the extract (i.e., after removal of the chloroform phase), peak A contained more than 60% of the total *n*-octylglucoside-stimulated MgATPase activity. When the sample was stored (0–4 °C) for 24 h or longer the latent MgATPase activity in peak A decreased to about 44% (Fig. 1 panel B). Furthermore, the relative apparent increase in intensity in peak C was confirmed to be an absolute increase in the amount of protein in that peak. That increase arose at the apparent expense of peak A and correlated to an absolute increase in the total amount of manifest (i.e., already activated) Mg^{2+}-dependent ATPase activity associated with peak C (Fig. 1 panel B). After extended periods following solubilization, the intensity of both peaks became smaller, reflecting the fact that the total amount of recovered protein (from peaks A and C) per application diminished, and both the total latent and manifest Mg^{2+}-dependent ATPase activities diminished. Although the data shown in Fig. 1 were obtained with the *C. reinhardtii* enzyme, qualitatively similar results have also been obtained for the purification of the chloroform-released spinach CF_1 (data not shown).

Fig. 2A shows the electrophoretic pattern of contiguous FPLC fractions from a typical *C. reinhardtii* CF_1 purification. Gel lanes a through f are from protein peak A (Fig. 2B) which contained latent, but not manifest, Mg^{2+}-dependent ATPase activity. Note that the



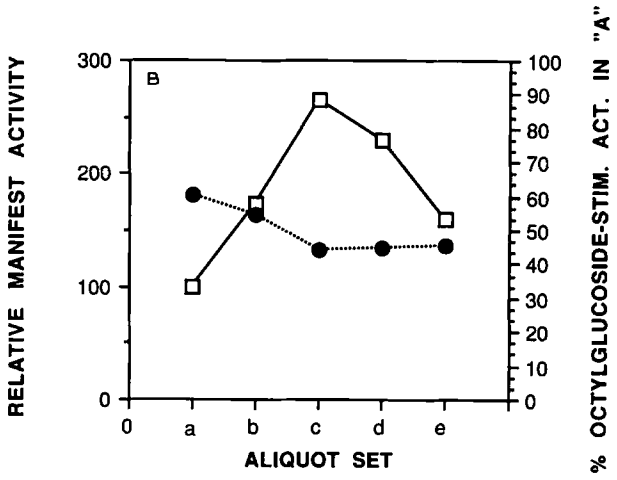


Fig. 1. Time-dependent changes in the isozyme distribution of *C. reinhardtii* CF_1 as analyzed by FPLC. 5-fold washed *C. reinhardtii* thylakoid membranes (215 mg Chl) were extracted as described in Materials and Methods. Equal volume aliquots were injected onto a Mono Q HR 5/5 using a 20 ml isocratic wash (40 mM NaCl) and a 50 ml (40–400 mM NaCl) continuous gradient. Profiles of absorbance at 280 nm are shown in panel A for the section of the chromatograph containing CF_1 isozymes purified from crude CF_1 injected at 1 h (a), 7 h (b), 24 h (c), 83 h (d) and 134 h (e) after chloroform extraction. The activity data subsequently obtained from the purified recovered isozymes is summarized in panel B. The total manifest (unstimulated) MgATPase activity is plotted relative to the total manifest activity recovered from the first chromatograph (open squares, solid line). The data represented by the filled circles (dashed line) is the percent of the total *n*-octylglucoside-stimulated MgATPase activity which was present in peak A.

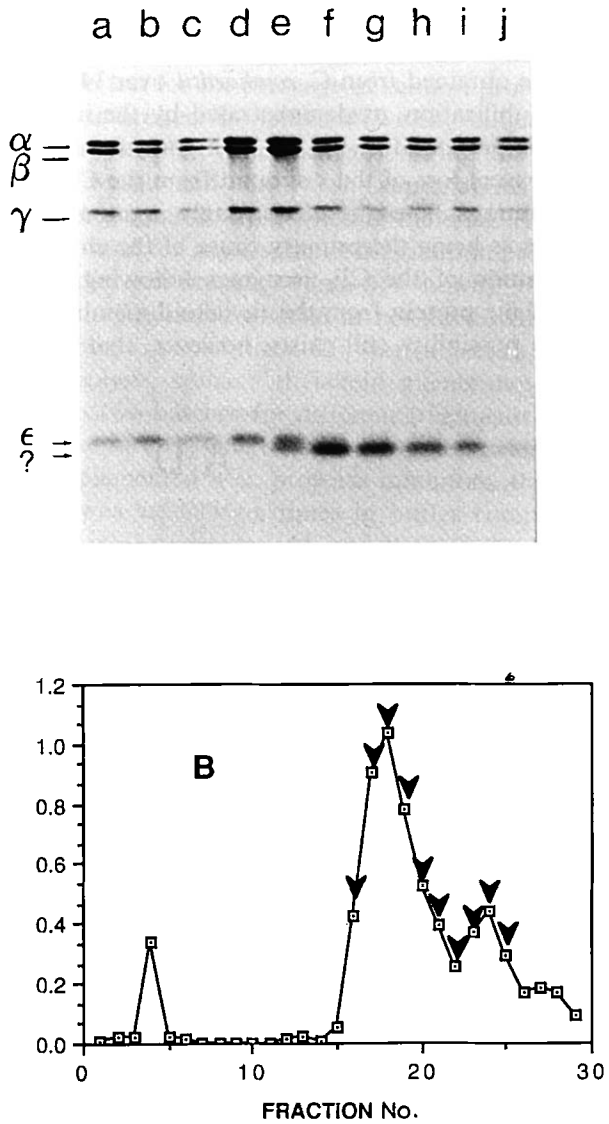


Fig. 2. SDS-PAGE of contiguous protein fractions of FPLC purified *C. reinhardtii* CF_1. Aliquots of contiguous FPLC fractions (identified with arrowheads in panel B) were prepared for SDS-PAGE analysis (panel A). The upper arrow points to the ε-subunit, the lower arrow points to an unidentified peptide which is not the ε-subunit (see text). The *C. reinhardtii* CF_1 sample was purified as in Fig. 1, except that a 120–400 mM NaCl gradient was used to develop the chromatograph, only a portion of which is shown in the figure. 50 μl of sample and 200 μl of Bradford reagent were mixed to obtain the protein profile shown in panel B.

ε-subunit (see upper arrow in figure) was present in all of these fractions. The identification of this peptide as the ε-subunit was based on the inhibitory effect of the ε-subunit on CF_1 ATPase activity [1,9,18,22]. Beginning in lane e there was a second peptide band (see lower arrow in figure) which migrated just below the ε-subunit from peak A. This peptide, which was not found in similar spinach preparations, continued to appear in subsequent fractions (including all of peak C) which had both manifest and latent ATPase activity. Though this band might be confused with the ε-subunit, several lines of evidence support that this peptide is not tightly associated with CF_1, and that it is probably not related to the ε-subunit. This evidence includes the resolution of this peptide from CF_1 isozymes by Superose 12 gel filtration chromatography, partial resolution by repeated Mono Q anion exchange chromatography, and a lack of significant cross-reactivity to polyclonal antibodies raised against the ε-subunit from *C. reinhardtii* CF_1 (data not shown). Thus, peak A (Fig. 1) represents the four subunit *C. reinhardtii* CF_1 lacking the δ-subunit whereas peak C represents a three subunit *C. reinhardtii* CF_1 lacking both the δ and ε subunits. As summarized in Table I, equivalent assignments can also be made for the spinach CF_1 isozymes.

Taken together, Figs. 1 and 2 show that when CF_1 is released from thylakoid membranes by chloroform extraction, there is a gradual conversion of ε-containing CF_1 isozymes into ε-deficient, ATPase activated CF_1 isozymes. This occurs regardless of the source of the enzyme (spinach data not shown). The nature of this conversion was investigated further.

Proteolysis does not significantly affect the isozyme distribution

Numerous workers have suggested that proteolysis is responsible for the loss of the δ-subunit from CF_1 when the enzyme is prepared by the chloroform extraction

Fig. 3. Immunoblot analysis of solubilized CF_1. Equal amounts of crude (unchromatographed) *C. reinhardtii* CF_1 were protected against further proteolysis as described in Materials and Methods, then electrophoresed via SDS-PAGE. The samples were then electroblotted onto a nitrocellulose sheet which was subsequently immunodecorated with antibodies raised against the isolated ε-subunit and visualized as described in Materials and Methods. Times elapsed between chloroform teatment and protease protection were 1 h (lane c); 4 h (lane d); 16 h (lane e); 24 h (lane f) and 143 h (lane g). Lanes b and h contain purified $CF_1(-\delta)$; lanes a and i contain pre-stained markers with approximate molecular masses of 130, 75, 50, 39, 27 and 17 kDa.

method (cf. Ref. 17). Therefore, proteolysis was considered as a potential cause for the apparent progression of ϵ-containing CF_1 to ϵ-deficient CF_1. In order to test this possibility, crude CF_1 extracts were allowed to stand for varying periods of time (up to 72 h) before aliquots were removed and protected against further proteolysis as described in 'Materials and Methods'. Comparison of the electrophoretic polypeptide patterns of these aliquots revealed no evidence for proteolytic degradation of either the δ-subunit from spinach CF_1 or the ϵ-subunit from both *C. reinhardtii* CF_1 and spinach CF_1 (data not shown). In addition, no ϵ-subunit degradation products were detected in the post-release crude CF_1 mixture obtained from *C. reinhardtii* over 143 h following solubilization, as demonstrated by the immunoblot analysis presented in Fig. 3. This analysis also showed no apparent loss of the ϵ subunit from the CF_1-containing solutions. These results strongly argue against proteolysis as being the primary cause of the change in the distribution of the CF_1 isozymes following solubilization of the protein from the thylakoid membranes.

The possibility still exists, however, that proteolysis

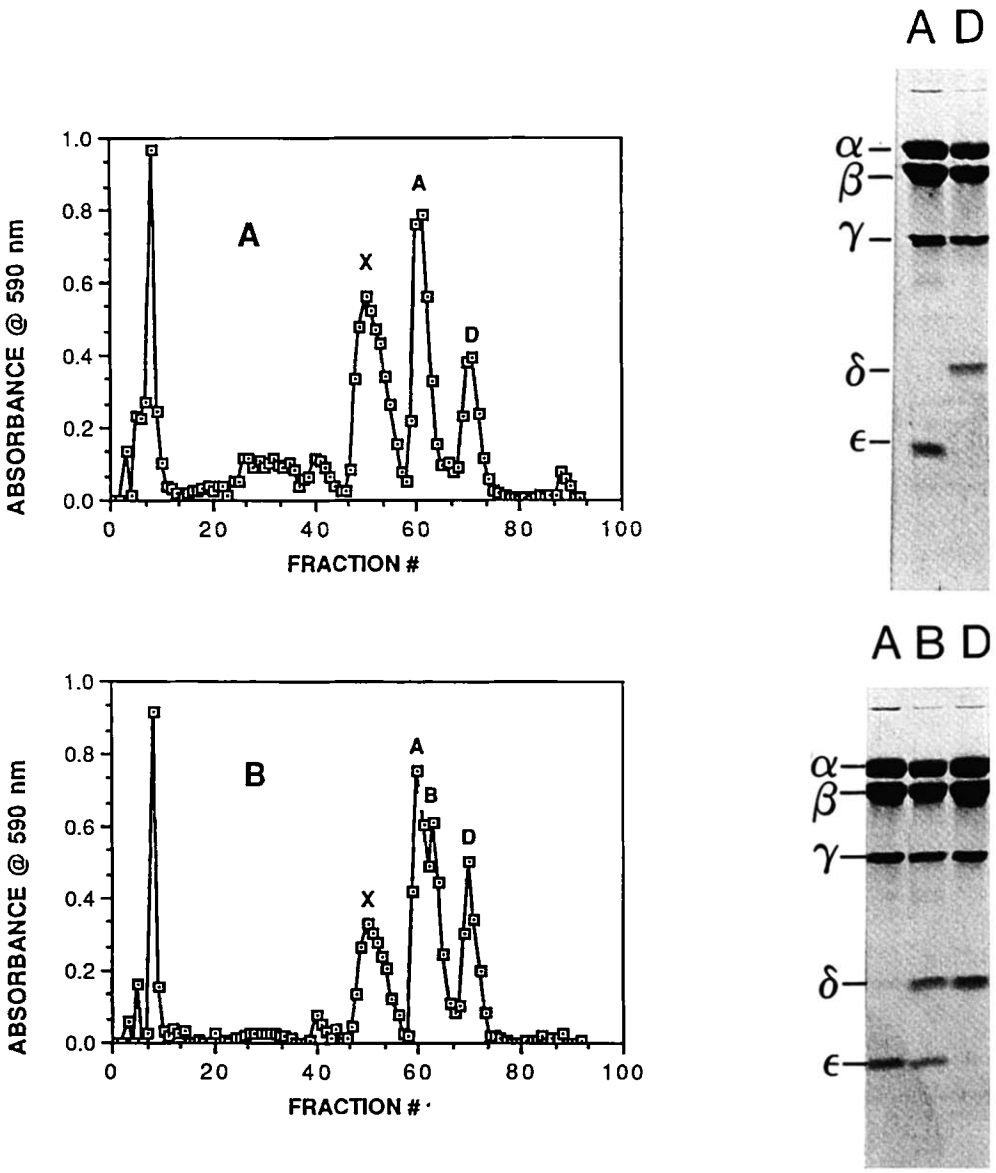


Fig. 4. The effect of the number of pyrophosphate washes on the yield of CF_1. Spinach leaves were ground in a buffer containing no protease inhibitors and membranes were divided into two equal portions. The first portion was washed twice with pyrophosphate buffer supplemented with 0.1 mM TLCK, 0.1 mM PMSF, and 0.1 mM *p*-aminobenzamidine. The thylakoid membranes (containing 164 mg chlorophyll) were chloroform extracted and the clarified crude CF_1 injected onto a Mono Q HR 10/10 1 h after solubilization (panel A; 100 ml isocratic wash; 500 ml continuous gradient). The second portion was washed six times in the absence of protease inhibitors. These thylakoid membranes (containing only 120 mg chlorophyll) were extracted and chromatographed (panel B) as above. The protein profiles shown were obtained after mixing 40 μl of sample with 210 μl of Bradford reagent. The total recovered CF_1 from the twice-washed membranes was 7.3 mg (from 164 mg chlorophyll) versus 9.5 mg (from 120 mg chlorophyll) from the six-fold washed case. (Note: Peak X is ribulose bisphosphate carboxylase.)

of the protein bound to thylakoid membrane prior to enzyme solubilization generated the initial ϵ-deficient isozymes. To test for the presence of subunit proteolytic fragments, unbroken *C. reinhardtii* cells, aliquots of membranes taken at various stages during the pyrophosphate wash, and the wash supernatants were analyzed for ϵ-subunit cross-reacting material. No evidence was found for any ϵ-subunit degradation products generated after cell breakage (data not shown).

In order to determine whether or not the distribution of the δ-subunit in solubilized CF_1 was subject to membrane proteolysis, spinach thylakoid membranes were divided into two batches for different treatments prior to the release of CF_1. One batch was washed twice with buffers supplemented with protease inhibitors, the second batch was washed six times in buffer completely deficient in inhibitors. Figs. 4A and B show the FPLC chromatographs, respectively, along with the SDS-PAGE profiles of the resolved peaks. Qualitatively, there were few notable differences in the distribution of CF_1 isozymes released from either batch of membranes. Both membranes yielded a high proportion of $CF_1(-\delta)$ (peak A) and $CF_1(-\epsilon)$ (peak D). One apparent difference, however, is a peak (peak B, CF_1 containing all five subunits) that arose from the membranes washed extensively in the absence of protease inhibitors (Fig. 4B). The lack of that peak in Fig. 4A appears to have been a consequence of the lower overall efficiency in enzyme yield from the twice-washed vs. six-fold washed thylakoid membranes and not the result of the inclusion of protease inhibitors. Therefore, if proteolysis did occur so as to affect the distribution of soluble isozymes, it must have occurred prior to the wash steps and have taken place even in the presence of protease inhibitors.

The effect of divalent cations on the initial distribution of isozymes

The difference in the efficiency of the extraction of CF_1 noted in Fig. 4 prompted an investigation of the effect of ionic strength on the profile of the isozymes extracted from washed membranes. Hesse and coworkers have clearly shown that the efficiency of CF_1 released by the Tris-Tricine-sucrose extraction technique is a function of the divalent metal concentration [10]. The release of CF_1 from spinach and from *C. reinhardtii* membranes by the chloroform extraction technique was also diminished by the inclusion of divalent cations in the extraction buffer. The addition of 4 mM $MgCl_2$ to the CF_1 extraction buffer caused a 27% reduction in the efficiency of the recovery of purified spinach CF_1 (normalized to chlorophyll units extracted). However, the distribution of CF_1 isozymes remained virtually the same for both control (40% $CF_1(-\delta)$, 19% CF_1 holoenzyme, 40% $CF_1(-\epsilon)$) and $MgCl_2$-supplemented (41% $CF_1(-\delta)$, 15% CF_1 holoenzyme, 43% $CF_1(-\epsilon)$) samples. The addition of 40 mM $CaCl_2$ to the CF_1 extraction buffer almost completely inhibited the recovery of purified CF_1 from *C. reinhardtii*.

Post-purification changes due to denaturation

Fig. 3 and other data discussed strongly suggest that proteolysis was not responsible for the loss of the ϵ-subunit following enzyme release. However, it was conceivable that the ϵ-subunit preferentially denatured and that this was the cause of the apparent conversion of ϵ-containing to ϵ-deficient enzyme. This possibility was tested using $CF_1(-\delta)$, $CF_1(-\epsilon)$, and CF_1 holoenzyme purified from spinach. Soluble and insoluble proteins were separated as described in Materials and Methods and the precipitate analyzed by SDS-PAGE. The compositions of the soluble and insoluble (denatured) isozymes did not appear to differ (data not shown), arguing against a selective denaturation of a particular subunit. Partial denaturation of all the isozymes does occur and would account for the loss of total protein observed in Fig. 1.

The conversion of ϵ-containing to ϵ-deficient isozymes: effect of DTT

When the purified spinach CF_1 isozymes containing the ϵ-subunit were extensively dialyzed against the CF_1 extraction buffer and then rechromatographed on a Mono Q column, they again resolved into different

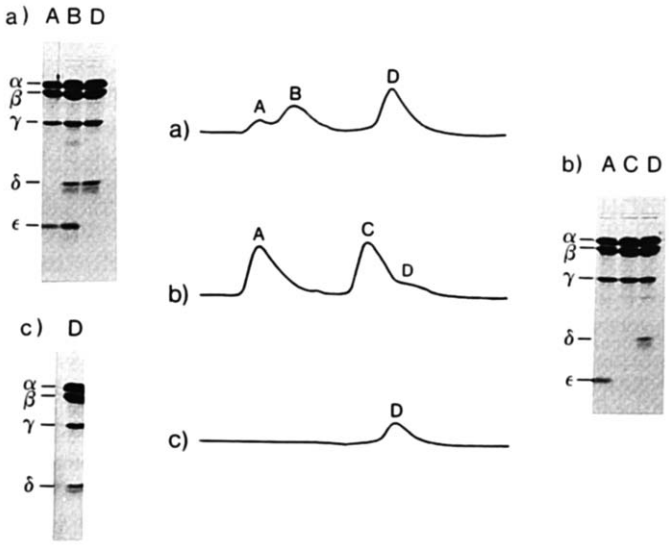


Fig. 5. Rechromatography of the isolated CF_1 isozymes. Spinach CF_1 isozymes which had been initially purified as in Fig. 4 (panel B) were dialyzed against an excess of CF_1 extraction buffer (see Materials and Methods). Following clarification, aliquots were injected onto a Mono Q HR 5/5 column (30 ml isocratic wash at 120 mM NaCl, 60 ml continuous gradient elution from 120 mM to 400 mM NaCl). Profile (a): rechromatography of CF_1 holoenzyme (B) redistributes as 4% $CF_1(-\delta)$ (A), 41% CF_1 holoenzyme (B), and 55% $CF_1(-\epsilon)$ (D). Profile (b): rechromatography of $CF_1(-\delta)$ (A) redistributes as 45% $CF_1(-\delta)$ (A), 42% $CF_1(-\delta,\epsilon)$ (C), and 13% $CF_1(-\epsilon)$ (D). Profile (c): rechromatography of $CF_1(-\epsilon)$ (D) appears to yield only $CF_1(-\epsilon)$ (D). The appearance of peak A in profile (a) and the presence of peak D in profile (b) are discussed in the text.

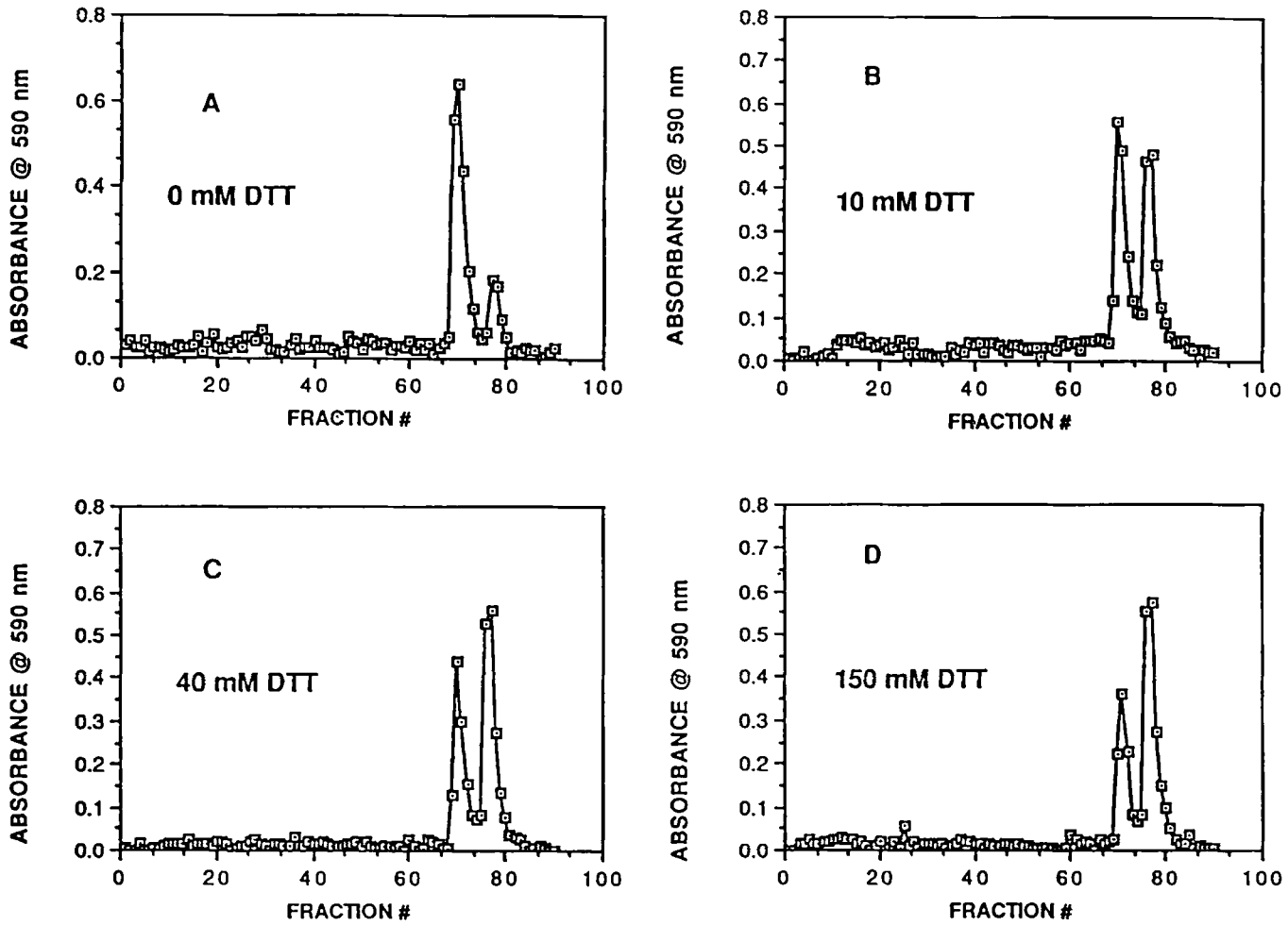


Fig. 6. The ability of DTT pre-incubation to lead to the formation of ϵ-deficient CF_1. Spinach $CF_1(-\delta)$ was concentrated by PM-10 ultrafiltration, then diluted to 0.05 mg/ml with 20 mM Tris-HCl (pH 7.5) in the presence of (A) 0 mM, (B) 10 mM, (C) 40 mM, or (D) 150 mM DTT and incubated for 3 h at approx. 25 °C. The enzyme was clarified by centrifuging at 30000 × *g* for 30 min, then injected (4 h after start of incubation) onto a Mono Q HR 5/5 anion exchange column using a 30 ml isocratic wash (120 mM NaCl) and a 60 ml continuous gradient (120–400 mM NaCl). Protein profiles were obtained by mixing 100 μl of sample and 150 μl of Bradford reagent. The recovered proteins were assayed for protein, and the isozyme identities were confirmed by SDS-PAGE. The resulting wt% of the $CF_1(-\delta,\epsilon)$ isozyme in each experiment was (A) 22%, (B) 38%, (C) 55%, (D) 58%.

TABLE I

Chromatographic elution data for CF_1 isozyme

Data were derived from the continuous (280 nm) monitoring of the effluent after application of the protein samples to the FPLC column. The nominal NaCl concentrations were chosen at the peak apexes and were based on the pump flow ratio. Chromatographs were obtained on the Mono Q HR 5/5 column (60 ml continuous gradient). The data ranges were selected from sets of chromatographs with varied isocratic wash volumes, varied initial NaCl concentrations (100–120 mM), and varied flow rates (0.5–2.0 ml/min).

Isozyme (species)	Elution concentration range (mM NaCl)
CF_1 holoenzyme (spinach)	315–325
$CF_1(-\delta)$ (spinach)	303–308
$CF_1(-\delta)$ (*C. reinhardtii*)	175–201
$CF_1(-\epsilon)$ (spinach)	342–350
$CF_1(-\delta,\epsilon)$ (spinach)	333–347
$CF_1(-\delta,\epsilon)$ (*C. reinhardtii*)	224–247

populations, as demonstrated in Fig. 5. Isozymes which originally contained the ϵ-subunit (CF_1 holoenzyme or $CF_1(-\delta)$) consistently yielded a corresponding isozyme which lacked the ϵ-subunit, in addition to residual amounts of the original parental isozyme (Fig. 5, UV profiles a and b). In contrast, rechromatography of the purified spinach $CF_1(-\epsilon)$ on the Mono Q anion exchange column resulted in a single peak of the parental isozyme (Fig. 5, UV profile c).

Similarly, rechromatography of dialyzed *C. reinhardtii* $CF_1(-\delta)$ yielded both the parental isozyme and the $CF_1(-\delta,\epsilon)$ isozyme. Rechromatography of the *C. reinhardtii* $CF_1(-\delta,\epsilon)$ yielded only the parental isozyme (data not shown). Thus, one can readily generate a three-subunit ($CF_1(-\delta,\epsilon)$) isozyme from the $CF_1(-\delta)$ isozyme by dialysis of the respective parent isozyme against extraction buffer. With the chromatography conditions used, the spinach $CF_1(-\delta,\epsilon)$ isozyme eluted very close to the spinach CF isozyme (Table I), so that

it was difficult to resolve the progression of δ-containing isozymes into δ-deficient isozymes. The appearance of 4% $CF_1(-\delta)$ in the rechromatography of CF_1 holoenzyme (Fig. 5, UV profile a) and the appearance of 13% $CF_1(-\epsilon)$ in the rechromatography of $CF_1(-\delta)$ (Fig. 5, UV profile b) was probably due to incomplete resolution of the original isozymes.

The factor in the dialysis buffer that caused the distribution of isozymes to re-equilibrate was DTT. Fig. 6 dramatically illustrates this for the spinach $CF_1(-\delta)$ isozyme. In this experiment, the enzyme was simply incubated with varying amounts of DTT for a total of 4 h prior to rechromatography. As seen in Fig. 6, increasing amounts of DTT in the buffer resulted in a progressive increase in the amount of $CF_1(-\delta,\epsilon)$ as a weight of the total recovered CF_1 isozymes.

Because the preceding data were obtained under chromatography conditions in which 1 mM DTT was present in the FPLC buffers, it was conceivable that the inclusion of DTT at a fixed concentration caused a shift from the initial isozyme distribution established in the 4 h incubation period. Therefore, the above experiment was repeated using a $CF_1(-\delta)$ isozyme as a starting material which had been rechromatographed in the absence of DTT to remove traces of the $CF_1(-\delta,\epsilon)$ isozyme. In addition, the DTT concentration was the same in both the FPLC and incubation buffers. These results, summarized in Table II, clearly demonstrated that increasing amounts of DTT lead to increasing percentages of $CF_1(-\delta,\epsilon)$ in the resolved isozyme mixtures. This experiment also showed that the dilute enzyme can exist as a population of ϵ-containing and ϵ-deficient enzymes even in the absence of DTT. This was the case for both the spinach and *C. reinhardtii* enzymes.

TABLE II

CF_1 isozyme redistributions at constant DTT concentrations

Spinach $CF_1(-\delta)$ or *C. reinhardtii* $CF_1(-\delta)$ was first rechromatographed using FPLC buffers lacking DTT to remove traces of ϵ-deficient CF_1. The $CF_1(-\delta)$ was then pre-incubated with varying amounts of DTT as described in Fig. 6. The chromatography conditions were exactly the same as in Fig. 6 with the exception that the DTT in the FPLC buffers was equal to the DTT concentration in the pre-incubation solution. The DTT concentrations and resulting wt% of the $CF_1(-\delta,\epsilon)$ isozyme in each experiment are shown in the table.

DTT concentration	Species	Weight percentage $CF_1(-\delta,\epsilon)$ as
0 mM	spinach	23
5 mM	spinach	32
40 mM	spinach	61
0 mM	*C. reinhardtii*	33
5 mM	*C. reinhardtii*	45
40 mM	*C. reinhardtii*	52

Discussion

Our experiments help to illustrate how subtle variations in the quality of CF_1 preparations can arise. Specifically, we have shown that the concentration of DTT and the length of time spent in a DTT-containing crude extract both play an important role in determining the composition of the putative 'homogeneous' coupling factor solution. Additionally, we have simplified the purification of the major stable CF_1 isozymes by bypassing the traditional low-resolution anion-exchange chromatography step and directly loading clarified crude CF_1 onto an FPLC column. The rapidly purified enzyme contains sufficiently low concentrations (if any) of proteases such that it may be stored in clean containers at room temperature for months, losing activity only due to the gradual loss of solubility.

With the chromatographic conditions that we used for this work, the three-subunit spinach $CF_1(-\delta,\epsilon)$ isozyme elutes in nearly the same volume as the $CF_1(-\epsilon)$ spinach isozyme (Table I). Thus it was difficult for us to quantitate in a reliable way the progression of the $CF_1(-\epsilon)$ isozyme into the $CF_1(-\delta,\epsilon)$ form. Within the current constraint, we report no significant progression of δ-containing to δ-deficient isozymes following solubilization of the enzyme from thylakoid membranes.

Our results demonstrate that it is not necessary to use special treatments to generate ϵ-deficient forms of CF_1, although such treatments are required if one wishes to obtain a stable form of the ϵ-subunit [22]. Our results serve to underscore the need to account for existing enzyme heterogeneity before making claims about generating subunit deficient forms by chromatographic techniques (when the actual process has been not dissociation and separation but rather resolution), and certainly before using a given enzyme preparation for kinetic determinations.

The effect of the number of membrane washes and of added divalent cations on the efficiency of enzyme recovery makes it clear that the chloroform extraction process is not merely a simple disruption of the membrane which leads to complete release of the CF_1 enzyme. Rather, this technique bears similarity to the EDTA [13] and Tris-Tricine-sucrose [10] extraction techniques, in that CF_1 yield decreases if the metal concentration increases. Beechey et al. [3] also observed that $MgCl_2$ inhibited the release of the F_1 from submitochondrial particles in the original description of the chloroform extraction technique. We interpret the slight increases in the proportion of spinach CF_1 holoenzyme obtained when thylakoid membranes are extracted under low ionic strength conditions as being within the limits of experimental uncertainty. If these increases are indeed real, they are consistant with the recent demonstration that sodium chloride diminishes the yield of

δ-containing isozymes solubilized via the EDTA extraction technique [14].

Pre-incubation of CF_1 with 100 mM DTT prior to chromatography in the presence of Mega 9 has been reported to resolve the $CF_1(-\delta,\epsilon)$ isozyme [8]. However, it was earlier demonstrated that detergent chromatography resulted in the loss of the ϵ-subunit [29], making it unclear as to whether loss of the ϵ-subunit in Ref. 8 was due to the detergent or the reductant. We have clearly shown that treating CF_1 with DTT prior to FPLC leads to the redistribution of isozymes, specifically an increase in those lacking the ϵ-subunit. There are at least two interpretations of this observation. One is that DTT is involved in enhancing the dissociation of the ϵ-subunit from the CF_1. An alternate interpretation consistent with the explanations proffered by Richter and McCarty [23] and by Andralojc and Harris [1] is that DTT (or light energization) causes a conformational change in CF_1 which decreases the affinity of the ϵ-subunit to the holoenzyme. Following such a change, the ϵ-subunit is then susceptible to removal by the column matrix or by glass surfaces. Since the original preparation of this manuscript, we have obtained evidence which supports the hypothesis that DTT enhances the reversible dissociation of the ϵ-subunit from CF_1.

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References

1 Andralojc, P.J. and Harris, D.A. (1988) FEBS Lett. 233, 403–407.
2 Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
3 Beechey, R.B., Hubbard, S.A., Linnett, P.E., Mitchell, A.D. and Munn, E.A. (1975) Biochem. J. 148, 533–537.
4 Berger, G., Girault, G., Andre, F. and Galmiche, J.-M. (1987) J. Liquid Chrom. 10, 1507–1517.
5 Binder, A., Jagendorf, A. and Ngo, E. (1978) J. Biol. Chem. 253, 3094–3100.
6 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
7 Engelbrecht, S., Lill, H. and Junge, W. (1986) Eur. J. Biochem. 160, 635–643.
8 Engelbrecht, S. and Junge, W. (1987) FEBS Lett. 219, 321–325.
9 Finel, M., Rubenstein, M. and Pick, U. (1984) FEBS Lett. 166, 85–89.
10 Hesse, H., Jank-Ladwig, R. and Strotmann, H. (1976) Z. Naturforsch. 31, 445–451.
11 Hicks, D.B. and Yocum, C.F. (1986) Arch. Biochem. Biophys. 245, 220–229.
12 Laemmli, U.K. (1970) Nature 227, 680–685.
13 Lien, S. and Racker, E. (1971) Methods Enzymol. 23, 547–555.
14 Lill, H., Engelbrecht, S. and Junge, W. (1988) J. Biol. Chem. 63, 14518–14522.
15 Magnussen, R.P., Portis, A.R., Jr. and McCarty, R.E. (1976) Anal. Biochem. 72, 653–657.
16 Merchant, S. and Selman, B.R. (1985) Photosynth. Res. 6, 3–31.
17 Moroney, J.V. and McCarty, R.E. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 169–174, Elsevier/North-Holland, Amsterdam.
18 Nelson, N., Nelson, H. and Racker, E. (1972) J. Biol. Chem. 247, 7657–7662.
19 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
20 Piccioni, R.G., Bennoun, P. and Chua, N.-H. (1981) Eur. J. Biochem. 117, 93–102.
21 Pick, U. and Bassilian, S. (1982) Biochemistry 24, 6144–6152.
22 Richter, M.L., Patrie, W.J. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7371–7373.
23 Richter, M.L. and McCarty, R.E. (1987) J. Biol. Chem. 262, 15037–15040.
24 Selman-Reimer, S., Merchant, S. and Selman, B.R. (1981) Biochemistry 20, 5476–5482.
25 Selman-Reimer, S., Finel, M., Pick, U. and Selman, B.R. (1984) Biochim. Biophys. Acta 764, 138–147.
26 Strotmann, H. and Bickel-Sandkotter, S. (1984) Ann. Rev. Plant Physiol. 35, 97–120.
27 Vignais, P.V. and Satre, M. (1984) Mol. Cell. Biochem. 60, 33–70.
28 Younis, H.M., Winget, G.D. and Racker, E. (1977) J. Biol. Chem. 252, 1814–1818.
29 Yu, F. and McCarty, R.E. (1985) Arch. Biochem. Biophys. 238, 61–68.
30 Yu, L.M., Merchant, S., Theg, S.M. and Selman, B.R. (1988) Proc. Natl. Acad. Sci. USA 85, 1369–1373.