PROBING BARLEY MUTANTS WITH A MONOCLONAL ANTIBODY TO A POLYPEPTIDE INVOLVED IN PHOTOSYNTHETIC OXYGEN EVOLUTION

by

LISBETH SKOU HØNBERG

Department of Physiology, Carlsberg Laboratory,
Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby

and

Institute of Genetics, University of Copenhagen
Øster Farimagsgade 2A, DK-1353 Copenhagen K

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A stable hybridoma line derived from the fusion of mouse myeloma cells with spleen cells from a mouse immunized with photosystem II vesicles has been isolated. The hybridoma cells secrete monoclonal antibodies to a 23 kD polypeptide present in isolated barley wild-type thylakoid membranes and known to be involved in water-splitting activity of photosystem II. No reaction between this monoclonal antibody to the 23 kD polypeptide and isolated internal etioplast membranes was detectable in immune-blot assays indicating that the synthesis of this polypeptide is stimulated by light. Immune-blot also revealed that the amount of 23 kD polypeptide in the photosystem II deficient mutants *viridis-zd*⁶⁹, *viridis-m*²⁹ and *viridis-115* is the same as in the wild-type. This indicates either that the oxygen evolving complex, of which the 23 kD polypeptide is a component, can be assembled independently of the photosystem II reaction centre, or that the 23 kD polypeptide, in the absence of the photosystem II reaction centre, is located in the thylakoid lumen and protected from protease attack. Furthermore it was shown that the cold sensitive barley mutant *tigrina-o*³⁴ contained the 23 kD polypeptide whether grown at 23 °C or 30 °C. The amount, on a chlorophyll basis, was reduced compared to that in the wild-type. Since the 23 kD polypeptide is present in *tigrina-o*³⁴ plants grown at 23 °C which lack chloroplast ribosomes, the 23 kD polypeptide must be coded for by a gene in the nucleus.

Abbreviations: BSA = bovine serum albumin; DMEM = Dulbecco's modified Eagles' medium; DMSO = dimethylsulphoxide; DTT = dithiothreitol; EDTA = ethylenediamine tetraacetate; EFs = endoplasmic fracture face (stacked); ELISA = enzyme linked immunosorbent assay; ESs = endoplasmic surface of stacked thylakoids; FCA = Freund's complete adjuvant; FiCA = Freund's incomplete adjuvant; Hepes = N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid; Ig = immunoglobulin; kD = kilodalton; Mr = molecular weight; NBC = newborn calf serum; PBS = phosphate buffered saline; PFs = protoplasmatic fracture face (stacked); PS I = Photosystem I; PS II = Photosystem II; SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis; Tricine = N-(tris(hydroxymethyl)methyl)-glycine; Wt = wild-type.

1. INTRODUCTION

The functional identification of various thylakoid membrane polypeptides has been achieved by correlating mutant polypeptide patterns with photosynthetic defects (7, 13, 23, 24, 29). Another successful approach has been the study of preparations of membrane vesicles enriched in either PS I or PS II activity (4, 12, 27, 28). Progress has been made in identifying the proteins involved in oxygen evolution of photosystem II. ÅKERLUND et al. (1) report that four polypeptides of M_r 34,000, 23,000, 21,000 and 16,000 can be released from inside-out PS II vesicles by high salt washing, resulting in an inhibition of oxygen evolution. Readdition of the four polypeptides to the washed inside-out vesicles resulted in partial reconstitution of oxygen evolution (1). MØLLER and HØJ (27) were able to release three polypeptides of M_r 32,000, 23,000 and 13,500 by high salt treatment of inside-out vesicles or a photosystem II preparation obtained with Triton X-100. The three polypeptides have been purified to homogeneity by chromatofocusing. Reconstitution of photosynthetic oxygen evolution was achieved by adding the purified 23,000 polypeptide to the salt washed photosystem II vesicles depleted of the 32,000, 23,000 and 13,500 molecular weight polypeptides. Reconstitution was not seen when the 32,000 or 13,500 polypeptides were added to the depleted membranes. Recently, a role for the 23 kD polypeptide in modifying the chloride requirement of oxygen evolution has been proposed (2).

Monoclonal antibodies (10, 21, 26) would appear to be excellent tools to extend such studies - specifically to determine the presence of polypeptides in photosynthetic mutants and the site of synthesis of the proteins involved in photosynthesis. Two major advantages of the technique are that purification of the antigen prior to immunization is not necessary, and the antibody obtained is monospecific.

In this study mice were immunized with a preparation of PS II vesicles (27), which contained a minimum of 10 polypeptides. By fusing mouse myeloma cells with spleen cells from an immunized mouse, a hybridoma line was obtained that secreted antibodies to a 23 kD polypeptide (15). This monoclonal antibody has

been used to determine the site of synthesis of the 23 kD polypeptide in the cold-sensitive mutant *tigrina-o*³⁴ (17). In addition, the occurrence of the 23 kD polypeptide in etioplast membranes and three PS II mutants *viridis-zd*⁶⁹, *viridis-m*⁷⁹ and *viridis-115* was determined.

2. MATERIALS AND METHODS

2.1. Plant material

Seeds of wild-type barley (*Hordeum vulgare* cv. Svalöf's Bonus) and the mutants *tigrina-o*³⁴, (5, 17, 18, 30), *viridis-zd*⁶⁹, (23, 24, 38, 43), *viridis-m*⁷⁹ (18, 24, 43) and *viridis-115* (18) were germinated in tap water moistened vermiculite. For the isolation of thylakoids from wild-type and mutants, seeds were grown under standard conditions in continuous white light (1,700 lux) at 23 °C and harvested after seven days. Additionally, seeds from wild-type and *tigrina-o*³⁴ were grown in continuous light at 30 °C. These seedlings were harvested after six days growth to achieve a comparable stage of development. Etioplasts were isolated from wild-type seeds grown in complete darkness for seven days at 23 °C.

2.2. Chemicals, culture media and cells

Myeloma cells (Sp 2/0- Ag 14) (36) were kindly provided by Dr. G. KÖHLER (Basel Institute for Immunology).

Myeloma and hybridoma cells were cultured in Dulbecco's modified Eagles' medium (DMEM) obtained from Flow Laboratories, Irvine, Scotland, supplemented with 2 mM-L-glutamine (Sigma Chemical Company, St. Louis, Mo., U.S.A.), 1 mM-sodium pyruvate (Boehringer, Mannheim, W. Germany), 100 units/ml penicillin-streptomycin solution (Gibco, Paisley, Scotland), 0.05 mM-2-mercaptoethanol (Merck, Darmstadt, W. Germany) and 10% (v/v) fetal calf serum (Gibco). This supplemented DMEM is called complete DMEM.

Hybridoma cells were selected in complete DMEM further supplemented with 1% (v/v) stock solution of 10 mM-hypoxanthine, 1.6 mM-thymidine and 0.04 mM-aminopterin. These chemicals were obtained from Sigma. This selective medium is called HAT medium.

The cells were frozen in 10% (v/v) dimethylsulphoxide (DMSO) purchased from Merck in complete DMEM.

Goat anti-mouse immunoglobulin G and M (IgG, IgM) were obtained from Meloy Laboratories, Inc., Diagnostics Division, Springfield, (USA). Peroxidase-conjugated rabbit anti-mouse immunoglobulin was a product of DAKO-immunoglobulins a/s Denmark. N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid (Hepes), dithiothreitol (DTT), N-(Tris(hydroxy-methyl)-methyl)-glycine (tricine) and 3-amino-9-ethylcarbazole were purchased from Sigma. Newborn calf serum (NBC) was supplied by Gibco. Bovine serum albumin (BSA) was obtained from Fluka AG, Switzerland. Sephacryl S-300 was purchased from Pharmacia, Uppsala, Sweden. For biosynthetic labelling L-³⁵S-methionine (specific activity approx. 1470 Ci · mmol⁻¹) was obtained from Amersham International, U.K. X-ray film (X-Omat RP 1) was from Kodak (USA). For immune-blot assays nitrocellulose filter (HAHY pore size 0.45 µm) was purchased from Millipore S.A. Molsheim, France.

Phosphate buffered saline (PBS) at pH 7.2 was prepared from 10 mM-phosphate and 140 mM-NaCl. Ethylenediamine tetraacetate (EDTA) and trichloroacetic acid were obtained from Merck. Freund's complete and incomplete adjuvant (FCA and FiCA) were products of Difco Laboratories, Detroit, Michigan, USA.

Isolated PS II vesicles and purified 23kD polypeptide were kindly supplied by Dr. B. LINDBERG MØLLER and P.B. HØJ, Carlsberg Laboratory, Copenhagen, Denmark.

2.3. Isolation of purified thylakoids and etioplasts

The isolation of barley thylakoids and etioplasts was performed according to published procedures (19), except that the isolation buffer contained 0.4 M-sucrose, 50 mM-tricine (pH 7.9) and 5 mM-MgCl₂. The purified organelles were shocked using 25 mM-Hepes and 10 mM-EDTA (pH 7.5), (shock-buffer). The released membranes were purified by flotation in a discontinuous sucrose gradient (19, 34), washed in shock-buffer and resuspended in medium con-

taining 0.1 M-Na₂CO₃ and 0.1 M-DTT pH 9.4. Chlorophyll was determined spectrophotometrically in 80% acetone extracts according to ARNON (3), and protein concentration according to LOWRY et al. (22). Thylakoids not used immediately were frozen in small aliquots at -20 °C.

2.4. Salt washing of barley thylakoids

Thylakoids from wild-type and mutant *tigrina-o*³⁴ were resuspended in 0.1 M-Na₂CO₃ plus 0.1 M-DTT pH 9.4 as described in section 2.3. The amount of thylakoids corresponding to 375 µg of chlorophyll were washed with 250 µl PBS pH 7.2 as described in (15). The membranes were centrifuged 15 min at 145,000 × g. The final supernatant, PBS-wash, was subjected to SDS-PAGE (sodium dodecyl sulphate polyacrylamide-gel electrophoresis).

2.5. SDS-PAGE and electrophoretic blotting

Thylakoid polypeptides were separated in an 11-15% gradient gel as described by CHUA and BENNOUN (7). The gels were stained with Coomassie blue R-250. Electrophoretic blotting of the polypeptides onto nitrocellulose filters was performed as described earlier (16, 39).

2.6. Immunization of mice with photosystem II vesicles

A female BALB/c mouse (Bomholtgaard Ry, Denmark) was injected intraperitoneally eight times with PS II vesicles. The immunization schedule was as follows: Initially the mouse was given PS II vesicles containing 230 µg chlorophyll in 100 µl FCA. Three weeks later a second injection with vesicles containing 230 µg chlorophyll in 100 µl FiCA was made, and the same antigen mixture was injected after a further three weeks. Thereafter injections were given weekly. The fourth, fifth and sixth injections contained vesicles with 45 µg chlorophyll and 80 µl PBS plus 100 µl FiCA. The last two times the mouse was given material corresponding to 68 µg chlorophyll and 70 µl PBS plus 100 µl FiCA. The mouse was bled after the last boost, and the blood was used in an immune-blot assay. The

antibody producing mouse was sacrificed three days after the eighth injection.

2.7. Preparation of monoclonal antibodies

Spleen cells (1.5×10^8) from the immunized mouse were fused with 1.8×10^8 myeloma cells (Sp 2/0-Ag 14) (36) essentially as described by FAZEKAS DE ST. GROTH and SCHEIDEGGER (10). The fused cells were distributed in ten microtest-plates (Teknunc, Inter Med, Roskilde, Denmark). Each well was previously supplied with 3×10^3 peritoneal mouse macrophages in 200 μ l HAT-media. The cells were cultured in a 37 °C humidified incubator supplied with 5% CO₂. One week after the fusion the cells were inspected and 50 μ l HAT medium was replaced by 37 °C fresh medium and thereafter 50-100 μ l HAT medium was renewed every second day. Supernatants of growing hybrids were tested for specific antibody production by an ELISA (enzyme linked immunosorbent assay) using thylakoid polypeptides as antigens. Positive clones were transferred to 24 well multi-dishes (Teknunc), and when the cells showed confluent growth they were tested for specific antibody production by an immune-blot assay employing either thylakoids or photosystem II vesicles as antigens. About one month after the fusion, the culture media was changed to complete DMEM. Positive clones were cloned by limited dilution (35). The resulting hybridoma clones were grown at 37 °C in culture bottles (Teknunc). Culture media containing 90% of the cells was removed every second day and replaced by fresh complete DMEM at 37 °C. This harvest was centrifuged 5 min at $200 \times g$ and the antibody containing supernatant was used directly in assays or stored at -20 °C for later purification. The hybridoma cells were resuspended in 10% DMSO in complete DMEM at a concentration of 10^7 cells/ml and placed at -70 °C overnight before they were stored in liquid nitrogen (10).

2.8. Screening assays

2.8.1. ELISA

ELISA were performed in a 96 well microtest plate (Teknunc) at room temperature. The pro-

A

tol included: a) Adsorption of the antigen to the well. To each well was added 50 μ l thylakoid membranes at a concentration of 10 μ g/ml in PBS which were incubated for two hours. b) Blocking with 3% BSA in PBS to eliminate nonspecific adsorption to the wells. c) Incubation for two hours with 50 μ l hybridoma supernatant. d) Washing five times with PBS. e) Incubation for one to two hours with 50 μ l peroxidase conjugated rabbit anti-mouse im-

B

Figure 1. Immune-blot assay for the identification of the 23 kD polypeptide using a monoclonal antibody.

The polypeptides were separated by SDS-PAGE and were then electrophoretically transferred to nitrocellulose filters. These were employed in immune-blot assays using a monoclonal antibody to the 23 kD polypeptide. As secondary antibody a rabbit anti-mouse coupled to peroxidase was employed and colour was developed with 3-amino-9-ethylcarbazole and H_2O_2 . Reacting polypeptides are marked with arrows. 1A: Thylakoid polypeptides (20 μ g chlorophyll applied). Lane 1: SDS-PAGE, lane 2: filter stained with Amido-black, lane 3: filter employed in an immune-blot assay. 1B: PS II vesicles (20 μ g chlorophyll applied) and purified 23 kD polypeptide.

munoglobulin at a concentration of 2 μ l/ml 10% NBC in PBS (v/v). f) Washing five times with PBS. g) Colour development with hydrogen peroxide and 3-amino-9-ethylcarbazole (11).

2.8.2. Immune-blot assay

Strips of nitrocellulose filters containing immobilized electrophoretically separated polypeptides (16, 39) were incubated in 10% NBC

Figure 2. Double immunodiffusion test according to Ouchterlony reveals that the hybridoma line to the 23 kD polypeptide is an IgM.

Hybridoma supernatant (40 μ l) was added to the centre well, and (15 μ l) goat anti-mouse IgG and IgM were added to peripheral wells.

(v/v) in PBS to prevent unspecific binding to the filters. The strips were then rinsed in PBS and incubated for two hours with either hybridoma supernatant or purified monoclonal antibodies (45 μ g/ml) in 10% NBC in PBS at 37 °C with occasional agitation. After washing five times during 30 min with 1% BSA (w/v) in PBS, the strips were incubated in 10% NBC containing peroxidase conjugated rabbit anti-mouse immunoglobulin (2 μ l/ml NBC). An additional five washes in PBS were made, and then the strips were stained with 3-amino-9-ethylcarbazole as described in (11).

2.9. Determination of the immunoglobulin class of the monoclonal antibodies

2.9.1. Double immunodiffusion in gels

The immunoglobulin class of the antibody was initially determined by an Ouchterlony double immunodiffusion (32, 33, 35) performed at room temperature in 1% (w/v) agarose containing 50 mM-Tris-HCl (pH 8.6) plus 0.1 M-NaCl. Hybridoma supernatant was added to the

centre well, and goat anti-mouse IgG and IgM (Meloy), were added to peripheral wells.

2.9.2. Biosynthetic labelling of antibodies

Hybridoma cells (1.5×10^6) were cultured overnight in 1 ml culture media supplied with 50 μ Ci 35 S-methionine instead of unlabelled methionine (35). The next day the cells were removed by centrifugation, and the antibody in the supernatant was reduced with mercaptoethanol and subjected to SDS-PAGE. After electrophoresis the gel was immersed in 50% methanol (v/v) for at least one hour and prepared for fluorography with 1 M-sodium salicylate in glycerol: methanol: water (3: 48.5: 48.5, v/v/v) for 15 - 20 min (6), dried and exposed overnight to RP 1 Royal X-Omat film at -70 °C. The immunoglobulin class was assigned after determining the molecular weight of the heavy chain (14).

2.10. Purification of the monoclonal antibodies

Monoclonal antibodies of the IgM class were purified by 50% (w/v) $(\text{NH}_4)_2\text{SO}_4$ precipitation overnight at 4 °C (35). After centrifugation the resultant pellet was dissolved in elution buffer (0.1 M-Tris-HCl at pH 7.8 plus 0.5 M-NaCl), and dialysed overnight in the same buffer which was changed twice. The dialysed sample was centrifuged to remove the denatured proteins before the sample was chromatographed on a Sephacryl S-300 column equilibrated in elution buffer. The eluted fractions were tested for specific antibody activity in an immune-blot assay (section 2.8). Positive fractions were pooled and dialysed against PBS (pH 7.2) at 4 °C. Protein concentration was determined according to LOWRY et al. (22).

3. RESULTS

3.1. Characterization of the monoclonal antibody to the 23 kD polypeptide

Initial experiments in which 10^8 spleen cells obtained from a mouse injected three times with the antigen were fused with 6×10^7 myeloma cells gave a fusion frequency of 50%. By changing the immunization schedule from three

Figure 3. Determination of the immunoglobulin class by biosynthetic labelling of antibodies.

Hybridoma cells were cultured overnight in a medium containing ^{35}S -methionine. The supernatant containing the secreted antibodies was subjected to SDS-PAGE, processed for fluorography according to CHAMBERLAIN (6) and the dried gel was exposed to RP 1 Royal X-Omat film overnight at -70°C . Lane 1: 40 μl hybridoma supernatant applied. Lane 2: 25 μl supernatant applied to the gel. The immunoglobulin class of the antibody was determined according to the molecular weight of the heavy chain (14). As molecular weight standards hybridomas secreting defined antibodies of the IgM and IgG classes were used.

to eight injections and raising the number of spleen cells from 10^8 to 1.5×10^8 and the number of myeloma cells from 6×10^7 to 1.8×10^8 cells, the fusion frequency was raised to between 80 and 90% (15). The hybridoma lines obtained were scored for the ability to secrete antibodies to polypeptides present in thylakoid mem-

branes. Initially, the hybridoma clones showing confluent growth were subjected to ELISA using thylakoids as antigens. The supernatants from the positive clones were subsequently tested by an immune-blot assay employing electrophoretically separated polypeptides from thylakoids and photosystem II vesicles as antigens. Approximately 10% of the hybridoma lines tested gave positive results. Among these, one of the clones reacted with a 23,000 molecular weight protein present in both the thylakoids and the PS II vesicles, as well as with the purified 23 kD polypeptide (Figures 1A and B, respectively). In the immune-blot assay of Figure 1A, the antibody has reacted only with the polypeptide of 23,000 molecular weight. Figure 1B lane 5 shows that the antibody also reacts with a polypeptide of 20 kD, which is sometimes present. The smaller polypeptide is thought to be a transformation product of the 23 kD polypeptide (15, 27). Increasing the antibody concentration six-fold gave an additional reaction with the 32 kD polypeptide (not shown). The immunoglobulin class of this hybridoma line was shown to be IgM by double immunodiffusion according to OUCHTERLONY (32) (Figure 2) and biosynthetic labelling of the antibodies (Figure 3). After cloning by limited dilution, the identified hybridoma line was cultured in large scale and the secreted monoclonal antibodies were purified from the culture media.

Figure 4. Immune-blot assay to test for the presence of the 23 kD polypeptide in internal chloroplast and etioplast membranes using the monoclonal antibody.

125 μg of protein from isolated internal chloroplast and etioplast membranes were employed as antigens in the assay.

Figure 5. Probing thylakoids isolated from wild-type and mutant *tigrina-o³⁴* grown at 23 °C and 30 °C with the monoclonal antibody to the 23 kD polypeptide in an immune-blot assay.

Thylakoids (20 µg chlorophyll) from wild-type (WT) grown at 23 °C and mutant grown at 23 °C and 30 °C were applied to the gel.

Figure 6. Immune-blot assay to test for the presence of the 23 kD polypeptide in mutant *viridis-zd⁶⁹* thylakoids. Thylakoids (20 µg chlorophyll) from wild-type (WT) and *viridis-zd⁶⁹* were applied to the gel.

3.2. Probing polypeptides from internal etioplast membranes with the antibody to the 23 kD polypeptide.

Figures 1A and B illustrate that the monoclonal antibody recognized a 23 kD polypeptide present in polypeptide preparations of thylakoid membranes and PS II vesicles isolated from light grown seedlings. An analogous experiment was carried out to determine if the 23 kD polypeptide is present in internal etioplast membranes. Equal amounts (125 µg) of protein of a chloroplast and an etioplast preparation were applied to an SDS gel, and after electrophoresis the polypeptides were transferred to a nitrocellulose filter. The 23 kD polypeptide was not detectable in the internal etioplast membranes by this immune-blot assay (Figure 4). A three-fold increase in the amount of internal etioplast membranes did not result in any detectable band due to reaction with the antibody, whereas it was possible to lower the amount of chloroplast proteins from 125 µg to 25 µg and still detect the 23 kD polypeptide. Thus the amount of this polypeptide in etioplasts is at most one-twentieth of that found in chloroplast membranes.

3.3. Probing polypeptides from the mutant *tigrina-o*³⁴ and three *viridis* mutants with the antibody to the 23 kD polypeptide

3.3.1. Immune-blot assays

Thylakoid polypeptides isolated from the cold-sensitive mutant *tigrina-o*³⁴ grown at 23 °C or 30 °C and separated by SDS-PAGE were transferred to nitrocellulose filters. These were probed with the monoclonal antibody to the 23 kD polypeptide. Figure 5, lanes 2 and 4 indicates that a 23 kD polypeptide was present in the mutant thylakoids when the plants were grown at 23 °C. This was confirmed by the immune-blot assay, which also demonstrated that the 23 kD polypeptide was present in thylakoids from mutant plants grown at 30 °C (Figure 5, lanes 6 and 7). Since equal amounts of chlorophyll (20 µg) were applied to the gel from wild-type and the mutant grown at both temperatures, it was concluded that the same amount of the polypeptide is present in *tigrina-o*³⁴ plants grown at the two temperatures. The

amount of the polypeptide, however, was clearly reduced in the mutant compared to the wild-type (Figure 5, lanes 5 and 6).

Additionally, thylakoid polypeptides isolated from the photosystem II mutants *viridis-zd*⁶⁹, *viridis-m*²⁹ and *viridis-115* were probed with the antibody to the 23 kD polypeptide in an immune-blot assay. The amount of chlorophyll applied to the gel was again the same in the mutants as in the wild-type (20 µg). Figures 6 and 7 reveal that the 23 kD polypeptide was present in equal amounts in the three mutants *viridis-zd*⁶⁹, *viridis-m*²⁹, *viridis-115* and the wild-type.

3.3.2. Salt washing of thylakoids isolated from wild-type and mutant *tigrina-o*³⁴ barley

Thylakoids isolated from seedlings of the wild-type and the nuclear gene mutant *tigrina-o*³⁴, grown at 30 °C were resuspended in Na₂CO₃-DTT as described in section 2.3. The mutant and the wild-type thylakoids containing 375 µg of chlorophyll were washed with PBS at pH 7.2. After centrifugation the supernatant was subjected to SDS-PAGE. The electrophoretogram of the Coomassie-blue stained gel showed that salt-washing of barley wild-type thylakoids releases at least eight bands including the α and β subunits of CF_I, plus major bands of M_r 32,000, 23,000 and 10,000 (Figure 8). No polypeptide bands were visible after Coomassie-blue staining of the supernatant of the salt-washed thylakoids from the mutant *tigrina-o*³⁴.

4. DISCUSSION

The mouse sacrificed in the fusion to obtain the monoclonal antibody to the 23 kD polypeptide had been immunized eight times with PS II vesicles. With this high stimulation it was expected that the resulting antibody response would yield antibodies of the IgG immunoglobulin class. The monoclonal antibody reacting specifically with the 23 kD polypeptide, however, was determined to be of the IgM class. This could be because the amount of protein injected was low, or that the 23 kD polypeptide does not stimulate the immunesystem of the

Figure 7. Probing thylakoids isolated from wild-type and the mutants *viridis-m²⁹* and *viridis-115* with the monoclonal antibody to the 23 kD polypeptide in an immune-blot assay.

Thylakoids (20 µg chlorophyll) from the wild-type (WT) and the two mutants were applied to the gel.

mouse to produce antibodies of the immunoglobulin G (IgG) class.

The monoclonal antibody reacting with the 23 kD polypeptide also weakly reacted with a polypeptide of apparent molecular weight

32,000 (15). Since the employed hybridoma line had been cloned by limited dilution (35), this cannot be due to a mixture of antibodies. Each monoclonal antibody has a unique binding site, so the cross reaction observed must be due to

Figure 8. Salt-washing of thylakoids isolated from wild-type and mutant *tigrina-o³⁴* grown at 30 °C.

Thylakoids from the wild-type and the mutant corresponding to 375 µg chlorophyll were washed with 250 µl PBS pH 7.2 (15). The membranes were centrifuged 15 min at 145,000 × g. The final supernatants, PBS-washes, were subjected to SDS-PAGE in parallel with thylakoids (20 µg chlorophyll) from wild-type and mutant germinated at 30 °C and the wild-type at 23 °C.

a similar antigenic site in the 23 kD and the 32 kD polypeptides (15, 17, 26).

Once an antibody producing hybridoma line has been established, unlimited amounts of the antibody directed against a single antigenic determinant is available. These antibodies can be employed in immune-blot assays to screen for the presence of the antigen in different polypeptide preparations e.g., isolated thylakoid preparations from mutants. The immune-blot assay in Figure 4 shows that the 23 kD polypeptide is present in barley chloroplasts, but is not detectable in internal etioplast membranes. Increasing amounts of etioplast membranes did not reveal the presence of the 23 kD polypeptide. This strongly implies that the synthesis of the 23 kD polypeptide and its incorporation into the thylakoids takes place during the light dependent development of the chloroplast.

The 23 kD polypeptide can be released together with two or three other polypeptides from inside-out PS II vesicles by high salt treatment resulting in an inhibition of oxygen evolution (1, 2, 20, 27). Photosynthetic oxygen evolution can be reconstituted by rebinding the 23 kD polypeptide to the salt washed PS II vesicles (1, 2, 27). The precise function of the 23 kD polypeptide is not clear, but it might have a structural or a regulatory function in binding chloride ions (2). Since the 23 kD polypeptide is a component of the oxygen evolving complex, it was of interest to study mutants defective in photosystem II activity. *Viridis-zd*⁶⁹, *viridis-m*²⁹ and *viridis-115* have greatly reduced or no photosystem II activity (24, 37, 43). The presence of the 23 kD polypeptide could be demonstrated in all three *viridis* mutants by an immune-blot assay. Thus the photosynthetic defects in these mutants cannot be due to a lack of this 23 kD polypeptide, which has been synthesized and transported through the thylakoid membranes (Figures 6 and 7). Freeze fracture electron microscopy of the grana membranes of the mutants has shown that the number of particles on the endoplasmic fracture face (EFs) is greatly reduced compared to wild-type, whereas the mutant membranes have a normal protoplasmic fracture face (PFs) (37, 42, 43). The reduction in the number of EFs particles has been shown to be proportional to the reduction in PS II

activity in mutants of barley as well as *Chlamydomonas* (31, 43). The ESs tetrameric particles, which are the surface projections of EFs particles, have been suggested as the likely site of the oxygen evolving complex. Assuming that the EFs particle is the site of the photosystem II reaction centre, the oxygen evolving complex of which the 23 kD polypeptide is a component might exist as a separate entity. That is, the oxygen evolving complex would be structurally distinct from the photosystem II reaction centre although functionally associated with it. Alternatively, it could be that the 23 kD polypeptide might be a component of the reaction centre of photosystem II perhaps located in the tetrameric ESs particles. In the absence of a nuclear-coded polypeptide normally associated with the reaction centre complex in these mutants, however, the components of the EFs particles are not assembled and are presumably destroyed by a protease located in the thylakoids. The 23 kD polypeptide would thus lack its normal binding site in the thylakoid and remain in the lumen of the thylakoids where the polypeptide is not accessible to protease attack.

To determine the site of synthesis of the 23 kD polypeptide, its presence in the temperature and light sensitive mutant *tigrina-o*³⁴ (5, 17) was studied. In white light at 23 °C, the chloroplast ribosomes of this mutant are destroyed, while cytoplasmic ribosomes are retained in normal amounts. The destruction of the chloroplastic ribosomes can be prevented by growing the mutant seedlings at 30 °C. At this permissive temperature, the mutant polypeptide pattern resembles that of the wild-type although normal photosynthetic function is not restored (17). The polypeptide composition of the mutant grown at the restrictive temperature reveals a reduction or complete absence of chlorophyll-*a* proteins 1, 2 and 3, cytochrome *f* and the subunits of coupling factor-1 (CF₁) (17). These proteins, except δ and γ subunit of CF₁, have been shown in other species such as spinach (25, 41), pea (9) and *Chlamydomonas reinhardtii* (8) to be synthesized in the chloroplasts (17, 25). This investigation has demonstrated that the 23 kD polypeptide is present in the mutant *tigrina-o*³⁴, although the protein is found in decreased amounts compared to wild-type both at the

restrictive and permissive temperatures. Since *tigrina-o³⁴* grown at 23 °C has no chloroplast ribosomes, no 23 kD polypeptide could be present in plants grown at the restrictive temperature if the polypeptide was coded in chloroplast-DNA. Its existence indicates that the polypeptide is coded by a nuclear gene, in agreement with results obtained by WESTHOFF et al. who report that the 34, 23 and 16 kD proteins are translated from poly-A RNA (40). The inability to recover these polypeptides with salt-washing from thylakoids of mutant *tigrina-o³⁴* at the restrictive temperature (figure 8, lane 2) indicates that they are not bound to the membranes in the mutant.

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