IDENTIFICATION OF COUPLING FACTOR SUBUNITS IN THYLAKOID POLYPEPTIDE PATTERNS OF WILD-TYPE AND MUTANT BARLEY THYLAKOIDS USING CROSSED IMMUNOELECTROPHORESIS

by

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The extrinsic part of coupling factor (CF₁) has been isolated from chloroplasts of barley and antibodies have been raised against the purified protein. The five different subunits of CF₁ were identified among the polypeptides of chloroplast and etioplast thylakoids using the technique of crossed immunoelectrophoresis. Three nuclear gene mutants of barley, *viridis-e*⁶⁴, *xantha-d*⁴⁹, and *xantha-b*¹², which are deficient in stroma lamellae were also studied by crossed immunoelectrophoresis. These mutants have reduced amounts of CF₁ which is consistent with the reported localization of CF₁ on the stroma lamellae.

1. INTRODUCTION

When barley thylakoids are solubilized in SDS and subjected to SDS-PAGE, at least 43 different polypeptide bands can be resolved (11). In an attempt to identify successively the function of these polypeptides we have studied the chloroplast coupling factor which consists of extrinsic (CF₁) and intrinsic (CF₀) components responsible for converting the energy of the

proton gradient established upon illumination of thylakoids into ATP (21). The extrinsic component CF₁ has previously been isolated from spinach, *Vicia faba* and tobacco (3, 13, 14, 29, 33). Two different methods of isolation have been used, one being based on the release of CF₁ from the thylakoid membrane by chloroform (33) and the other on its removal by EDTA treatment (3, 14). Like the mitochondrial coup-

Abbreviations: CF₁ = extrinsic part of chloroplast coupling factor, RuBP' case = ribulose-1,5-bisphosphate carboxylase, SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis, DTT = dithiothreitol, tricine = N-(tris-(hydroxymethyl)-methyl)glycine, Tris = tris-(hydroxymethyl)-aminomethane, kD = kilodalton.

ling factor F_1 , CF_1 appears generally to be composed of five types of subunits named α , β , γ , δ and ϵ in order of decreasing molecular weight (21). The enzyme is known to be cold labile (19) and the final stages of isolation are therefore carried out at room temperature which frequently leads to proteolytic degradation of the protein (3). Storage or repeated freezing and thawing causes changes in the electrophoretic properties of bacterial coupling factor probably due to proteolytic attack (24, 30). It is actually suggested that the release of bacterial coupling factor from the membrane by treatment with EDTA involves proteolytic cleavage (4). Column chromatography also may result in loss of specific subunits due to dissociation (33). Final analysis of purified CF_1 by SDS-PAGE may therefore reveal the molecular weights of partially degraded subunits and the loss of CF_1 subunits by dissociation may result in the erroneous assignment of impurities in the preparation as components of CF_1 .

These difficulties can be overcome by the technique of crossed immunoelectrophoresis (7, 8). The polypeptides separated in the first dimension by SDS-PAGE are electrophoresed in a second dimension into an agarose gel containing specific antibodies raised against individual purified polypeptides. The presence of an antigen in the first dimension gel is then registered as a precipitation arch in the antibody containing gel. The position of the immunoprecipitation peak in the agarose gel enables the precise localization of the antigenic polypeptide in the first dimension gel. In the present paper the use of this technique in the identification of the five subunits of CF_1 in the total membrane polypeptide pattern of chloroplasts and etioplasts from barley is reported. The antibody against barley CF_1 was found only to cross react with the α and β subunits of other plant species and could therefore not be used to identify all five different subunits in the total thylakoid polypeptide pattern of these species. The total polypeptide pattern of purified thylakoids from several nuclear gene mutants of barley show a reduction in the amount of polypeptides present in the region corresponding to the α and β subunits of CF_1 (17, 25, 26). Three such mutants, *viridis-e*⁶⁴, *xantha-d*⁴⁹, and *xantha-b*¹², were examined by crossed immunoelectrophoresis to determine whether CF_1 was

present in reduced amounts or present at wild-type levels but with altered electrophoretic mobility.

2. MATERIALS AND METHODS

2.1. Plant material

Seeds of wild-type barley (*Hordeum vulgare* cv. Svalövs Pallas) were germinated in tap water moistened vermiculite. Seedlings were harvested after growing 7 days at 20 °C in continuous white light (1700 lux), except for etiolated seedlings which were grown in the dark. The recessive mutants *viridis-e*⁶⁴, *xantha-d*⁴⁹, and *xantha-b*¹² were grown in the light under the same conditions. Because these mutants do not produce seeds, stocks are maintained in the heterozygous state. The mutant seedlings arising are easily separated from the wild-type phenotype by their paler green (*viridis-e*⁶⁴) or yellow (*xantha-d*⁴⁹ and *-b*¹²) color.

2.2. Isolation of thylakoids

Thylakoid membranes for use in the isolation of CF_1 were prepared in a grinding medium consisting of 0.4 M-sucrose, 20 mM-Tricine/NaOH and 10 mM-NaCl, pH 7.8. In a typical experiment 1 kg of precooled leaves were homogenized in 4 volumes of grinding medium by use of a kitchen blender with replaceable razor blades (12). The homogenate obtained was filtered through two layers of 31 μ m nylon gauze and centrifuged for 5 min at 6.000 g. The chloroplast pellet was suspended and washed with 10 mM-sodium pyrophosphate, pH 7.5 and recentrifuged for 5 min at 6.000 g. After 4 additional washings involving a total of 2500 ml pyrophosphate buffer, the chloroplast pellet was suspended and washed with 10mM-NaCl, 2 mM-Tricine/NaOH, pH 8.0. A final thylakoid pellet containing approx. 190 mg of chlorophyll was then obtained by centrifugation at 10.000 g for 10 min. All operations were carried out at 4 °C.

When used as standards in SDS-PAGE, thylakoid membranes were prepared as earlier described (11), solubilized partly in Na_2CO_3 /DTT, and stored in small aliquots at -20 °C until used.

2.3. Preparation of CF_I by chloroform extraction

Freshly prepared barley thylakoids (approx. 190 mg of chlorophyll) were resuspended at 3.0 mg chlorophyll/ml in 0.25 M-sucrose, 10 mM-Tris/SO₄ (pH 7.6), 1 mM-EDTA, 2mM-ATP, and 5mM-DTT. To this suspension one half volume of chloroform was added. After gentle shaking for 15 sec. the emulsion obtained was broken by centrifugation for 3 min at 2.500 g (33). The aqueous layer was removed with a pasteur pipette and centrifuged at 48.000 g for 30 min. The yellow supernatant (approx. 45 mg protein) was collected and recentrifuged at 226.000 g for 2 hrs to pellet residual amounts of RuBP'case present. The supernatant was then centrifuged at 226.000 g for 6 hrs. After this centrifugation CF_I can be collected with a syringe as a yellow oily material (approx. 16 mg protein) at the bottom of the tube. A very tiny brownish and densely packed precipitate also obtained contained CF_I contaminated with RuBP'case and was discarded. All operations were carried out at room temperature.

2.4. Preparation of CF_I by use of DEAE-cellulose chromatography

CF_I was obtained from freshly prepared barley thylakoids (approx. 190 mg chlorophyll) essentially by the method of BINDER et al. (3). Upon ammonium sulfate precipitation of the major CF_I containing fractions from the DEAE-cellulose column, a total of 85 mg protein were recovered. The protein was dialysed overnight in a nitrogen atmosphere against 50 mM-Tris/HCl, 1 mM-ATP, 2 mM-EDTA, and 20 mM-β-mercaptoethanol, pH 7.8, and then centrifuged for 20 min at 40.000 g. The clear supernatant was loaded on to four linear (10 to 40 %) sucrose gradients prepared in 20 mM-Tris/HCl, 2 mM-EDTA, and 20 mM-β-mercaptoethanol, pH 7.8. After centrifugation for 4 hrs at 155.000 g in a Beckman 60 Ti rotor, 1.25 ml fractions were removed from the top of the gradient by use of a Searle Auto Densi Flow IIC instrument. Pure CF_I banded in the upper half of the gradient while CF_I contaminated with RuBP'case was concentrated near the bottom of the gradient. A total of 65 mg of protein was recovered from the major CF_I fractions.

2.5. Preparation of CF_I depleted thylakoids

Thylakoids depleted of CF_I were obtained after 10 consecutive washings of thylakoids with 0.7 mM-EDTA followed by centrifugation at 10.000 g for 5 min.

2.6. Assays

Chlorophyll was determined spectrophotometrically in 80 % acetone extracts according to ARNON (1). Protein concentration was measured by the method of LOWRY et al. (16) with bovine serum albumin as a standard.

During initial isolations of CF_I, the degree of purification was monitored by measurements of the latent Ca²⁺-ATPase activity of CF_I (14), and by measurements of the fluorescence intensity ratio between 300 and 350 nm (3).

2.7. SDS-PAGE and crossed immunoelectrophoresis

SDS-PAGE was performed according to published procedures (5). The molecular weights of separated polypeptides were determined by the use of the following molecular weight markers: bovine serum albumin (68.000), ovalbumin (43.000), aldolase (40.000), chymotrypsinogen A (25.000), and cytochrome c (12.400).

Non-denaturing PAGE was performed as described by DAVIS (9) using a 4 % stacking gel and a 7 % separation gel.

Crossed immunoelectrophoresis was carried out as described by CHUA and BLOMBERG (7), except that the detergent Brij 30 was used as a replacement for Lubrol PX in the bridge-gel connecting the SDS-gel strip with the antibody containing agarose gel.

2.8. Preparation of CF_I antibody

Antibodies were raised by injecting rabbits subcutaneously in the back with purified CF_I containing all five subunits. The immunisation program consisted of an initial injection of 200 µg of purified CF_I mixed with 0.5 ml complete Freund's adjuvant followed by four weekly injections of 200 µg of CF_I mixed with 0.5 ml incomplete Freund's adjuvant. Five weeks after the first injection, the rabbits were bled from the ear. Three weeks after bleeding, the rabbits were

boosted with 200 µg CF_I mixed with 0.5 ml incomplete Freund's adjuvant and bled ten days later. This bleeding program was repeated several times. The serum was purified as described by HARBOE and INGILD (10).

2.9. Electron microscopy

Leaf tissue was prepared for electron microscopy by fixation in 2% glutaraldehyde in 0.06 M-sodium phosphate buffer at pH 7.0 and postfixed in 1% OsO₄ in 0.06 M-sodium phosphate buffer at pH 7.0 as described earlier (11).

2.10. Chemicals

Ammonium sulfate p.a. was obtained from Riedel-de Haën AG, Seelze, Hannover, Brij 30 from Sigma Chemical Co., Saint Louis, Missouri, preswollen DEAE-cellulose (DE 52) from Whatman Ltd., Springfield Mill, Maidstone, Kent, and Freund's adjuvant (complete and incomplete) from Difco Laboratories, Detroit, Michigan.

3. RESULTS

3.1. Preparation of CF_I by chloroform extraction

CF_I can be released from spinach thylakoids by brief exposure to chloroform and a procedure for isolation and purification of the protein has been reported (33). When this method was adopted directly to prepare CF_I from barley, the preparations obtained were highly contaminated with RuBP'case. This was indicated by SDS-

Figure 1. Polypeptide patterns of a) CF_I depleted wild-type thylakoids obtained after repeated washes with EDTA, b) wild-type barley thylakoids and c) CF_I obtained by a brief exposure of the chloroplast membranes to chloroform.

Separation by SDS-PAGE was carried out on an 11–15% gradient gel. Apart from the CF_I subunits, the positions of chlorophyll a-protein 1 (Chl_a-P1), the apoprotein of light-harvesting chlorophyll a/b-protein 2 (Chl_{a/b}-P2) and the apoprotein of chlorophyll a-protein 3 (Chl_a-P3) are indicated. The nomenclature system used for the chlorophyll-proteins is defined in (18).

PAGE showing co-migration of polypeptides from the CF₁ preparation with those of the large and small subunit of RuBP'case purified from barley (23). Also monospecific antibodies raised against the large and small subunits of RuBP'case produced precipitation peaks when tested by the immunoelectrophoresis procedure against the CF₁ preparation. Additional steps were therefore necessary to obtain CF₁ preparations not contaminated with RuBP'case. First, the thylakoids were repeatedly washed with a 10 mM solution of sodium pyrophosphate which releases RuBP'case from the thylakoids (28). Secondly, residual amounts of RuBP'case were removed from CF₁ by differential ultracentrifugation. The CF₁ preparation obtained in this manner is free of RuBP'case as judged by SDS-PAGE and by crossed immunoelectrophoresis. This final centrifugation also removes the impurities observed in the 25 to 35 kD molecular weight range upon SDS-PAGE (Figure 1c). Tentative identification of the polypeptides in the preparation as subunits of CF₁ were based on comparison of the molecular weights of the observed polypeptide bands after SDS-PAGE with the reported values from spinach (33). Additional attempts to purify barley CF₁ by either ion exchange chromatography (33) or by gel filtration using Sephadex G-200 (33) or Sephadex 6B resulted in preferential loss of the γ , δ and ϵ subunits and were therefore abandoned. The subunit stoichiometry of the CF₁ preparation obtained is somewhat variable from one experiment to the next. When large amounts of leaf material (more than 1 kg) were processed, the δ and ϵ subunits were almost always lost. An important factor determining the loss of the δ subunit, is the length of the chloroform treatment (Figure 2). When chloroform was added to the thylakoid suspension without agitation followed by immediate centrifugation, high amounts of the δ subunit were

Figure 2. Electrophoretogram showing the polypeptides present in the aqueous phase obtained after treatment of a suspension of barley thylakoids with chloroform and immediate centrifugation (a). The position of the five subunits of CF₁ are indicated. After 15 sec exposure to chloroform, the yield of the δ subunit is significantly decreased (b). (11–15% SDS-PAGE).

recovered in the aqueous supernatant. When the exposure time was prolonged to 15 sec, the yield of the δ subunit was markedly decreased.

3.2. Preparation of CF₁ by EDTA extraction

CF₁ is released from the thylakoids when these are suspended in a dilute EDTA solution. DEAE-cellulose is added to the suspension and the DEAE-cellulose with CF₁ bound to it is recovered by filtration, poured into a column, and CF₁ eluted by use of a high salt buffer and precipitated with ammonium sulfate (3). The dialyzed material was finally purified on a sucrose gradient. After the column chromatography step, 20 mM- β -mercaptoethanol was included in all buffers to diminish proteolytic breakdown.

The absence of RuBP'case in the CF₁ preparation was monitored by crossed immunoelectrophoresis of the preparation against RuBP'case antibody. No precipitation peaks were obtained. Non-denaturing PAGE produced only one polypeptide band with an electrophoretic mobility different from that of purified RuBP'case (Figure 3). Analysis of the preparation by SDS-PAGE revealed the presence of 5 major polypeptides with molecular weights of 58, 57, 37, 21, and 13.5 kD, respectively (Figure 4 c). These were tentatively identified as the α , β , γ , and ϵ subunits of CF₁ due to the close similarity of these

Figure 3. Electrophoresis of a) pure CF₁ and b) RuBP'case from barley on a 7% non-denaturing polyacrylamide gel.

Figure 4. Polypeptide pattern of a) CF₁ depleted wild-type membranes obtained after repeated washes with EDTA b) wild-type barley thylakoids c) barley CF₁ purified by the EDTA extraction method d) barley CF₁ before the last step of purification, i.e. the final sucrose gradient centrifugation. The position of the five subunits of CF₁ are indicated. (5–15% SDS-PAGE).

molecular weights with those reported for the subunits of CF_I isolated from spinach (59.5, 56, 37, 17.5, and 13 kD) (21). Analysis of the CF_I preparation by SDS-PAGE before purification on the sucrose gradient revealed the presence of additional polypeptide bands which may derive from proteolytical degradation of the γ subunit (Figure 4 d).

3.3. Thylakoids depleted of CF_I

The SDS-PAGE polypeptide pattern of thylakoids depleted of CF_I by repeated EDTA washes is shown on Figure 1a and 4 a. When this pattern is compared with that of isolated CF_I, it is apparent that the polypeptides which are preferentially removed in the EDTA extracted membranes have the same electrophoretic mobility as those found in isolated CF_I. This experiment therefore provides additional support to the assignment of these polypeptides as subunits of CF_I.

3.4. Secondary modification of CF_I during isolation, storage, and handling

Storage of five subunit CF_I preparations with 0.02 % sodium azide at either 4 °C or -20 °C resulted in changes in the SDS-PAGE patterns of the samples. Often the polypeptide band corresponding to the δ subunit completely disappeared (3). This phenomenon was also observed upon SDS-PAGE analysis of thylakoids which had been repeatedly frozen and thawed (Figure 7a compared to Figure 1b). Besides the disappearance of the δ subunit, an additional polypeptide band at 55 kD appeared (Figure 5). This probably represent a proteolytical degradation product of either the α and/or β subunit. Similarly, one or multiple bands with electrophoretic mobilities slightly faster than the γ subunit were observed (Figure 5). It is interesting that these same additional bands were present in CF_I preparations before the final purification step on the sucrose gradient (Figure 4 d). This could indicate that these polypeptides originate by proteolytic cleavage of the CF_I complex and therefore can be separated from the intact complex on the sucrose gradient. The same degradation products were also observed when CF_I were prepared according to the chloroform method (Figure 1c and 2).

Figure 5. The effect of chloroform and MgCl₂ on the mobility of CF_I subunits isolated by the EDTA method; a) and d) CF_I, b) same CF_I sample treated with 50 % v/v chloroform and water phase applied, c) same CF_I sample containing 25 mM-MgCl₂. The positions of the five subunits are indicated.

The additional polypeptide bands are proteolytic break-down products.

In a separate study the effect of EDTA or chloroform on the electrophoretic mobility of the subunits of purified CF_I was investigated. The addition of chloroform to CF_I purified by the EDTA method (3) did neither change the electrophoretic mobility nor the proteolytical breakdown pattern (Figure 5). Also the addition of EDTA to CF_I prepared by the chloroform method had no effect. When magnesium ions were added to either of the CF_I preparations a

decrease in electrophoretic mobility was observed. This is in agreement with the reported effect of magnesium ions on the mobility of chlorophyll proteins (2) and thus seems to be a general effect of high ion strength.

3.5. Crossed immunoelectrophoresis

Due to the possible proteolytic breakdown of CF_1 during isolation and storage, the molecular weights of the native CF_1 subunits may be different from those of the isolated subunits and thus identification of the subunits in the total polypeptide pattern based on comparison of electrophoretic mobilities ambiguous. Crossed immunoelectrophoretograms obtained by SDS-PAGE of the purified CF_1 preparation in the first dimension and with the purified antibody in the second, showed five precipitation peaks corresponding to each of the isolated subunits. When the antibody was tested against the total thylakoid polypeptide pattern, five immunoprecipitation peaks were observed (Figure 6 A). These peaks were superimposable with the five polypeptides which based on their electrophoretic behaviour were considered as subunits of CF_1 . This experiment thus reveals these five polypeptides in the total thylakoid pattern to be the α , β , δ , and ϵ subunits of CF_1 . When the antibody was reacted against EDTA extracted thylakoids, only very small precipitation peaks in the position of the α and β subunits were observed. This is in agreement with the depletion of CF_1 from such membranes (Figure 1a and 4 a).

The polypeptide composition of etioplast thylakoids (11) differs greatly from that of chloroplast thylakoids (Figure 6 B), and the presence of the subunits of CF_1 in etioplasts can therefore not be determined by direct comparison of SDS-PAGE patterns. However, when the

Figure 6. Crossed immunoelectrophoresis of A) wild-type chloroplast thylakoid proteins and B) etioplast thylakoid proteins with antibodies to CF_1 purified by the EDTA procedure.

The position of the five subunits is indicated and in B) the polypeptide pattern of chloroplast thylakoids is included for reference. The 11–15 % SDS-PAGE strip in the first dimension contained approx. 50 μ g of protein and the agarose gel contained purified rabbit serum IgG at a concentration of 0.44 mg cm^{-2} .

antibody to isolated CF_1 was reacted against total etioplast thylakoid polypeptides, five precipitation peaks superimposable with the positions of the five CF_1 subunits from thylakoids were obtained (Figure 6 B). Thus all five subunits of CF_1 are present in etioplasts. In addition, a shoulder on the δ subunit precipitation peak was repeatedly observed, apparently representing the presence of a proteolytic degradation product of the δ subunit.

The polypeptide patterns of the three nuclear gene barley mutants *viridis-e*⁶⁴, *xantha-d*⁴⁹, and *xantha-b*¹² from the Copenhagen Stoch Collection showed an apparent deficiency of CF_1 (Figure 7). These mutants were therefore examined by crossed immunoelectrophoresis to determine if this apparent deficiency was actually due to the presence of only very small amounts of CF_1 or if the CF_1 polypeptides of these mutants had altered electrophoretic mobilities. All three mutants only produced a very weak reaction with the CF_1 antibody as illustrated for *viridis-e*⁶⁴ in Figure 8. The precipitation peaks observed corresponded to the electrophoretic mobilities of the α , β , and δ subunits of wild-type thylakoid polypeptides. The area of the peak is proportional to the amount of antigen present (8) and it can therefore be concluded that the thylakoids of these mutants actually are deficient in CF_1 .

It was hoped that the antibody raised against barley CF_1 also could be used to localize the subunits of CF_1 in the total thylakoid polypeptide patterns of other plant species. However, when spinach, maize, tomato, and potato thylakoid polypeptides were tested in crossed immunoelectrophoresis with the barley CF_1 antibody, only the α and β subunits produced precipitation peaks. Different batches of antibody obtained by injection of different rabbits invariably produced the same result. Since the α and β subunits are the CF_1 subunits most easily identified based solely on electrophoretic mobility, the use of the barley CF_1 antibody for this purpose was not further pursued.

4. DISCUSSION

The identification of thylakoid polypeptides with respect to their function can be approached in various ways. Comparison of electrophoretic

Figure 7. Electrophoretogram of chloroplast thylakoid polypeptides from a) wild-type barley and the barley nuclear gene mutants, b) *viridis-e64*, c) *xantha-d49*, d) *xantha-b12* and e) CF_I depleted wild-type membranes obtained after repeated washes with EDTA.

The positions of Chl_a-P1, Chl_{a/b}-P2 apoprotein and the five CF_I subunits are shown. (11–15 % SDS-PAGE).

Figure 8. Crossed immunoelectrophoresis of the thylakoid polypeptides from the barley mutant *viridis-e64* with CF_I antibodies.

Experimental conditions as described in legend to Figure 6.

grams of wild-type thylakoid proteins with those of mutants with known photosynthetic defects have been employed (17). The main difficulty with this method is that the majority of mutants available are missing several polypeptides (17, 25, 26, 32), and therefore do not allow the unambiguous assignment of one polypeptide to one function. Another approach is to isolate and purify proteins of known function and compare the electrophoretic mobility of these proteins with those in the total thylakoid polypeptide pattern. Although more than 40 thylakoid polypeptides can be resolved by SDS-PAGE (11) there are probably many more polypeptides present in the chloroplast membranes. This means that some bands in the patterns represent more than one polypeptide species. Moreover, polypeptides present in low amounts might not be detected by current staining methods. These disadvantages are likely to be overcome by identification of the polypeptides with the technique of crossed immunoelectrophoresis (7, 8).

The usefulness of this method in identifying the location of specific polypeptides in a complex pattern is demonstrated by the identification of all five subunits of CF_I in thylakoid and etioplast membrane patterns. This is the first report to demonstrate that all five subunits of CF_I are present in etioplasts with the same electrophoretic mobilities and antigenic properties as observed for chloroplast thylakoids. Earlier the presence of the α and β subunits in maize etioplasts have been indicated by comparison of the total etioplast polypeptide pattern with that of isolated

Figure 9. Electron micrograph of a thin section through a *xantha-d⁴⁹* chloroplast showing giant grana (gg) and the deficiency in stroma thylakoids (arrows). $\times 37,000$. (Bar = 1.0 μm).

CF₁ (15). Because of the presence of a major polypeptide with electrophoretic mobility similar to that of the γ subunit (Figure 6 B) such a direct comparison of SDS-polypeptide patterns can not be used to determine the presence or the absence of the γ subunit in etioplasts. This problem was easily overcome by use of crossed immunoelectrophoresis. An additional advantage of using this method is that antigenic polypeptides not resolved in the first dimension chromatogram can be separated in the second dimension due to their presence in different amounts or due to their different antigenic properties. This is exemplified in Figures 6 A and 6 B. The 11 to 15% polyacrylamide gradient gel shown produces an even distribution of the thylakoid polypeptides throughout the gel. However, in order to obtain a satisfactory separation of the α and β subunits of CF₁ a 5 to 15% gel should be used (Figure 4 b and c). Nevertheless, immunoelectrophoresis by

use of an 11 to 15% first dimension gel produces two clearly distinct immunoprecipitation peaks.

The sensitivity of crossed immunoelectrophoresis in establishing the presence and identity of a particular polypeptide is an advantage when dealing with material available only in small quantities such as mutant thylakoids and in the analysis of mutants in which thylakoid polypeptides with similar electrophoretic mobilities may mask missing polypeptides. Also the presence of a polypeptide with an altered electrophoretic mobility from that of the wild-type can be detected if its antigenic properties have remained similar (6). In this study the barley mutants *viridis-e⁶⁴*, *xantha-d⁴⁹*, and *xantha-b¹²* were examined and were found to be highly deficient in CF₁. The localization of CF₁ in chloroplasts has earlier been demonstrated to be restricted to the end grana discs and the stroma lamellae (20),

Figure 10. Polypeptide pattern obtained by SDS-PAGE of thylakoids from wild-type barley seedlings.

CF₁ extrinsic component of coupling factor. CF₀ membrane component of coupling factor. Chl_a-P1 chlorophyll a-protein 1. Chl_{a/b}-P2 light harvesting chlorophyll a/b-protein 2. Chl_a-P3 chlorophyll a-protein 3 belonging to the reaction centre of photosystem II. (11–15 % SDS-PAGE).

the latter being particular deficient in the mutants examined (Figure 9) (22, 27). Thus the reduced area of these membranes has apparently not been compensated for by a higher packing density of CF₁ as was shown for etioplast primary thylakoids of oats and barley (31).

In an earlier study several barley polypeptides were shown to cross-react with antibodies raised against total polypeptides from *Chlamydomonas reinhardtii* (25). It was hoped that the antibodies raised to barley CF₁ would also cross react with CF₁ from other species. However, total thylakoid polypeptides from spinach, maize, tomato, and potato as well as purified CF₁ from spinach only produced two precipitant peaks corresponding to the α and β subunits. The catalytic sites of CF₁ are located on these two subunits (21) and one may speculate that the primary structure of the α and β subunits therefore is more conserved than that of the smaller subunits. A second explanation for the limitation of the cross-reactivity to the α and β subunits could be that these two subunits are much larger than the others and therefore can admit more changes in the primary structure without the loss of all antigenic determinants.

A prerequisite for the use of crossed immunoelectrophoresis in identifying polypeptides in complex patterns is the availability of a pure protein against which a specific antiserum may be prepared. In this study CF₁ was attempted purified from barley by release of the protein from the thylakoids by treatment with either chloroform or EDTA. Release of CF₁ by chloroform treatment gave preparations which partly had lost the low molecular weight subunits (Figure 1c and 2). Preparations of CF₁ containing all five subunits were obtained by release of the protein by EDTA (Figure 4 c). Besides proteolytic breakdown products of the different subunits, the main contaminant during

purification was RuBP'case. This has also been experienced in previous studies (3, 13, 33). The molecular weights of the large and small subunits of RuBP'case are 55 and 14 kD, respectively (23) and thus similar to those of the β and ϵ subunits of CF₁. Complete removal of RuBP'case from the CF₁ preparation was therefore necessary and checked by use of non-denaturing polyacrylamide gel electrophoresis and by immunoelectrophoresis using antibodies raised against purified RuBP'case.

The present status in the assignment of functions to individual polypeptides in the SDS-PAGE pattern of barley thylakoids are summarized in Figure 10. Besides the localization of the CF₁ subunits as reported in this paper and the earlier reported characterization of the chlorophyll proteins (18), the additional assignments made are based on electrophoretic mobilities of isolated proteins (CF₀) and of isolated photosystem I particles (iron sulfur centres) as well as a specific staining method for cytochrome f (32).

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REFERENCES

1. ARNON, D. I.: Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24, 1-14 (1949)
2. ARO, E.-M. & N. VALANNE: Effect of Magnesium on Chlorophyll-Protein Complexes. *Physiol. Plant.* 43, 261-265 (1978)
3. BINDER, A., A. JAGENDORF & E. NGO: Isolation and composition of the subunits of spinach chloroplast coupling factor protein. *J. Biol. Chem.* 253, 3094-3100 (1978)
4. BRAGG, P. D. & C. HOU: Proteolysis of the delta subunit is required for release of the Ca²⁺, Mg²⁺-activated ATPase from the cell membrane of *Escherichia coli*. *FEBS Letters* 103, 12-16 (1979)
5. CHUA, N.-H. & P. BENNOUN: Thylakoid membrane polypeptides of *Chlamydomonas reinhardtii*: Wild-type and mutant strains deficient in Photosystem II reaction center. *Proc. Nat. Acad. Sci. USA* 72, 2175-2179 (1975)
6. CHUA, N.-H.: A uniparental mutant of *Chlamydomonas reinhardtii* with a variant thylakoid membrane polypeptide. In *Genetics and Biogenesis of Chloroplasts and Mitochondria*. Eds. Th. Bücher et al. Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 323-330 (1976)
7. CHUA, N.-H. & F. BLOMBERG: Immunochemical studies of thylakoid membrane polypeptides from spinach and *Chlamydomonas reinhardtii*. A modified procedure for crossed immunoelectrophoresis of dodecyl sulfate protein complexes. *J. Biol. Chem.* 254, 215-223 (1979)
8. CONVERSE, C. A. & D. S. PAPERMASTER: Membrane protein analysis by two-dimensional immunoelectrophoresis. *Science* 189, 469-472 (1975)
9. DAVIS, B. J.: Disc Electrophoresis-II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.*, pp. 404-427 (1964)
10. HARBOE, N. & A. INGILD: Immunization, isolation of immunoglobulins, estimation of antibody titre. In *A Manual of Quantitative Immunoelectrophoresis Methods and Applications*. Eds. N. H. Axelsen, J. Krøll & B. Weeke. Universitetsforlaget, Oslo, pp. 161-164 (1973)
11. HØYER-HANSEN, G. & D. SIMPSON: Changes in the polypeptide composition of internal membranes of barley plastids during greening. *Carlsberg Res. Commun.* 42, 379-389 (1977)
12. KANNANGARA, C. G., S. P. GOUGH, B. HANSEN, J. N. RASMUSSEN & D. J. SIMPSON: A homogenizer with replaceable razor blades for bulk isolation of active barley plastids. *Carlsberg Res. Commun.* 42, 431-439 (1977)
13. KWANYUEN, P. & S. G. WILDMAN: Evidence that genetic information for chloroplast coupling factor I is shared by nuclear and chloroplast DNA. *Biochim. Biophys. Acta* 502, 269-275 (1978)
14. LIEN, S. & E. RACKER: Preparation and assay of chloroplast coupling factor CF₁. In *Methods of Enzymology*, Vol. XXIII. Ed. A. San Pietro, A. P., pp. 547-561 (1971)
15. LOCKSHIN, A., R. H. FALK, L. BOGORAD & C. L. F. WOODCOCK: A coupling factor for photosynthetic phosphorylation from plastids of light- and dark-grown maize. *Biochim. Biophys. Acta* 226, 366-382 (1971)

16. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275 (1951)
17. MACHOLD, O. & G. HØYER-HANSEN: Polypeptide composition of thylakoids from viridis and xantha mutants in barley. *Carlsberg Res. Commun.* 41, 359–366 (1976)
18. MACHOLD, O., D. J. SIMPSON & B. L. MØLLER: Chlorophyll-proteins of thylakoids from wild-type and mutants of barley (*Hordeum vulgare* L.). *Carlsberg Res. Commun.* 44, 235–254 (1979)
19. MCCARTY, R. E. & E. RACKER: Effect of coupling factor and its antiserum on photophosphorylation and hydrogen ion transport. *Brookhaven Symp. Biol.* 19, 202–214 (1966)
20. MILLER, K. R. & L. A. STAEHELIN: Analysis of the thylakoid outer surface. Coupling factor is limited to unstacked membrane regions. *J. Cell Biol.* 68, 30–47 (1976)
21. NELSON, N.: Structure and function of chloroplast ATPase. *Biochim. Biophys. Acta* 456, 314–338 (1976)
22. NIELSEN, N. C., K. W. HENNINGSEN & R. M. SMILLIE: Chloroplast membrane proteins in wild-type and mutant barley. In *Proc. Third Int. Congr. Photosynthesis*. Ed. M. Avron. Elsevier Scientific Publishing Company, Amsterdam, pp. 1603–1614 (1974)
23. POULSEN, C., S. STRØBÆK & B. G. HASLETT: Studies on the primary structure of the small subunit of ribulose-1,5-diphosphate carboxylase. In *Genetics and Biogenesis of Chloroplast and Mitochondria*. Eds. Th. Bücher et al., Elsevier/North Holland, Biomedical Press, Amsterdam, pp. 17–24 (1976)
24. RITZ-GOLD, C. J. & A. F. BRODIE: Limited proteolysis of coupling factor-latent ATPase from *Mycobacterium phlei*. Effects of different enzymes and modifying agents. *Biochim. Biophys. Acta* 547, 18–26 (1979)
25. SIMPSON, D., G. HØYER-HANSEN, N.-H. CHUA & D. von WETTSTEIN: The use of gene mutants in barley to correlate thylakoid polypeptide composition with the structure of the photosynthetic membrane. In *Proc. Fourth Int. Congr. Photosynthesis*. Eds. D. O. Hall, J. Coombs & T. W. Goodwin, London: The Biochemical Society, pp. 537–548 (1977)
26. SIMPSON, D. J., B. L. MØLLER & G. HØYER-HANSEN: Freeze-fracture structure and polypeptide composition of thylakoids of wild-type and mutant barley plastids. In *Symposium on Chloroplast Development*, Spetsai 1978. Eds. G. Akoyunoglou et. al. Elsevier/North-Holland Biomedical Press, pp. 507–512 (1978)
27. SMILLIE, R. M., N. C. NIELSEN, K. W. HENNINGSEN & D. von WETTSTEIN: Development of photochemical activity in chloroplast membranes. I. Studies with mutants of barley grown under a single environment. *Aust. J. Plant. Physiol.* 4, 415–438 (1977)
28. STROTMANN, H., H. HESSE & K. EDELMANN: Quantitative determination of coupling factor CF₁ of chloroplasts. *Biochim. Biophys. Acta* 314, 202–210 (1973)
29. SÜSS, K.-H.: Identification of chloroplast thylakoid membrane polypeptides: Coupling factor of phosphorylation (CF₁) and cytochrome f. *FEBS Letters* 70, 191–196 (1976)
30. VOGEL, G. & R. STEINHART: ATPase of *Escherichia coli*: Purification, dissociation, and reconstitution of the active complex from the isolated subunits. *Biochemistry* 15, 208–216 (1976)
31. WELLBURN, A. R.: Distribution of chloroplast coupling factor (CF₁) particles on plastid membranes during development. *Planta* 135, 191–198 (1977)
32. WETTSTEIN, D. von, B. L. MØLLER, G. HØYER-HANSEN & D. SIMPSON: Genetic manipulation of photosystem I and II in chloroplast membranes. EC Solar Energy R&D Programme. 3rd Coordination Meeting of Contractors, June 18–20, Brussels (1979)
33. YOUNIS, H. M., G. D. WINGET & E. RACKER: Requirement of the δ subunit of chloroplast coupling factor I for photophosphorylation. *J. Biol. Chem.* 252, 1814–1818 (1977)