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CHLOROPLAST MEMBRANES OF THE GREEN ALGA *ACETABULARIA MEDITERRANEA*

I. ISOLATION OF THE PHOTOSYSTEM II

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SUMMARY

1. In the presence of Triton X-100, chloroplast membranes of the green alga *Acetabularia mediterranea* were disrupted into two subchloroplast fragments which differed in buoyant density. Each of these fractions had distinct and unique complements of polypeptides, indicating an almost complete separation of the two fragments.

2. One of the two subchloroplast fractions was enriched in chlorophyll *b*. It exhibited Photosystem II activity, was highly fluorescent and was composed of particles of approx. 50 Å diameter.

3. The light-harvesting chlorophyll-protein complex of the Photosystem II-active fraction had a molecular weight of 67 000 and contained two different subunits of 23 000 and 21 500. The molecular ratio of these two subunits was 2 : 1.

INTRODUCTION

One of the major goals of current photosynthetic research is the purification and characterization of Photosystems I and II. Photosystem I particles have been isolated from a number of plant species after mechanical disruption or detergent treatment of chloroplast membranes. However, the Photosystem II fractions obtained by these methods were not pure; they were enriched in Photosystem II activity but still contained a significant amount of Photosystem I (see ref. 1). Only recently, by using further purification steps, have Photosystem II-active fractions been isolated which were devoid of Photosystem I activity [2–5]. It has been suggested that two of these Photosystem II-active preparations contain the reaction center of Photosystem II [3, 4] because they lacked some properties ascribed to Photosystem II. Based on these results it was proposed that Photosystem II contained a reaction-center chlorophyll-protein complex, which binds chlorophyll *a*, and a light-harvesting chlorophyll-protein complex, which is enriched in chlorophyll *b* [3].

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

In the present study we describe the isolation of a purified Photosystem II fraction from the chloroplast membrane of the green alga *Acetabularia mediterranea* by conditions which in higher plants allow only the isolation of a purified Photosystem I. The purified Photosystem II fraction was characterized and its properties compared to those described for Photosystem II of higher plants. The light-harvesting chlorophyll-protein complex of the Photosystem II in *A. mediterranea* had a molecular weight of 67 500. The chlorophyll-binding polypeptide of 30 000 – 35 000 daltons described for other plants [6–11] and identified previously as the light-harvesting chlorophyll-protein complex of Photosystem II [11] was also found in *A. mediterranea.* This polypeptide was shown to be a subunit of the 67 500-dalton chlorophyll-protein complex of Photosystem II.

METHODS

Cells

A. mediterranea was grown by the method of Hämmerling [19].

Isolation of chloroplasts

Approx. 800–1000 plants were washed several times with cold sea water, dried on filter paper, cut into pieces and homogenized with a glass tissue grinder in 40 ml chloroplast buffer 2 (see ref. 13). The homogenate was first passed through two layers of cheesecloth, then through one layer of Miracloth and was then centrifuged for 10 min at 2000 × *g*. The supernatant was discarded. The sediment was then suspended in 40 ml 0.1 M Tris·HCl (pH 7.6). After centrifugation for 10 min at 1000 × *g*, the sediment was washed again with the Tris buffer. This sediment was designated "washed chloroplast membranes."

Fractionation with Triton X-100

The washed chloroplast membranes were incubated with 1 % Triton X-100 for 30 min at 0 °C in a modification of the procedure of Briantais [14]. After the treatment with Triton X-100, the membrane fragments were centrifuged through a sucrose gradient (11 ml) made by layering a 10 % to 40 % linear gradient onto a 1-ml 55 % sucrose cushion. The gradient was centrifuged for 22 h at 190 000 × *g* in a Beckman Spinco SW 40 rotor. The green layers were removed from the gradient with a pipette, dialyzed against distilled water and freeze-dried. For photochemical assays the green material was dialyzed against Tris·HCl (pH 7.5).

Photochemical assays

Photosystem II activity was assayed by the method of Vernon and Shaw [15] using diphenyl carbazide as electron donor and 2,6-dichlorophenolindophenol (DCIP) as acceptor. The reaction mixture contained in 3 ml, membrane fragments containing 10–15 μg chlorophyll and 200 μmol Tris·HCl (pH 7.5) and 30 nmol DCIP.

Photosystem I activity was measured by photoreduction of $NADP^+$. The mixture contained in 3 ml, membrane fragments containing 10–15 μg chlorophyll and in (μmol) Tris·HCl (pH 7.5), 200; $NADP^+$, 0.18; and saturating amounts of ferredoxin, plastocyanin and $NADP^+$ reductase. Diphenyl carbazide (1.5 μmol) or sodium ascorbate (6 μmol) plus DCIP (0.15 μmol) were added, respectively, as electron donors.

The changes in absorbance at 590 nm (DCIP reduction) or at 340 nm ($NADP^+$ reduction) were measured after illuminating with saturating incandescent white light for 30 s at 20 °C. Plastocyanin was isolated by the method of Gorman and Levine [16] and ferredoxin and $NADP^+$ reductase were prepared according to San Pietro and Lang [17].

Fluorescence measurements

Fluorescence emission spectra were recorded on a Perkin-Elmer, type MPF-2a, fluorescence spectrophotometer. Fluorescence recordings at liquid nitrogen temperature were carried out in 60 % glycerol.

Chlorophyll determination

Chlorophyll was extracted into 80 % acetone and spectra were recorded with a Cary Model 14 spectrophotometer. Total chlorophyll and chlorophylls *a* and *b* were determined by the method of Arnon [18].

Electrophoretic separation of proteins

The membrane fractions were dissolved in a solution containing 0.2 % sodium dodecylsulfate, 10 % glycerol, 0.1 % 2-mercaptoethanol, 65 mM Tris–HCl (pH 6.8) and were applied to a 10–15 % linear polyacrylamide gradient gel. Electrophoresis was for 3 h at 20 mA constant current using the apparatus described by Studier [19] and the buffer system described by Laemmli [20]. All steps were carried out at 4 °C. The gel was stained with Coomassie Blue and then destained according to Fairbanks et al. [21].

Isolation of the chlorophyll-protein complexes

In the presence of EDTA and 2-mercaptoethanol more than 40 % of the protein of the washed chloroplast membranes was solubilized and could be separated from the remaining membrane fragments. The EDTA-insoluble chloroplast membranes contained all the chlorophyll (Apel, K., in preparation). After polyacrylamide gel electrophoresis of the solubilized EDTA-insoluble chloroplast membranes, the gel sections containing chlorophyll bands were cut out and freeze-dried overnight. Then the lyophilized material was pulverized with a mortar and pestle, suspended in a small volume of 0.1 % sodium dodecylsulfate, 10% glycerol, 0.1 % 2-mercaptoethanol, 65 mM Tris·HCl (pH 6.8) and dialyzed for 2 h against the same buffer at 4 °C. The suspension was then centrifuged for 10 min at 17 000 × *g* and the resulting supernatant was subjected to polyacrylamide gel electrophoresis. Absorbances of Coomassie Blue-stained bands in the gels were scanned at 608 nm with a Gilford 2000 spectrophotometer.

Molecular-weight estimation of the chloroplast membrane proteins

Molecular weights of the membrane proteins were estimated from a calibration curve obtained with the following proteins: *Escherichia coli* RNA polymerase subunits β' (165 000), β (155 000) and α (39 000), β-galactosidase (130 000), bovine serum albumin (69 000), carbonic anhydrase (29 000), ribonuclease (13 700) and cytochrome *c* (12 500). β-Galactosidase was purchased from Worthington, ribonuclease from

Calbiochem, and *E. coli* RNA polymerase core enzyme was a gift of D. Ratner. The other proteins were obtained from Sigma.

Electron microscopy

For electron microscopy, the subchloroplast fractions from the sucrose density gradients were dialyzed against 10 mM phosphate buffer (pH 7.6) and negatively stained with 2 % aqueous uranyl acetate. Grids were examined in a Philips 200 microscope and micrographs were taken at an initial magnification of 60 000.

RESULTS

Washed chloroplast membranes

The membranes were able to photoreduce $NADP^+$ or DCIP if provided with plastocyanin, ferredoxin, $NADP^+$ reductase and diphenyl carbazide (Table I).

TABLE I

PHOTOSYNTHETIC ACTIVITIES OF VARIOUS CHLOROPLAST MEMBRANE FRACTIONS OF *A. MEDITERRANEA*

Fractions	Rate (μmol photoreduced/mg chlorophyll per h)		
	DCIP		$NADP^+$
	−DCMU	+DCMU (10^{-5} M)	
Washed chloroplast membranes	57		38*
Fraction B of washed chloroplast membranes	25	2	0**
Fraction C of washed chloroplast membranes	0	0	0**

* Diphenyl carbazide as electron donor.
** Ascorbate plus DCIP as electron donor.

The chlorophyll *a*/*b* ratio of the washed chloroplast membrane in *A. mediterranea* was approx. 1.2 and was significantly lower than that of chloroplast membranes in *Chlamydomonas reinhardi* [22] or higher plants (see ref. 1).

The washed chloroplast membrane of *A. mediterranea* solubilized by 0.2 % sodium dodecylsulfate could be resolved electrophoretically into four chlorophyll-containing bands (Fig. 1F). The fastest migrating band contained free chlorophyll, while in the three other bands the chlorophyll was associated with proteins. The molecular weights of the proteins in the three chlorophyll-containing bands were 125 000, 67 000 and 21 500 based on their relative electrophoretic mobilities. Besides these three chlorophyll-binding proteins, the washed chloroplast membranes contained at least 51 additional polypeptides.

Fractionation with Triton X-100

The washed chloroplast membranes were disrupted by incubation with Triton X-100, and the resulting suspension of subchloroplast fragments was subjected to sucrose-gradient centrifugation. The suspension was resolved into three different

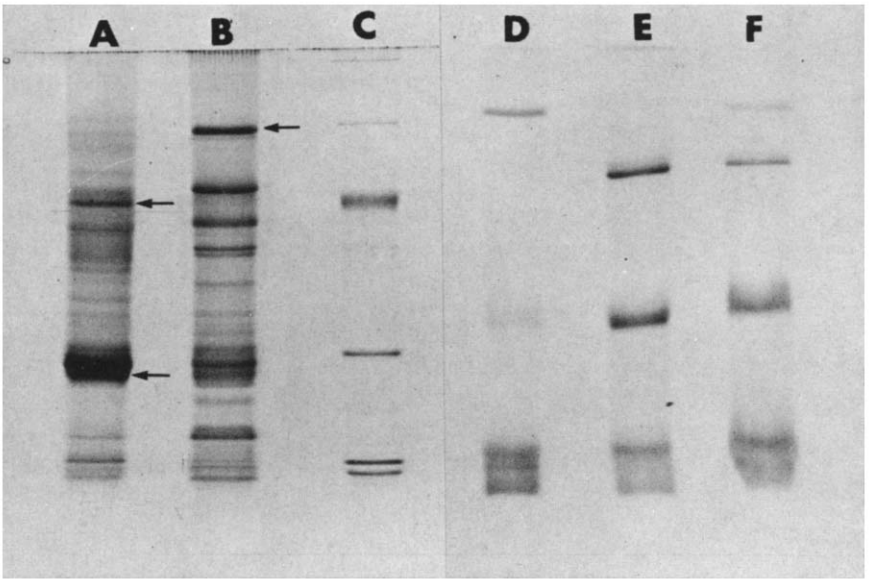

Fig. 1. Chlorophyll and protein pattern after electrophoresis of the solubilized washed chloroplast membranes and its Subfractions B and C on polyacrylamide gels before (D–F) and after (A, B) Coomassie Blue staining. Subfraction B (A and E), Subfraction C (B and D), the washed chloroplast membrane (F) and the standard proteins (C) β-galactosidase (130 000), bovine serum albumin (69 000), carbonic anhydrase (29 000), ribonuclease (13 700) and cytochrome *c* (12 500) were solubilized and separated electrophoretically as described in Methods. The arrows indicate the position of chlorophyll-binding proteins.

chlorophyll-containing bands, which were designated A, B and C (Fig. 2). Fraction A contained free chlorophyll; the ratio of chlorophyll *a* to *b* was the same as in the unfragmented membrane. Under the electron microscope no structure could be detected.

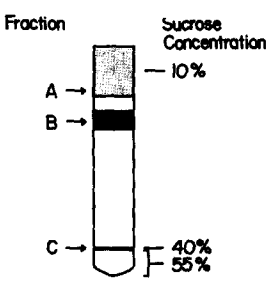

Fig. 2. Schematic representation of the distribution of subchloroplast fragments of *A. mediterranea* after sucrose density-gradient centrifugation. Washed chloroplast membranes were treated with Triton X-100 and centrifuged as described in Methods.

Fraction C was enriched in chlorophyll *a* (chlorophyll $a/b = 3.9$). It was weakly fluorescent at room temperature (Table II). Under the electron microscope a semi-

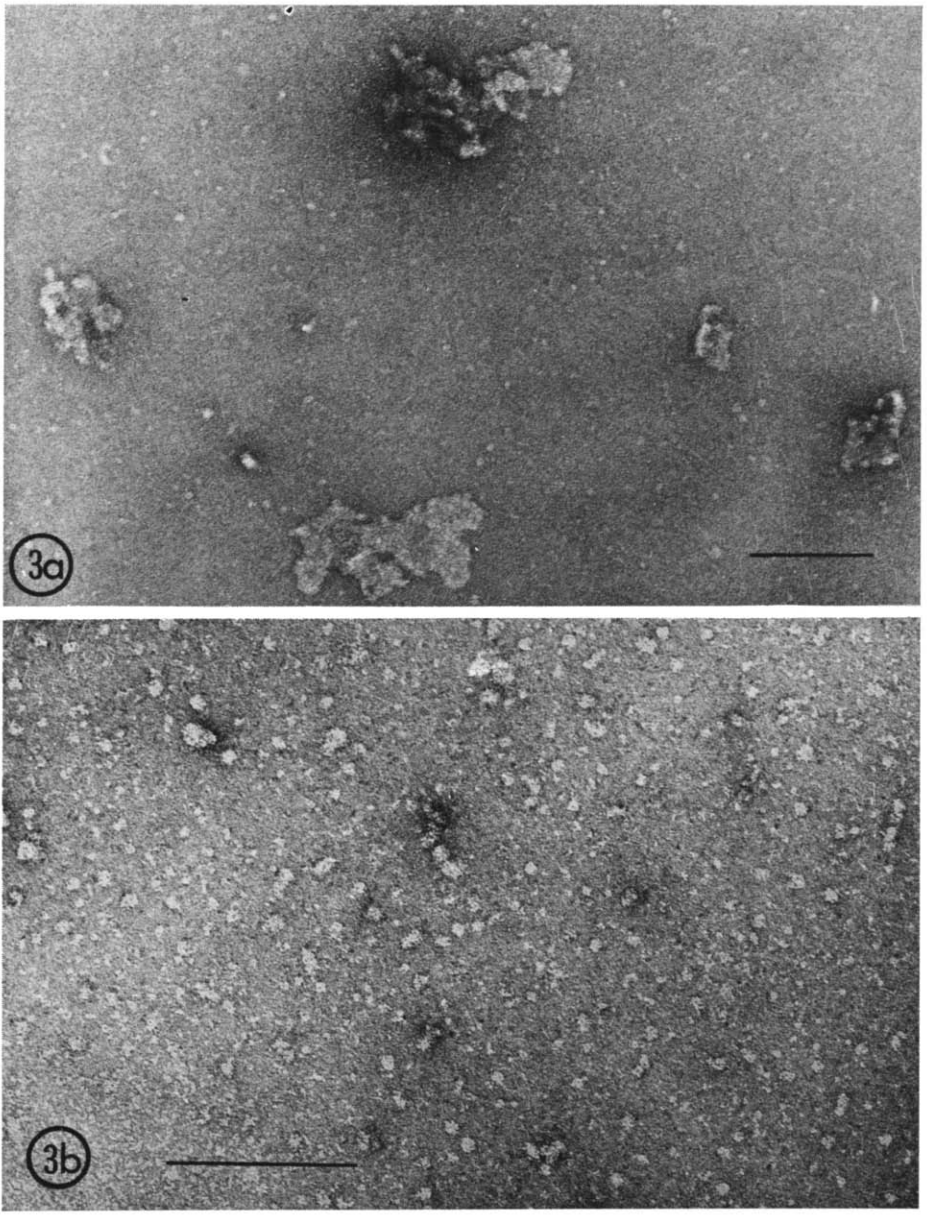

Fig. 3. Electronmicrographs of the Fraction B (b) and Fraction C (a) of the washed chloroplast membranes of *A. mediterranea*. The fractions were negatively stained with 2 % aqueous uranyl acetate. Bars represent 100 nm.

membraneous structure could be seen (Fig. 3a). After gel electrophoresis of the solubilized Fraction C, only the 125000 dalton chlorophyll-binding protein appeared in addition to a fast-migrating band of free chlorophyll (Fig. 1D). When the gel was

TABLE II

FLUORESCENCE YIELD AT 20 °C

The fluorescence yield was measured with an excitation wavelength of 436 nm and an emission wavelength of 680 nm. Values are expressed as arbitrary units per 0.1 absorbance units at 436 nm.

Fractions	Fluorescence yield
Washed chloroplast membranes	3.2
Fraction B	10.3
Fraction C	0.9

overloaded with sample, sometimes traces of the 67 000- and 21 500-dalton membrane proteins could be detected. After Coomassie Blue staining of the gel, at least 32 polypeptides appeared (Fig. 1B).

Neither Fraction A nor C was photochemically active. Among the three subchloroplast fractions of the washed chloroplast membrane, only Fraction B exhibited photochemical activity. Fraction B photoreduced DCIP, but not $NADP^+$ (Table I), thus showing Photosystem II activity [15]. The DCIP reduction was inhibited by 10^{-5} M 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU). The Photosystem II-active Fraction B was characterized in more detail.

Characterization of the Photosystem II-active fraction

Fraction B had a chlorophyll *a*/*b* ratio of 0.64; low compared to a ratio of 1.2 for the unfragmented membrane. It was strongly fluorescent at room temperature (Table II). The fluorescence emission spectra at 77 °K are shown in Fig. 4. The unfragmented washed chloroplast membrane had a small absorption band in the red region at 680 nm and a more intense, broader band at 698 nm. Fraction B was highly fluorescent, and its low-temperature emission spectrum contained very intense bands

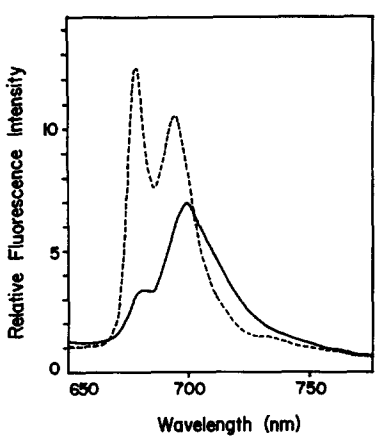

Fig. 4. Fluorescence emission spectra of the washed chloroplast membranes (—) and the Photosystem II-active Fraction B (- - -) of *A. mediterranea* at 77 °K.

at 678 and 694 nm which seem to be similar to the two main bands observed in the low-temperature fluorescence emission spectrum of Photosystem II-enriched fractions from higher plants [23, 24]. None of the membrane fractions showed fluorescence bands in the far-red region between 700 and 800 nm, which have been described for higher plants [1, 5, 23].

The Fraction B could be solubilized by 0.2% sodium dodecylsulfate and separated on polyacrylamide gradient gels. Before Coomassie Blue staining of the gel, three chlorophyll-containing bands appeared (Figs 1E, 5D). These bands corresponded to the free chlorophyll zone and protein-containing bands at 67 000 and 21 500 daltons.

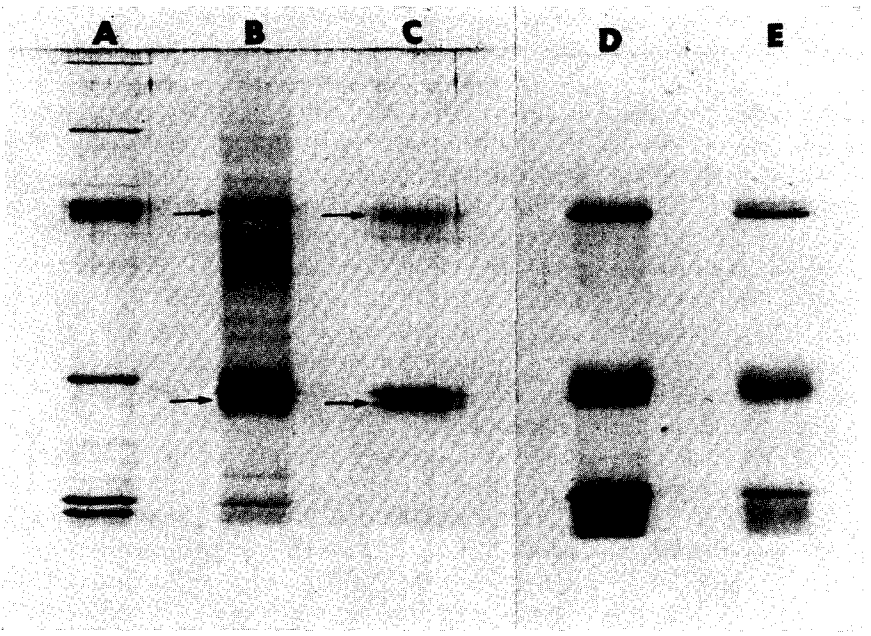

Fig. 5. Electrophoresis of the solubilized proteins of the Photosystem II-active Fraction B of *A. mediterranea* chloroplasts. (D, B) Chlorophyll and protein pattern of the solubilized total fraction before (D) and after (B) Coomassie Blue staining of the gel. (E, C) Chlorophyll and protein pattern after reelectrophoresis of the isolated 67 000-dalton chlorophyll-protein complex before (E) and after (C) Coomassie Blue staining of the gel. The arrows indicate the position of chlorophyll-binding proteins. (A) Marker proteins as shown in Fig. 1.

The chlorophyll *a*/*b* ratios of the 67 000-dalton and the 21 500-dalton chlorophyll-protein complexes were 0.65 and 0.61, respectively, and were very similar to the chlorophyll *a*/*b* ratio of the subchloroplast Fraction B (0.64). Even when the gel was overloaded with sample, the high-molecular-weight (125 000) protein which was part of Fraction C, could not be detected among the proteins of the chlorophyll-containing complex of Fraction B (Figs 1E, 5D).

Following electrophoretic separation of the solubilized Fraction B, Coomassie Blue staining of the gel revealed at least 22 different proteins (Figs 1A, 5B). Besides the 67 000-dalton and 21 500-dalton chlorophyll-binding proteins, 20 additional bands in the molecular-weight range of 130 000 to 10 000 appeared in the electrophoretic

pattern of the Fraction B. Most of the polypeptides of Fraction B did not appear in Fraction C. This indicates an almost complete separation of the Fraction B from the Fraction C.

Under the electron microscope the structure of the Photosystem II-active Fraction B appeared uncontaminated by fragments of Fraction C. Fraction B consisted of particles of an approximate size of 50 Å (Fig. 3b).

The chlorophyll-protein complex of the Photosystem II-active fraction

Attempts were made to isolate and purify the two chlorophyll-binding proteins of Fraction B. After electrophoresis of the solubilized membrane proteins, gel sections containing the 67 000- and 21 500-dalton chlorophyll-protein complexes were cut out and the chlorophyll-binding proteins were eluted and reapplied to polyacrylamide gels.

During reelectrophoresis of the 21 500-dalton chlorophyll-protein material, the sample was resolved into two chlorophyll-containing bands. Besides the 21 500-dalton chlorophyll-protein band, a second, faster migrating band containing only free chlorophyll was present. Coomassie Blue staining of the gel further revealed that the 21 500-dalton chlorophyll band contained two polypeptides. In addition to the 21 500-dalton polypeptide, a 23 000-dalton polypeptide also appeared which apparently could not be separated from the 21 500-dalton chlorophyll-protein complex by the isolation method used. The 23 000-dalton and 21 500-dalton polypeptides were still present when the sample was dissolved with a high concentration of sodium dodecylsulfate (3 %). Under these conditions all the chlorophyll was dissociated from the 21 500-dalton protein and appeared during electrophoresis only as a single band of free chlorophyll. Thus the 23 000-dalton protein was not derived from the 21 500-dalton polypeptide during solubilization and represented a distinct protein.

Reelectrophoresis of the 67 000-dalton chlorophyll-protein complex revealed three chlorophyll-containing bands (Fig. 5E). Besides the chlorophyll associated with the 67 000-dalton protein, there appeared a band of free chlorophyll and a third chlorophyll-containing band, which had the same electrophoretic mobility as the 21 500-dalton chlorophyll-binding protein. After Coomassie Blue staining of the gel, three

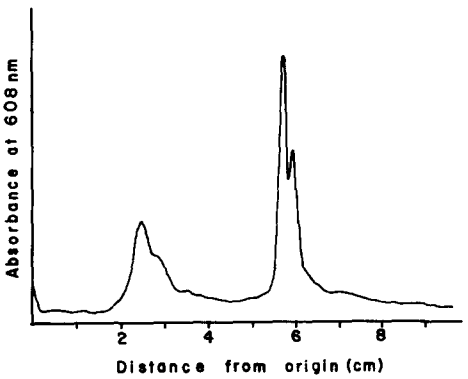

Fig. 6. Densitometric tracing curve of the Coomassie Blue-stained chlorophyll-protein complex and its subunits as in Fig. 5C. The 67 000-dalton chlorophyll-protein complex was isolated as described in Methods.

major protein bands were detected (Fig. 5C). The 67 000-dalton protein band was diffuse and sometimes a smaller component of approx. 59 000 daltons was seen which could not be detected in the electrophoretic pattern of the original solubilized fraction of EDTA-insoluble membranes (Apel, K., in preparation). In addition to the diffuse protein zone of 67 000 daltons, a 23 000-dalton polypeptide and a chlorophyll-binding 21 500-dalton polypeptide appeared. Both these proteins had the same electrophoretic mobility as the 21 500-dalton chlorophyll-binding protein and the prominent 23 000-dalton protein of the membrane Fraction B. Obviously, the 67 000-dalton chlorophyll-protein complex of *A. mediterranea* was unstable under the conditions employed and was dissociated into two subunits of 23 000 and 21 500 daltons. The absorbances of the stained protein bands were scanned and the relative amounts of the dissociated components were estimated from the areas underneath the tracing curves (Fig. 6). Assuming the amount of stain to be proportional to the mass of protein, the molecular ratio of the 23000-dalton to the 21 500-dalton subunits was approx. 2:1 (2:0.97). This ratio indicated a minimum molecular weight of 67 500 for the undissociated chlorophyll-protein complex. This value is in agreement with the molecular weight of 67 000 calculated from the electrophoretic mobility of the chlorophyll-protein complex.

DISCUSSION

In the green alga *A. mediterranea* Triton X-100 treatment of the chloroplast membrane released a particle fraction which seemed to represent purified Photosystem II. The results of our work showed strong evidence for both the identification of the particles as Photosystem II and its purity.

Fraction B of the washed chloroplast membranes photoreduced only DCIP, but not $NADP^+$, thus showing Photosystem II activity. Its physicochemical properties also were those characteristic of Photosystem II. It was enriched in chlorophyll *b* and showed strong fluorescence at room temperature. Furthermore, the chlorophyll-protein of 21 500 daltons, which seems to be identical to the low-molecular-weight chlorophyll-protein complex of Photosystem II in other plants [6–11], was found only in the Photosystem II-active Fraction B. The large chlorophyll-protein complex of 125 000 daltons, which had a similar chlorophyll *a*/*b* ratio and molecular weight as the chlorophyll-protein complex of Photosystem I [6–9] was found only in Fraction C. This fraction was enriched in chlorophyll *a* and exhibited a very low fluorescence. These properties coincide with reported characteristics of Photosystem I (see ref. 1).

The contamination of the Photosystem II fraction by Photosystem I could not be estimated directly, since none of the subchloroplast fragments showed Photosystem I activity. However, additional criteria demonstrated that the Photosystem II-active fraction was uncontaminated by fragments of Fraction C. In electronmicrographs the particle-like appearance of the Photosystem II-active fraction seemed to be uncontaminated by Fraction C material. After electrophoretic separation of the two solubilized Fractions B and C distinct and unique complements of polypeptides, including different chlorophyll-binding proteins, were found for each fraction. We assume therefore that the Photosystem II-active Fraction B was not contaminated by other subchloroplast fragments and represented the purified Photosystem II.

In some respects, the effects of the detergent treatment on the chloroplast membrane in *A. mediterranea* differed from those reported for other organisms. The treat-

ment of higher-plant chloroplast membranes with detergents usually separates only a Photosystem I particle from a membrane fraction which still exhibits both Photosystem I and II activities (see ref. 1). Such a separation resulting from detergent treatment has been related to the arrangement of thylakoids in grana in higher plants [25]. In *A. mediterranea*, grana-like structures are formed only when the cells are kept in the dark for several days [26]. Differences in the chloroplast membrane organization of higher plants and *A. mediterranea* could thus account for differences in the effects of detergent treatment.

After the separation of a purified Photosystem II particle from the chloroplast membrane of *A. mediterranea*, we have investigated the organization and protein composition of this membrane fraction.

It has been shown in higher plants and *Chl. reinhardi* that Photosystem II-preparations contain a low-molecular-weight chlorophyll-protein complex of about 30 000 to 35 000 daltons [6–10] which has been previously identified as the light harvesting chlorophyll-protein complex of Photosystem II [11]. Recently, Anderson and Levine [9] were able to resolve this chlorophyll-protein band into two polypeptides. In *A. mediterranea* after electrophoretic separation of the solubilized Photosystem II fraction, the low-molecular-weight chlorophyll-protein complex was also resolved into two polypeptides of 23 000 and 21 500 daltons. Our results clearly showed that the 21 500-dalton polypeptide together with the 23 000-dalton polypeptide is part of the 67 000-dalton chlorophyll-protein complex. This 67 000-dalton complex seems to represent the light-harvesting chlorophyll-protein complex of Photosystem II in *A. mediterranea*.

From the densitometric tracing curves of the Coomassie Blue-stained proteins, the molecular ratio of the 23 000- and the 21 500-dalton subunits in the 67 000-dalton complex has been estimated to be 2 : 1. This ratio corresponds to a minimum molecular weight of 67 500 which is in agreement with the calculated value of 67 000. However, since it has been shown that Coomassie Blue does not always correlate with the mass of proteins [27], the molecular-weight estimation based on staining intensity can only be tentative and has to be confirmed by other methods.

The nature of the chemical bonds which maintain the integrity of the 67 000-dalton chlorophyll-protein complex is unknown. Since on one hand the omission of 2-mercaptoethanol from the solvent did not influence the stability of the solubilized protein (Apel, K., unpublished results), apparently disulfide bonds were not involved in the binding of the subunits. On the other hand, in the presence of sodium dodecylsulfate, dissociation of the 67 000-dalton chlorophyll-protein complex was always paralleled by the appearance of free chlorophyll. This could perhaps mean that lipid-protein interactions are necessary for maintaining the integrity of this protein.

Recently, Photosystem II-active particles were isolated from chloroplast membranes of higher plants and were suggested to be the reaction center of Photosystem II [3, 4]. During the preparation of these particles, most of the chlorophyll *b* was removed [3, 4, 28]. Based on these data, it was proposed that Photosystem II contains a light-harvesting chlorophyll-protein complex, which is enriched in chlorophyll *b*, and a reaction center, which binds chlorophyll *a* [3]. The Photosystem II particles of the washed chloroplast membranes in *A. mediterranea* were photoactive and hence contained the Photosystem II reaction-center chlorophyll-protein. However, except for the light-harvesting chlorophyll-protein complex of 67 000 and its chlorophyll-binding

subunit of 21 500 daltons, which both are enriched in chlorophyll *b*, no other chlorophyll-proteins could be detected in the Photosystem II fraction. Either the chlorophyll of the reaction-center complex had been separated from the protein under our experimental conditions or the relative amount of the reaction center within the Photosystem II-active membrane fraction was too low to be detected by electrophoresis.

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