Thylakoid Membrane Polypeptides of *Chlamydomonas reinhardtii*: Wild-Type and Mutant Strains Deficient in Photosystem II Reaction Center

(Mendelian mutants/temperature-sensitive mutation/gel-concentration gradient electrophoresis/ fluorescence induction kinetics/electron transport)

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ABSTRACT   Unstacked thylakoid membrane vesicles were obtained from a homogenate of *Chlamydomonas reinhardtii* by flotation in a 1.8 M sucrose layer containing 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid)–10 mM EDTA (pH 7.5). Sodium dodecyl sulfate-gradient gel electrophoresis showed that the wild-type membranes have a total of at least 33 polypeptides ranging in molecular weights from 68,000 to less than 10,000. The wild-type and three non-photosynthetic mutant strains were studied with respect to their photosynthetic electron transport properties, their fluorescence rise kinetics, and their membrane polypeptide compositions. The results showed a strong correlation between the presence of a membrane polypeptide (molecular weight = 47,000) and the activity of the photosystem II reaction center. This polypeptide is missing from F34 (a Mendelian mutant lacking Q, the primary electron acceptor of photosystem II), but is partially restored in a suppressed strain of F34 in which there is an incomplete recovery of photosystem II activity. In a thermosensitive mutant, T4, the same polypeptide is present in reduced amount only in cells grown at 35° but not in those grown at 25°. Evidence from fluorescence rise kinetics and partial photochemical reactions show that the cells grown at 25° are similar to wild-type cells but the cells grown at 35° are greatly deficient in Q.

Although the mechanisms of the photosynthetic electron transport reactions have been intensively studied, relatively little is known about the molecular architecture of the thylakoid membranes on which these reactions are localized (compare ref. 1). Chemical analysis revealed that the thylakoid membranes are made up of approximately 50% lipids and 50% proteins (2). There is evidence that there are at least 10 to 20 polypeptides of different molecular weights in these membranes (3–11).

Several approaches are available for the identification of the functions of the thylakoid membrane polypeptides. One approach is to fractionate the membranes by either detergents (5, 6, 9) or passage through a French pressure cell (9, 11) into small fragments enriched in either photosystem I (PS I) or photosystem II (PS II) activities. The polypeptide components of these subchloroplastic fragments or pigment–protein complexes can then be identified by sodium dodecyl sulfate-gel electrophoresis. Another approach is to analyze the membrane polypeptides of mutant strains which are either pigment-deficient (12–16) or have specific lesions in the electron transport pathway (6, 17, 18). The missing or altered

polypeptides can then be correlated with the deleted functions in the mutant.

In this paper, we have adopted the mutant approach and compared the polypeptide profile of thylakoid membrane of wild-type *Chlamydomonas reinhardtii* with those of mutant strains lacking or deficient in PS II activity. Our results suggest that a membrane polypeptide of molecular weight 47,000 is required for the activity of PS II reaction centers.

MATERIALS AND METHODS

Conditions of Cell Culture. The wild-type (*137c*, *mt⁺*) and three mutant strains (F34, F34SU1, and T4) of *Chlamydomonas reinhardtii* were grown in Tris–acetate–phosphate medium under conditions described by Gorman and Levine (19). F34 and T4 were derived from the wild-type (WT) strain by mutagenesis with methyl methane sulfonate and selected as high fluorescence mutants (20). F34 has been characterized and shown to have no PS II activity (21, 22). F34SU1 was obtained by irradiating the parental strain, F34, with ultraviolet light and was selected for its ability to grow slowly on minimal medium. This strain has partially restored PS II activity (23). T4 is a conditional mutant: it can grow on minimal medium at 25° but requires acetate for growth at 35°. The photosynthetic properties of this mutant will be described here for the first time.

Isolation of a Thylakoid Membrane Fraction. Thylakoid membranes were purified from cell-free homogenates by a modification of the flotation procedure described previously (24). One liter cultures were harvested during the exponential phase of growth (3 to 5 × 10⁶ cells per ml) by centrifugation at 2500 × *g* for 5 min at 0°. The following operations were carried out in the cold (0–4°). The pelleted cells were washed once in 0.3 M sucrose/25 mM HEPES-KOH (pH 7.5)/1mM MgCl₂ and resuspended in 20 ml of the same buffer. Cells were disrupted by passing the suspension (2 × 10⁸ cells per ml) through a chilled French pressure cell maintained at 4000 lb/in² (27.58 MPa) and the homogenate was centrifuged at 2000 × *g*ₘₐₓ for 10 min. The supernatant, containing almost all of the soluble proteins, most of the mitochondria, and some small chloroplast membrane vesicles, was discarded. To unstack thylakoid membranes (25), release Ca⁺⁺-dependent ATPase (26) and trapped starch granules, we resuspended the 2,000 × *g*ₘₐₓ pellet in 30 ml of 0.3 M sucrose/5 mM HEPES-KOH (pH 7.5)/10 mM EDTA by homogenizing with a motor-driven teflon pestle. The membrane vesicles were collected by centrifugation at 50,000 × *g*ₘₐₓ for 10 min. The pellet was resuspended in 15 ml of 1.80 M sucrose/5 mM HEPES-KOH (pH 7.5)/10 mM EDTA, and

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Abbreviations: WT, wild-type; PS I, photosystem I; PS II, photosystem II; DCMU, 3,4-dichlorophenyl dimethylurea; PBQ, *p*-benzoquinone; DPIP, 2,6-dichlorophenol indophenol; MV, methyl viologen; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.
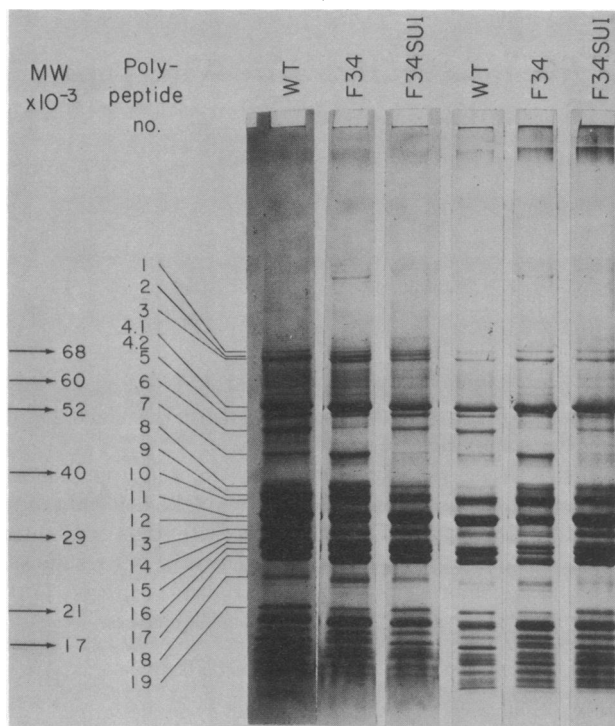
FIG. 1. Sodium dodecyl sulfate-gel electrophoretogram of thylakoid membranes from wild-type (WT), F34, and F34SU1. The first three slots from the left contained unextracted thylakoid membranes (25 μg of chlorophyll), whereas the remaining three slots contained thylakoid membranes (37.5 μg of chlorophyll) which had been extracted with 90% acetone. The relationship between electrophoretic mobilities and molecular weights was established with the following markers: bovine serum albumin (68,000), catalase (60,000), α-amylase (52,000), creatine kinase (40,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,000), and myoglobin (17,000).
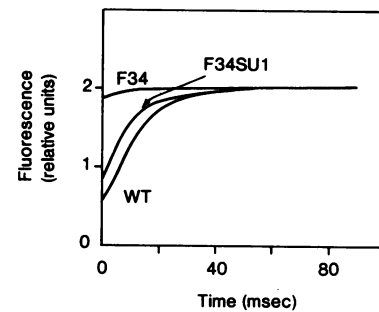

FIG. 2. Fluorescence rise curve of wild-type, F34, and F34SU1. Experiments were performed with dark-adapted intact cells in the presence of 10 μM of DCMU as described under *Materials and Methods.*

5 ml of the suspension were overlaid with 2 ml of 1.30 M sucrose/5 mH HEPES–KOH (pH 7.5)/10 mM EDTA and then with 5 ml of 0.5 M sucrose/5 mM HEPES–KOH (pH 7.5.). The discontinuous sucrose gradient was centrifuged at 40,000 rpm for 1 hr at 4° in an SB 283 rotor of the IEC centrifuge (model B 60). After this centrifugation, thylakoid membrane vesicles and eye-spot materials floated to the 1.30 M sucrose layer and the 0.5 M sucrose layer, respectively, whereas unbroken cells, nuclei, cell wall materials, pyrenoids, and starch granules were pelleted at the bottom. The 1.30 M sucrose layer, containing the thylakoid membranes, was collected and diluted with 3 volumes of 5 mM HEPES–KOH (pH 7.5)/10 mM EDTA and the membranes were pelleted by centrifugation at 50,000 × g_{max} for 10 min. Approximately 70–85% of the chlorophyll present in the homogenate was recovered in this fraction which had a protein to chlorophyll (w/w) ratio of about 5. Electron microscopic examination of the thylakoid membrane fraction revealed that it consisted primarily of thylakoid membrane vesicles which are all unstacked, with a few contaminating eye-spot materials, and occasional broken mitochondria (Chua and Ojakian; in preparation).

Sodium Dodecyl Sulfate-gel Electrophoresis of Membrane Polypeptides. Thylakoid membranes were solubilized in a mixture containing 0.05 M Na_2CO_3, 0.05 M dithiothreitol, 2% sodium dodecyl sulfate, 12% sucrose, and 0.04% brom-

phenol blue to a final chlorophyll concentration of 1 mg/ml and a ratio of sodium dodecyl sulfate to chlorophyll of 20:1 by weight. To remove photosynthetic pigments, we extracted thylakoid membranes twice with 90% acetone at room temperature before the precipitate was solubilized as described above. Both the acetone-extracted and nonextracted samples were heated in a boiling waterbath for 1 min immediately after solubilization.

Electrophoresis of membrane polypeptides was carried out in a slab-gel apparatus modified from the design of Studier (27); essentially the discontinuous alkaline buffer system of Neville (28) was used with a stacking gel of 1–2 cm and a separating gel of about 20 cm. The stacking gel was made up of 6% acrylamide, whereas the separating gel was made up of a linear concentration gradient of acrylamide (7.5–15%) as described by Alvares and Siekevitz (29) accompanied by a 5–17.5% sucrose gradient in the gel. The ratio of acrylamide to N,N'-methylenebisacrylamide for both gels was 30:0.8. The following buffers were used: upper reservoir buffer, 0.04 M boric acid–0.041 M Tris–0.1% sodium dodecyl sulfate (pH 8.64); stacking gel buffer, 0.0267 M H_2SO_2–0.0541 M Tris–0.1% sodium dodecyl sulfate (pH 6.10); separating gel buffer, 0.0308 M HCl–0.4244 M Tris–0.1% sodium dodecyl sulfate (pH 9.18); lower reservoir buffer, same as the separating gel buffer except that the sodium dodecyl sulfate was omitted. Electrophoresis was performed at a constant current of 17.5 mA for about 12 hr at room temperature. Gels were stained for 3–5 hr with 0.25% Coomassie brilliant blue in 50% methanol–7% acetic acid and excess dye was removed by repeated washings in 30% methanol–7% acetic acid. Stained gels were scanned at 550 nm with a Gilford spectrophotometer (model 240) equipped with a linear transport gel scanner.

Measurements of Fluorescence and Electron Transport Reactions. Fluorescence and electron transport reactions were measured at room temperature as described previously (30, 22). Chloroplast fragments used in the assays were prepared according to Gorman and Levine (19). Chlorophyll and protein concentrations were determined according to Arnon (31) and Lowry *et al.* (32), respectively.

Chemicals. All chemicals were of analytical grades when available.

RESULTS

In the electrophoretic experiments in which the sodium dodecyl sulfate-gel concentration gradient (7.5–15%) was used, a linear relationship exists between R_F and log molecular weight in the 70,000–15,000 range (results will be published).
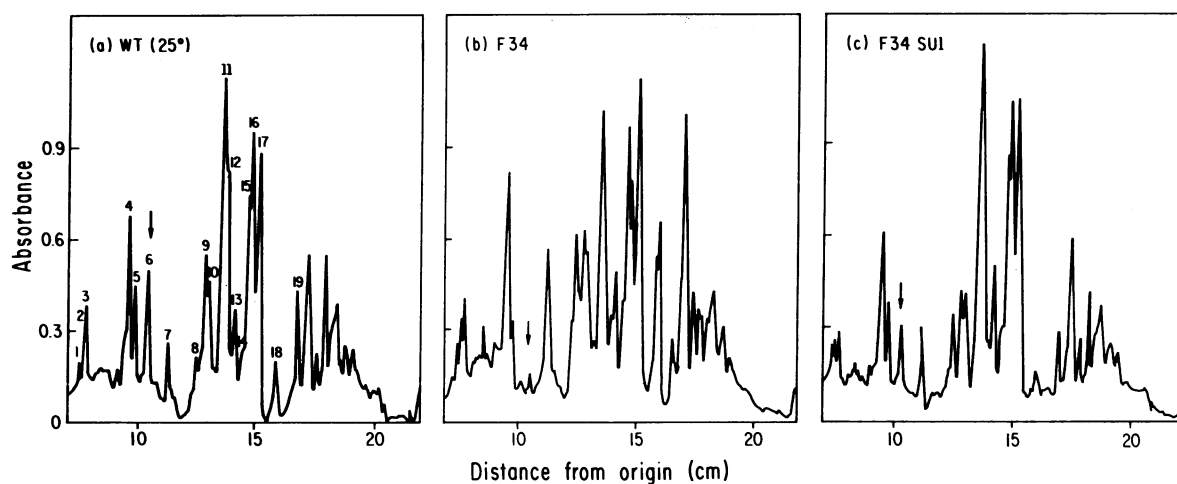
FIG. 3. Densitometric tracings of thylakoid membrane polypeptides prepared from wild-type, F34, and F34SU1. Gels of unextracted thylakoid membranes from Fig. 1 were scanned at 550 nm as described under *Materials and Methods*. The vertical arrow indicates the position of polypeptide 6.

Using this system, we found that the WT thylakoid membranes consisted of a total of at least 33 polypeptides (Fig. 1). These membrane polypeptides fall roughly into three groups on the basis of their electrophoretic mobilities. There are eight polypeptides ranging in molecular weight from 68,000 to 40,000, 12 polypeptides between 40,000 and 20,000, and a group of minor polypeptides with molecular weights of less than 20,000. The separation of the last group of polypeptides was severely interfered with by the presence of sodium dodecyl sulfate–pigment complexes which had similar electrophoretic mobilities (Fig. 1). Upon extraction of the photosynthetic pigments with 90% acetone, at least 13 to 15 polypeptides became evident in the low-molecular-weight range (Fig. 1). We have enumerated the membrane polypeptides in the electrophoretogram by consecutive numbers beginning from the high-molecular-weight region. In addition to the 7.5–15% gel concentration gradient, membrane polypeptides were also separated in sodium dodecyl sulfate-gels containing the following acrylamide gradients: 5–10%, 7.5–10%, 7.5–12.5%, and 10–15% (data not shown). These gradients were designed to provide optimal resolution of polypeptides at different molecular weight ranges. Control experiments with purified marker proteins (bovine serum albumin, catalase, α-amylase, creatine kinase, myoglobin, cytochrome *c*, lysozyme, and RNase) showed that each protein migrated as a single band in all of these gradient gels including the 7.5–15% gel system. We found that although band 4 moved as a single band in 7.5–15%, 7.5–12.5%, and 10–15% gradient gels, it was split into two distinct bands in 5–10% and 7.5–10% gradient gels, both of which gave good resolution between 68,000 and 25,000. All other polypeptide bands obtained with the 7.5–15% gel system were not split in other gel systems and, therefore, we assume that each of these bands represents only one polypeptide. However, the possibility that some of these bands may contain more than one polypeptide cannot be ruled out.

Thus, it can be seen that although acetone extraction allowed the visualization of low molecular weight (<20,000) polypeptides, it also resulted in a selective loss of polypeptide 2 and a weakening of the intensities of other polypeptides (e.g., 5, 6, 7, 8, and 14). Whether these polypeptides were wholly or partly soluble in 90% acetone or whether they failed to penetrate the stacking gel (compare Fig. 1) after the ace-

tone treatment is not known. However, it is clear from these experiments that the 90% acetone-extracted membranes can only be used for the display of the low-molecular-weight polypeptides.

In addition to the polypeptides numbered, there are some faint bands in the high-molecular-weight region as well as in other parts of the gel, but since the recovery of these bands is highly variable we assume they are contaminants. The recovery of polypeptide 7 (molecular weight 41,000) is also variable in the WT as well as the mutant strains (see later) examined, but due to its relative abundance, as assessed by its staining intensity with Coomassie blue, it could be a polypeptide that is loosely bound to the membrane.

In an attempt to identify the functions of some of these membrane polypeptides, we have examined the membrane polypeptide compositions of mutant strains of *C. reinhardtii* which have specific lesions at the PS II reaction center. One such mutant is F34 which has been characterized previously (21, 22). The data in Table 1 confirm previous observations that this mutant is unable to perform any PS II reaction but has normal PS I activity. The lack of Hill reaction activity could be due to a block either in the oxidizing or the reducing side of PS II. If the block is on the oxidizing side, i.e., between H₂O and PS II, the fluorescence yield should be low (33), whereas if it is on the reducing side of PS II, the fluorescence yield should be high (34, 35). Fluorescence measurements of F34 showed that it has a high level of initial fluorescence with no variable portion, and the fluorescence yield is not affected by the addition of 10 μM 3,4-dichlorophenyl dimethylurea (DCMU) (Fig. 2). These results demonstrate that the primary electron acceptor of PS II, designated Q by Duysens (35), is either missing or inactive and hence all the PS II reaction centers could not function. Examination of the membrane polypeptides of F34 (Fig. 3b) revealed that the mutation also led to a loss‡ of polypeptide 6 (molecular weight 47,000) and an about 50% reduction in the amount of polypeptide 5 (molecular weight 50,000) (Figs. 1 and 3). In addition, there is a slight increase in polypeptide 16 relative to

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‡ The faint band seen in Fig. 1 is not identical to polypeptide 6 but has a slower electrophoretic mobility, as shown in this and other experiments. This band can be better resolved from polypeptide 6 in a 10–15% gradient gel.
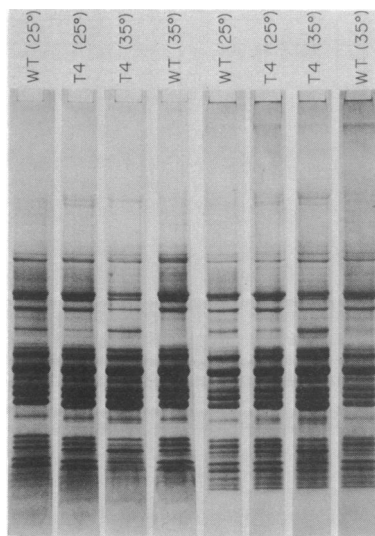
FIG. 4. Sodium dodecyl sulfate-gel electrophoretogram of thylakoid membranes from wild-type and T4 strains grown at both 25° and 35°. The first four slots from the left contained unextracted thylakoid membranes (25 μg of chlorophyll), whereas the remaining four slots contained thylakoid membranes (37.5 μg of chlorophyll) which had been extracted with 90% acetone.
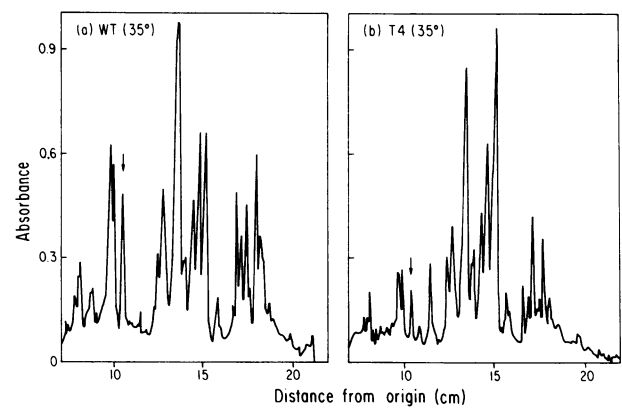

FIG. 5. Densitometric tracings of thylakoid membrane polypeptide prepared from wild-type and T4 strains, both cultured at 35°. Gels of unextracted thylakoid membranes from Fig. 5 were scanned at 550 nm as described under *Materials and Methods*. The vertical arrow indicates the position of polypeptide 6.

polypeptides 15 and 17, and an increase in the amounts of some small polypeptides which move slightly ahead of polypeptide 19. The increase in polypeptide 18 (Figs. 1 and 3b) was not seen in other experiments and therefore must be due to contaminating polypeptides present in this particular experiment. Since F34 has no PS II activity at all, we tentatively conclude that polypeptide 6 is required for the normal functioning of the PS II reaction center.

The PS II activity in F34 could be partially restored to that observed for the WT strain, by a suppressor mutation induced by UV irradiation (23). Several suppressed strains of F34 have been isolated and the membrane polypeptides of one of them were studied. One suppressed strain, F34SU1, has about 50% of the Hill-reaction activity found in the WT strain (Table 1), and the half-time of the fluorescence rise curve of this strain is 1.2 times faster than that of the WT strain (Fig. 2). Estimation of the area circumscribed by the fluorescence rise curve and the maximum level of fluorescence provides a reliable method for computing the number of active PS II reaction centers (23, 30). With this method, we estimated that F34SU1 has about 60% of the active quencher and hence active PS II reaction centers found in the WT strain. To see if this partial phenotypic suppression of the loss of PS II activity was paralleled by a partial restoration of the amount of polypeptide 6, we examined thylakoid membranes of F34SU1. Figs. 1 and 3 show that this is indeed the case. The amount of polypeptide 5 is approximately the same as that in the WT strain but polypeptide 6, which is entirely missing from membranes of F34, is restored to approximately one half of that found in the WT strain. This correlation between partial restoration of active PS II quencher and the partial recovery of polypeptide 6 strongly suggests that the latter is essential for the reaction center activity of PS II.

We next examined the membrane polypeptide profile of a thermo-sensitive mutant, T4. When cultured at 25°, this mutant is very similar to the WT strain in its electron transport properties (Table 1) and fluorescence induction kinetics (data not shown), but when grown at 35°, it is similar to F34

and has normal PS I but greatly reduced PS II activity (Table 1). From fluorescence measurements, we estimated that T4 (35°) has about 10% of the number of active PS II reaction centers as has the WT strain (35°) (data not shown). To see if the temperature-sensitive deficiency in Q was also accompanied by a similar conditional change in thylakoid membrane phenotype, we analyzed the membrane polypeptides of T4 grown at both the permissive (25°) and restrictive (35°) temperatures. Fig. 4 shows that the membrane polypeptide composition of T4 at 25° is similar to that of the WT strain at 25° except that there is a greater amount of polypeptide 17 (compare Fig. 1) in the mutant. Thylakoid membranes isolated from T4 grown at 35°, however, show deficiencies (Figs. 4 and 5) in polypeptides 4.1 and 4.2 (molecular weight 52,000), 5 (molecular weight 50,000), and 6 (molecular weight 47,000) (compare Fig. 1).

Genetic analysis of F34 and T4 show that both mutations are Mendelian and therefore of nuclear origin (results to be published). However, it is not known whether these two loci are allelic or not.

DISCUSSION

The polypeptide composition of *Chlamydomonas* thylakoid membranes has been previously examined with single pore gel electrophoresis in either acetic acid–urea (4) or sodium dodecyl sulfate (3, 6), and in both systems approximately 18 to 20 membrane polypeptide bands are resolved. We have improved on the resolution of thylakoid membrane polypeptides by combining the sodium dodecyl sulfate-disc system of Neville (28) with a gel concentration gradient (36). The sulfate–borate system used by Neville (28) is capable of stacking sodium dodecyl sulfate–protein complexes over a wide range of molecular weights, thus providing very sharp bands, whereas the pore gradient allows the separation and resolution of polypeptides with widely different molecular weights on the same gel. With this system, we have found that the thylakoid membrane of wild-type *C. reinhardtii* is composed of at least 33 polypeptides ranging in molecular weights from 68,000 to less than 10,000. An identical polypeptide profile could be obtained with thylakoid membranes prepared by the method of Hoober (3). Therefore, the increase in the number of polypeptide bands observed in this system as compared to those reported previously (3, 4, 6) cannot be explained by the dif-

TABLE. 1   *Photochemical reactions of chloroplast fragments*
prepared from wild-type and mutant strains

| | Rate of reaction (μmol of O_2 evolved or consumed/mg of chlorophyll per hr) | | | |
Strain	PBQ-Hill	Ferri-cyanide-Hill	MV-Hill	DPIPH$_2$ → MV
WT (25°)	210	90	75	495
F34 (25°)	<2	<2	<2	511
F34SU1 (25°)	125	42	56	520
WT (35°)	193	80	72	370
T4 (25°)	230	70	92	360
T4 (35°)	21	10	8	380

The PBQ-Hill reaction was carried out in a reaction mixture containing whole cells (15 μg of chlorophyll/ml), 10 mM potassium phosphate buffer (pH 7.0), and 2 mM *p*-benzoquinone. For the ferricyanide-Hill reaction, the mixture contained chloroplast fragments (30 μg of chlorophyll/ml), 40 mM HEPES-KOH (pH 7.0), 20 mM KCl, 2.5 mM MgCl$_2$, 2 mM NH$_4$Cl, and 5 mM potassium ferricyanide. The reaction mixture for the MV-Hill reaction contained chloroplast fragments (30 μg of chlorophyll/ml), 40 mM HEPES-KOH (pH 7.0), 20 mM KCl, 2.5 mM MgCl$_2$, 2 mM NH$_4$Cl, 0.2 mM MV, and 1 mM NaN$_3$. The photoreduction of MV with DPIP/ascorbate coupler as the electron donor was performed with a reaction mixture containing chloroplast fragments (7.5 to 15 μg of chlorophyll/ml), 40 mM HEPES-KOH (pH 7.0), 20 mM KCl, 2.5 mM MgCl$_2$, 2 mM NH$_4$Cl, 0.2 mM MV, 0.1 mM DPIP, 3 mM sodium ascorbate, 1 mM NaN$_3$, and 10 μM DCMU.

ferent methods of membrane purification but must be due to the greater resolving power of the gradient gel electrophoresis.

Analysis of the membrane polypeptide profiles of a group of mutant strains shows that the presence of polypeptide 6 (molecular weight 47,000) is associated with the proper functioning of PS II reaction center. Thus, this polypeptide is completely missing from the thylakoid membrane of F34, which lacks active PS II reaction centers, but is partially restored to the WT level in suppressed strains of F34 in which there is partial recovery of PS II activity. A similar correlation exists between the amount of this polypeptide and PS II activity in T4, a temperature-sensitive mutant which shows conditional defects in the PS II reaction center.

In addition to polypeptide 6, polypeptide 5 is present in reduced amounts in F34, whereas in T4 cultured at 35°, polypeptides 5, 4.1, and 4.2 are also affected. Whether one or all of these polypeptides play a role in PS II activity is not known. However, it is clear from our results that they are not important for PS I activity since reductions in the amounts of these polypeptides in F34 or T4 grown at 35° do not affect the photoreduction of MV with DPIP/ascorbate as the electron donor (compare Table 1).

Both F34 and T4 grown at 35° are similar to two previously characterized mutants, ac-115 and ac-141 (34, 37), in that their lesions in the electron transport pathway are similar to those of ac-115 and ac-141. Levine *et al.* (6) have reported that a thylakoid membrane polypeptide designated IIa is present in reduced amount in ac-141 as compared to the amount present in the WT strain. However, recent evidence by Levine and Durham (16) indicates that polypeptide IIa as well as polypeptides IIb and c are not required for PS II activity *in vivo* since they lack a mutant strain, ac-5, which

is able to carry out the PBQ-Hill reaction. The group II polypeptides probably correspond to polypeptides 8-17 and these polypeptides have molecular weights smaller than that of polypeptide 6.

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