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OBSERVATIONS ON PHOTOSYSTEM II MUTANTS OF SCENEDESMUS: PIGMENTS AND PROTEINACEOUS COMPONENTS OF THE CHLOROPLASTS*

NORMAN I. BISHOP AND JAMES WONG

Department of Botany, Oregon State University, Corvallis, Oreg. (U.S.A.)

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SUMMARY

Nine mutants of the green alga, *Scenedesmus obliquus*, which are blocked in the Photosystem II portion of photosynthesis were analyzed for possible deletion or alteration of (1) various components of the photosynthetic electron transport system, (2) of chloroplast lipids, (3) of total chlorophyll or of the chlorophyll *a*/chlorophyll *b* ratio, and (4) of their content of carotenes and carotenoids. No changes in content or activity of ferredoxin, ferredoxin–$NADP^+$ reductase, plastocyanin, cytochrome *c*-552, and the membrane-bound *b*-type or *c*-type cytochromes were observed. The most consistent differences noted between the mutant strains and the wild-type strain were in the molar ratio of chlorophyll/plastoquinone A, the total chlorophyll content, and a decreased content of α- and β-carotene with a concomitant increase of carotenoids. The loss of Photosystem II activity in these mutant strains, as observed either with whole cells or with isolated chloroplast fragments, may be accounted for by their decreased content of plastoquinone A. Their decreased chlorophyll content and altered carotene/xanthophyll ratio also suggests possible alteration of chloroplast membranes resulting in increased internal oxidation of the photosynthetic pigments.

INTRODUCTION

Within recent years, a number of mutations of a variety of photosynthetic organisms have been employed to study specific aspects of photosynthesis such as the mechanism of the formation of chloroplast pigments[1–3], the details of chloroplast development[4–6], and the role of certain pigments in different partial reactions of photosynthesis[7–10]. Specific studies on the mechanism of function of Photosystem I and Photosystem II with mutations have been limited largely to the green algae, *Chlamydomonas reinhardi*[11–13] and *Scenedesmus obliquus*[14–16]. In these algae photosynthetic mutants have been isolated wherein the activity of Photosystem II has been specifically suppressed. In more detailed studies in this laboratory, and in cooperation with other research groups, it has been shown that although a normal

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

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Photosystem I reaction is extant in these Scenedesmus mutants, no evidence for Photosystem II activity has been observed. These observations include the following: the light-initiated EPR signal has only the characteristics of the fast signal[17]; isolated chloroplasts only show reactions typical of Photosystem I[16]; certain light-induced absorbance changes show principally the function of the long-wavelength photoreactions[16–18]; and no characteristic delayed light emission typical of the normal phenotype has been detected[19]. Also, derivative absorption spectroscopy (at room and liquid nitrogen temperatures) revealed that no major alteration has occurred in the *in vivo* forms of chlorophyll[21] and more recent studies with the electron microscope have revealed no conspicuous alteration of chloroplast structure (N. I. BISHOP, unpublished data).

In attempts to explore further the nature of the mutation which results in the loss of Photosystem II activity, we have examined more closely the various chloroplast pigments and a number of the proteinaceous components of the chloroplasts. It will be reported that in nine individual Photosystem II mutants, the most consistent differences noted between them and the normal phenotype of Scenedesmus are a decrease in the total amounts of chlorophylls and of plastoquinone A synthesized.

METHODS AND MATERIALS

Culture and maintenance of algal strains

Normal wild-type *Scenedesmus obliquus* (strain D_3) and various photosynthetic mutants were grown heterotrophically on a basal nitrate medium[16] which was supplemented with glucose (0.5 %) and yeast extract (0.25 %). Screw-topped 500-ml erlenmeyer flasks, each containing 250 ml of culture medium, were inoculated with the appropriate algal strain, placed on a thermostated rotary shaker[28], and grown for a maximum of 3 days. Myxotrophic cultures were grown on the same medium, but in culture tubes in an illuminated growth chamber as previously described[21]. The packed cell volume of each culture was determined by centrifugation in "protein constable tubes" (Cenco Scientific). From such determinations it was possible to obtain equal cell volumes of each of the mutants and of the wild type for comparison studies.

Isolation and purification of algal plastoquinone

Approx. 10–15 g wet weight of washed cells were extracted 4 times with 30-ml portions of hot methanol. The extracts were combined in a 250-ml volumetric flask and brought to volume. An aliquot of this extract was used to determine the total chlorophyll according to the method outlined by HOLDEN[22]. Chlorophyll *a* and *b* amounts were determined from the appropriate extinction coefficients obtained by MACKINNEY[23].

The methanol extract was evaporated to dryness and washed onto a 2.5 cm × 15 cm column of 100 mesh silicic acid with two 50-ml washes of isooctane (2,2,5-trimethylpentane). Upon adsorption of the extract, the β-carotene was eluted from the column with 125 ml of $CHCl_3$–isooctane (15:85, v/v). Subsequent elution with 150 ml $CHCl_3$–isooctane (75:25, v/v) removed the plastoquinones. The column chromatography procedure was expedited by using a water-aspirator vacuum and collecting the fractions in 250-ml suction flasks. The plastoquinone fraction was evaporated to dryness for further purification by thin-layer chromatography.

Purification of the plastoquinone was achieved by a 3-step thin-layer chromato-

graphy procedure: (1) The extract was taken up in chloroform, streaked onto a 20 cm × 20 cm silica gel plate and developed in a benzene–heptane solvent (60:40, v/v ($R_f = 0.45$). (2) The plastoquinone was scraped off, eluted, streaked on a paraffin treated silica gel plate (immersed in a 5 % solution of liquid paraffin in ligroine, b.p. 66–75°) and developed in 3 % H_2O-acetone solvent ($R_f = 0.38$). The plastoquinone was located on the plate as a purple band when the plate was sprayed with a 0.1 % rhodamine in absolute ethanol solution and viewed under ultraviolet light. (3) The plastoquinone was eluted free of the dye and the silica gel with redistilled chloroform, restreaked onto a 5 cm × 20 cm silica gel plate and developed in redistilled petroleum ether (b.p. 36–43°) until all the paraffin was removed from the origin. The plastoquinone, which did not move from the origin, was removed from the silica gel with chloroform and then evaporated to dryness. The purified sample was taken up in buffered ethanol (0.01 M ammonium acetate, pH 5.0, in absolute ethanol) for spectrophotometric analysis.

The $\Delta E^{1\%}_{1\,cm}$ of 210 (at 255 nm) for the reduced–oxidized spectrum of plastoquinone was used to calculate the plastoquinone concentration of each of the mutant strains[24]. From this calculation, molar concentrations were estimated and a molar ratio of quinone to chlorophyll determined. For such calculations, an average molecular weight for chlorophyll was calculated based upon the ratio of chlorophyll *a* to chlorophyll *b* in the samples, which was 3.4 ± 0.4 in wild type cells.

The method described for the purification of plastoquinone effectively separates all of the algal quinones, including the various plastoquinones, vitamin K, and tocopherylquinones. Moreover, the plastoquinone is purified from a number of components which interfere with its determination; these components are most likely a variety of fatty acids which also have ultraviolet absorption and show alteration of spectra upon treatment with borohydride. When pure authentic plastoquinone A was run through the complete purification procedure, 75 % of the original amount was recovered.

Preparation and purification of chloroplast proteins

For studies on the electron transport components of the chloroplast, large-scale cultures were grown in 3-l Fernbach flasks; a total of 22 l of culture medium was required to provide about 120 g fresh weight of packed cells. The cells were washed with 0.2 M Tris–HCl (pH 7.5) and treated according to the method employed by POWLS *et al.*[25]. Further purification of ferredoxin–$NADP^+$ reductase, ferredoxin, plastocyanin, and cytochrome 552 was secured by chromatography on DE-32, Sephadex G-100, and hydroxylapatite according to the procedures of GORMAN AND LEVINE[26,27] and MATSUBARA[28]. The activity of each of the components, except for the cytochrome *c*-552, was determined with isolated spinach chloroplasts that had been freed from soluble proteins by extraction with 0.8 M Tris (pH 8.0). This treatment was essentially the same as the reported by YAMASHITA AND BUTLER[29] except that the extraction procedure was continued for 3 h at 4°. Since this procedure effectively removes any Photosystem II activity, either the electron donor system dichlorophenolindophenol–ascorbate or diphenylcarbazide[30], was employed in analysis of $NADP^+$ photoreduction. The activity of reduced cytochrome *c*-552 as an electron donor in a photochemical system was tested by measuring the rate of its photooxidation by a digitonized fraction of spinach chloroplast[31].

Assay of membrane-bound components

The isolation of insoluble chloroplast components and anaylsis for bound cytochrome *c*-552, cytochrome *b*-559 and cytochrome *b*-562 were performed according to the method previously published[25].

Lipid analysis

1 ml of packed cells of each of the phenotypes was extracted with 50 ml of hot methanol, centrifuged at 2000 × *g*, the pellet extracted with 50 ml of methanol–chloroform (1:1, v/v), and centrifuged. The pellet from this step was extracted once more with chloroform, centrifuged and the entire extracts combined and evaporated to dryness.

The various lipids and pigments contained in the extract were separated by either one or two-dimensional thin-layer chromatography on Silica Gel G. For one-dimensional analysis, 20 cm × 20 cm plates were spotted with 0.2 ml of extract and run in a solvent consisting of chloroform–methanol–water (65:25:4, by vol.). For two-dimensional analysis, the first solvent was the same as above and the second solvent consisted of *n*-butanol–acetic acid–water (40:25:5, by vol.). Lipid-like components on the chromatograms were detected by exposing the plates to iodine vapor or by spraying the plates with 0.1 % rhodamine B (in ethanol) and viewing the quenched fluorescence under ultraviolet light.

Further characterization of the lipid components was performed by growing the algae in the presence of either $NaH_2\,{}^{32}PO_4$ or uniformly ^{14}C-labeled glucose prior to the extraction and chromatography. Labeled substrates were added during the logarithmic growth phase for a period of 4 h and the cells were subsequently harvested and extracted as indicated above. After chromatographic separation of the various components, the thin-layer chromatography plates were exposed to "No-Screen" X-ray film for a period of 7 days for the ^{32}P-labeled extracts and for 14 days for the ^{14}C-labeled extracts. The thin-layer chromatography plates were then sprayed with rhodamine B and compared to the patterns observed on the developed X-ray film.

Carotene and carotenoid analysis

For a quantitative analysis of the total complement of carotenoids in mixotrophic or heterotrophically grown cells of Scenedesmus, it was essential to select cells that were in the logarithmic growth phase, which was obtained by inoculating fresh medium with 50 µl of cells and harvesting after 2 days of growth. 1 ml of packed cells was extracted 3 times with 50 ml portions of hot methanol and the combined extract saponified by the addition of 20 ml of 5 M NaOH. The saponification was accelerated by heating the extract to boiling and then quickly cooling to room temperature.

The saponified extract was diluted to approx. 80 % methanol with water and the carotenoids were partitioned into petroleum ether. The partitioning was expedited by the addition of 10 % NaCl; partitioning with petroleum ether was repeated until all carotenoids had been removed from the lower phase. The petroleum ether fractions were then combined, washed with water, dried with anhydrous Na_2SO_4, evaporated to dryness under vacuum and redissolved in 5 ml of chloroform.

1 ml of the extract was streaked onto 20 cm × 20 cm thin-layer chromatography plates of Silica Gel G and developed in a solvent composed of petroleum ether–

isopropanol–water (100:10:0.5, by vol.). Prior to development, the plates were preincubated with this solvent in order to obtain better resolution of the several carotenoid bands. After development, individual bands were removed from the plates and extracted from the silica gel with acetone. Individual samples were evaporated to dryness and the appropriate solvent added for the spectrophotometric determination of the concentration of each of the components. Although some 10–11 separate compounds or bands can be separated by this procedure, only those values for $\alpha + \beta$-carotene and lutein + zeaxanthin will be reported in this paper.

The procedure outlined above does not separate the isomeric forms of carotene (α- and β-carotene) and carotenoid (lutein and zeaxanthin). Their separation was obtained by rechromatographing the bands containing the isomers on thin-layer chromatography plates on an adsorbent consisting of $CaCO_3$–$Ca(OH)_2$–MgO (29.5:5.0:6.0, by weight). Effective separation of α- and β-carotene was obtained with a solvent consisting of 1 % isopropanol in isooctane; by increasing the isopropanol concentration to 10 %, adequate separation of lutein and zeaxanthin was obtained.

The amounts of carotene and carotenoids contained in each of the samples were determined spectrophotometrically by employing the $E^{1\%}_{1\,cm}$ values for the predominant isomer, *i.e.* β-carotene and lutein, in either petroleum ether or in ethanol respectively[22].

RESULTS AND DISCUSSION

General characteristics of mutant strains employed

For this study, nine of the mutants of Scenedesmus in our collection which have the general characteristics required of a Photosystem II mutant were examined. Three other mutant types were used for comparison against the Photosystem II mutants as well as against the wild-type strain. The additional strains employed were ScD_3-8, ScD_3-26, ScD_3-50. The characteristics of each of these strains has been described in part elsewhere. In particular, ScD_3-8 is deficient in the reaction center chlorophyll of Photosystem I, *i.e.* it has no detectable P-700[17,20]; ScD_3-26 lacks membrane-bound cytochromes[25], has poorly developed chloroplasts, and appears to lack chloroplast ribosomes; ScD_3-50 lacks cytochrome *c*-552[25]. None of these mutants possesses, the *in vivo* capacity for photoreduction in contrast to the Photosystem II mutants but through chloroplast studies and fluorescence analyses it is known that they possess some Photosystem II activity. In Table I, the general *in vivo* reaction characteristics of the mutant forms employed in this study are compared to the wild-type Scenedesmus. In Table II, the ability of chloroplasts isolated from the various strains to carry out the photoreduction of either $NADP^+$, of cytochrome *c*, or of DCIP (with electrons from water or from 2,5-diphenylcarbazide) or of $NADP^+$ with DCIP–ascorbate as the electron donor system are summarized. Consideration of the data in Tables I and II reveals the clear distinction between the Photosystem II mutants and the other types used for comparison. The inability of chloroplasts isolated from the Photosystem II mutants to photoreduce DCIP (with diphenylcarbazide as the electron donor) indicates that all of these mutants lack the reducing end of Photosystem II.

Variations in the capacity for photophosphorylation in some of these mutant strains have been discussed previously[16].

TABLE I

THE PHOTOCHEMICAL ACTIVITY OF WHOLE CELLS OF VARIOUS MUTATIONS OF *Scenedesmus obliquus* (HETEROTROPHIC)

Photosynthesis, the quinone-Hill reaction, and photoreduction were measured manometrically employing the all-glass differential respirometer (Gilson Medical Electronics). Photosynthesis measurements were made in No. 9 carbonate–bicarbonate buffer pre-equilibrated with 1 % CO_2–air. Freshly sublimed *p*-benzoquinone was retained in the sidearm of a reaction flask prior to initiating the Hill reaction which was performed in 96 % N_2–4 % CO_2. Photoreduction was determined in cells that had been adapted for 6 h to an atmosphere of 96 % H_2–4 % CO_2; for the measurement of photoreduction in the wild type DCMU, at a final concentration of $4 \cdot 10^{-6}$ M, was added prior to initiating rate measurements. All manometric experiments were done with freshly harvested cells which were selected from cultures 50–56 h old. The variable yield fluorescence data represent the augmentation of 686 nm fluorescence emission (as excited by 436-nm wavelength light) caused by the addition of a supplemental 650-nm wavelength actinic light. Further considerations on the measurements of fluorescence are included in the following paper.

Algal strain	*Photosynthesis* (*μl O_2/h per mg chlorophyll*)	*Hill reaction* (*μl O_2/h per mg chlorophyll*)	*Photoreduction* (*μl CO_2/h per mg chlorophyll*)	*Variable yield fluorescence* (*% increase*)
ScD_3 wild-type	880	530	378	20
Photosystem II mutants				
ScD_3-a′	11	15	400	1
ScD_3-4	24	25	387	0
ScD_3-5	20	25	350	1
ScD_3-10	20	17	360	0
ScD_3-11	16	20	411	0
ScD_3-15	62	30	366	0
ScD_3-40	16	23	397	1
ScD_3-42	10	13	430	0
ScD_3-67	54	38	342	3
Photosystem I or electron transport mutants				
ScD_3-8	25	280	13	110
ScD_3-26	0	20	0	57
ScD_3-50	0	23	0	74

Pigment analyses

In earlier publications concerning some of the general features of the Photosystem II mutants used in this study, it was reported that no major change in pigmentation was observed. The more detailed analysis that has been made recently revealed a minor but consistent difference between the Photosystem II mutants and the wild-type strain. The most significant difference noted in the analysis of the chlorophylls was an approx. 25 % decrease in the total chlorophyll in all of the Photosystem II mutants. A similar decrease was not seen in the other mutants studied. Although total chlorophyll is apparently decreased, and most strikingly in mutant 42, no significant difference in the production of chlorophyll *a* over that of chlorophyll *b* was observed, *i.e.* the ratio of these chlorophylls in the mutants remained essentially the same as in the wild type. These data are summarized in Table III along with other information on the carotene content of the mutants.

From the data of this table, it is also apparent that the Photosystem II mutants accumulate a lesser amount of α- + β-carotene and lutein + zeaxanthin than the wild-type. Additional information (to be published elsewhere) will show that the

TABLE II

THE PHOTOCHEMICAL ACTIVITY OF ISOLATED CHLOROPLAST FRAGMENTS OF WILD-TYPE AND VARIOUS MUTATIONS OF *Scenedesmus obliquus* (HETEROTROPHIC)

The methodology employed for the measurement of the algal chloroplast reactions summarized below was essentially the same as described by PRATT AND BISHOP[16] except that younger cells were employed (see METHODS). Measurements of the reduction of DCIP with 2,5-diphenylcarbazide as the hydrogen donor were performed according to the procedure of VERNON AND SHAW[30]. Photosystem II activity of the wild-type chloroplast was inactivated by heating for 10 min at 50° prior to measuring the photoreduction of DCIP with diphenylcarbazide (DCP) as the hydrogen donor.

Chloroplast source	*Reaction mixture*			
	DCIP	*NADP+*	*DCIP–ascorbate–NADP+*	*DCIP–DPC*
ScD_3-wild-type	98	238	141	92
ScD_3-a′	0	5	128	0
ScD_3-4	1	5	130	0
ScD_3-5	1	5	110	2
ScD_3-10	2	5	98	3
ScD_3-11	0	5	146	0
ScD_3-15	1	5	137	2
ScD_3-40	3	5	128	0
ScD_3-42	4	5	110	10
ScD_3-67	6	5	140	4
ScD_3-8	46	5	5	88
ScD_3-26	3	5	10	2
ScD_3-50	1	5	78	2

TABLE III

PIGMENT CONTENT OF WILD-TYPE AND VARIOUS MUTANT FORMS OF *Scenedesmus obliquus* (HETEROTROPHIC)

Algal strain	*Total chlorophyll (mg)*	*Chlorophyll a (mg)*	*Chlorophyll b (mg)*	*Chl a / Chl b*	*α- + β-Carotene (mg)*	*Lutein + zeaxanthi (mg)*
Wild-type	5.90 ± 0.78	4.56 ± 0.54	1.34 ± 0.22	3.40 ± 0.43	0.12 ± 0.02	0.23 ± 0
System II mutants						
ScD_3-a′	4.88 ± 0.44	3.80 ± 0.30	1.07 ± 0.27	3.74 ± 0.79	0.07 ± 0.01	0.17 ± 0
ScD_3-4	4.54 ± 0.74	3.65 ± 0.58	0.90 ± 0.17	4.11 ± 0.40	0.09 ± 0.02	0.18 ± 0
ScD_3-5	4.23 ± 0.90	3.35 ± 0.71	0.88 ± 0.20	3.82 ± 0.42	0.07 ± 0.02	0.19 ± 0
ScD_3-10	4.32 ± 0.35	3.43 ± 0.28	0.88 ± 0.17	3.96 ± 0.54	0.07 ± 0.01	0.20 ± 0
ScD_3-11	4.48 ± 0.38	3.49 ± 0.24	0.99 ± 0.17	3.45 ± 0.34	0.06 ± 0.02	0.13 ± 0
ScD_3-15	4.10 ± 0.85	2.90 ± 0.38	1.00 ± 0.20	2.94 ± 0.32	0.04 ± 0.01	0.18 ± 0
ScD_3-40	4.90 ± 0.70	3.74 ± 0.41	1.07 ± 0.27	3.74 ± 0.79	0.06 ± 0.02	0.14 ± 0
ScD_3-42	3.07 ± 0.44	2.33 ± 0.32	1.16 ± 0.29	3.13 ± 0.41	0.05 ± 0.01	0.14 ± 0
ScD_3-67	4.57 ± 0.52	3.68 ± 0.57	0.74 ± 0.13	4.92 ± 0.82	0.09 ± 0.02	0.18 ± 0
System I or electron transport mutants						
ScD_3-8	5.13 ± 0.62	3.97 ± 0.52	1.16 ± 0.26	3.42 ± 0.34	0.10 ± 0.02	0.24 ± 0
ScD_3-26	5.85 ± 0.41	4.52 ± 0.26	1.12 ± 0.11	4.04 ± 0.17	0.08 ± 0.01	0.28 ± 0
ScD_3-50	6.06 ± 0.35	5.02 ± 0.34	1.18 ± 0.06	4.20 ± 0.45	0.09 ± 0.02	0.14 ± 0

Photosystem II mutants produce a greater amount of the secondary carotenoids, such as hydroxyechininone, than the wild-type or the other mutant strains. It is to be stressed that the above analyses were made on dark-grown cells wherein the opportunity for photooxidation is minimal. The apparent increased oxidation status of the carotenoid fractions may indicate an alteration in chloroplast structure which is more accessible for degradatory oxidations in the Photosystem II mutants.

Total lipid analysis

Considerable importance has been given to the possible role of certain chloroplast lipids in the functioning of Photosystem II. Most critical attention has been given to those lipids which are abundant in the chloroplasts, the galactolipids, and particularly those that are enriched in the fatty acids, linolenic and linoleic acid[32,33]. Extensive effort has been spent on our part in attempts to determine if the Photosystem II mutants of Scenedesmus have an altered content of lipids; compounds studied included, in addition to the galactolipids, various phospholipids, sulfolipids, and neutral lipids. Repeated analyses with either ^{32}P- or ^{14}C-labeled algal lipid, or with non-radioactive fractions, have failed to show any major difference in the various lipid fractions. Both the amounts and the apparent rates of synthesis of the lipids in the wild-type and the mutants were similar. The various lipids separated from whole cells of Scenedesmus by the thin-layer chromatography procedure included the following: phosphatidyl inositol, sulfolipid, digalactosyl diglyceride, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidic acid, monogalactosyl diglyceride and lecithin. No attempt was made to determine if any variation in fatty acid composition occurred in the various lipid fractions from either the wild-type or the various mutant strains.

Quinone analysis

Many researches have established that plastoquinone (most likely plastoquinone A (PQ-A)) functions in the electron transport system of photosynthesis. A more specific site of its function appears to be as one of the initial electron acceptors for Photosystem II. SMILLIE AND LEVINE[11] in studies on mutants of Chlamydomonas demonstrated that the plastoquinone content of two mutant strains, ac-115 and ac-141, (which have been shown to be Photosystem II mutants possessing many characteristics similar to those described here for the Photosystem II mutants of Scenedesmus) is decreased by about 80 %. Analyses on the plastoquinone content of the Scenedesmus mutants revealed a striking similarity in both mixotrophically and heterotrophically grown cells. All of the mutants, when grown heterotrophically, with the exception of ScD_3-42, contain approx. 50 % less plastoquinone than the wild type. When grown myxotrophically, the wild-type cells synthesize significantly more of the chloroplast pigments with a noticeable shift in the molar ratio of plastoquinone/chlorophyll. Only in mutants 40 and 42 was this ratio changed; the ratio in the other mutants remains essentially the same as that noted for heterotrophic growth. The data showing these results (Table IV) were obtained from 10 separate determinations on each of the algal strains. No significant differences were noted in the content of α-tocopherol or of α-tocopherol quinone. The low concentrations of vitamin K_1 made it extremely difficult to assess any variation between the wild-type and the mutant strains examined.

TABLE IV

MOLE RATIO PLASTOQUINONE A/CHLOROPHYLL IN HETEROTROPHICALLY AND MYXOTROPHICALLY GROWN CELLS OF SCENEDESMUS

Algal strain	*Heterotrophic*	*Mixotrophic*
Wild-type	0.0049 ± 0.0007	0.0093
System II mutants		
a′	0.0023 ± 0.0002	0.0027
4	0.0030 ± 0.0009	0.0024
5	0.0031 ± 0.0005	0.0028
10	0.0032 ± 0.0006	0.0029
11	0.0014 ± 0.0003	0.0028
15	0.0024 ± 0.0008	0.0025
40	0.0017 ± 0.0006	0.0044
42	0.0043 ± 0.0002	0.0056
67	0.0028 ± 0.0004	0.0038
System I or electron transport mutants		
8	0.0046 ± 0.0002	N.D.*
26	0.0029 ± 0.0001	N.D.
50	0.0044 ± 0.0002	N.D.

* N.D. = not determined.

Soluble protein components

The general properties of purified ferredoxin, ferredoxin–$NADP^+$ reductase, plastocyanin and cytochrome *c*-552 of *Scenedesmus obliquus* were examined and were found to be essentially identical to those obtained from other sources such as spinach chloroplasts[34], and Chlamydomonas[26]. No deficiency in any of the above listed factors was observed in the Photosystem II mutants. Following the isolation and purification of the individual factors, each of them was tested for its appropriate activity with chloroplasts isolated from wild-type cells[16] along with the appropriate cofactors also obtained from wild-type cells.

The data of Table V summarize our observations on these cofactors potentially involved in the reduction of $NADP^+$. It is clearly apparent that those derived from the Photosystem II mutants are fully active.

These findings substantiate the *in vivo* assays for photoreductive activity of the Photosystem II mutants where it was observed that a normal capacity for the reduction of carbon dioxide with hydrogen gas as the electron donor existed.

Membrane-bound electron transport components

The assay procedure employed for studying the water-soluble electron transport components of the chloroplasts of algae detects a number of other components which are probably not of chloroplastic origin. Cytochrome *c*-549, cytochrome *b*-562, and cytochrome *b*-558 are routinely detected during the purification of the other cofactors listed previously. The cytochrome *c* is undoubtedly of mitochondrial origin, but the source of the *b*-type cytochromes is questionable. Examination of the "lamellar float" obtained during the $(NH_4)_2SO_4$ fractionation of the soluble protein fraction reveals the electron transport components that remain bound to insoluble chloroplast mem-

TABLE V

THE ACTIVATION OF $NADP^+$ PHOTOREDUCTION OR CYTOCHROME PHOTOOXIDATION BY INDIVIDUAL ELECTRON TRANSPORT COMPONENTS OF CHLOROPLAST DERIVED FROM WILD TYPE OR MUTANT STRAINS OF SCENEDESMUS

The complete reaction mixture for the determination of NADP photoreduction contained wild type chloroplasts equivalent to 40 μg chlorophyll, 10 nmoles ferredoxin, 10 nmoles $NADP^+$ reductase, 5 nmoles plastocyanin, 6 μmoles $MgCl_2$, 50 μmoles Tris–HCl buffer (pH 7.2), and 0.8 nmole $NADP^+$, in 3 ml of 0.16 M sucrose–0.01 M KCl. Reduction rates are expressed as percent of control (wild type chloroplasts and cofactors) which was 228 μmoles $NADP^+$ reduced per h per mg chlorophyll. The reaction mixture for the measurement of the rate of photooxidation of cytochrome *c*-552 contained 5 nmoles reduced plastocyanin, 20 nmoles reduced cytochrome 552, 50 μmoles phosphate buffer (pH 7.0), 50 μmoles $(NH_4)_2SO_4$ and digitonized spinach chloroplasts equivalent to 10 μg of chlorophyll in a final volume of 3.0 ml. Oxidation rates are expressed as percent of control with cytochrome *c*-552 derived from wild-type Scenedesmus. Control rate was 72 μmoles cytochrome oxidized per h per mg chlorophyll.

Source of cofactor	*Limiting cofactor*			
	Ferredoxin	*$NADP^+$ reductase*	*Plastocyanin*	*Cytochrome f*
Wild-type	100	100	100	100
System II mutants				
ScD_3-a′	103	93	97	98
ScD_3-11	98	96	100	102
ScD_3-15	89	102	93	97
ScD_3-40	104	87	103	94
System I or electron transport mutants				
ScD_3-8	100	102	97	94
ScD_3-26	104	87	90	104
ScD_3-50	98	95	88	—

brane components. We have previously shown this fraction to contain bound cytochrome *f* (552 nm), and potentially two *b*-type cytochromes (559 and 562 nm)[25]. Only the cytochrome components contained in either acetone powders of the "lamellar float" or in acetone powders of the total algal extract (see METHODS AND MATERIALS) of mutants a′, 11, and 40 were examined. Slight amounts of chlorophyll retained in the acetone powders often obscured minor absorbance changes in the 520–530 nm region. However, as is shown in Fig. 1, the ferricyanide and dithionite difference spectra of the mutant strains compare favorably to those of the wild-type. The principle absorption maximum of the *b*-type cytochromes (as obtained in the dithionite difference spectrum) occurs at approx. 560 nm. With the techniques employed only this rather broad single peaked spectrum was obtained. Examination of the dithionite difference spectra in the Soret region showed only one peak at 431 nm in mutant and wild-type strains. To date, we have not seen a variation in either the bound or soluble forms of either the *b*-cytochromes or of cytochrome *f* comparable to that noted by SMILLIE AND LEVINE[11] for the Photosystem II mutants of Chlamydomonas. The difficulties involved in our assay procedures preclude any categorical statement about the possible interplay between plastoquinone A (membrane bound) and the *b*-type cytochromes which are thought to be involved in Photosystem II activity.

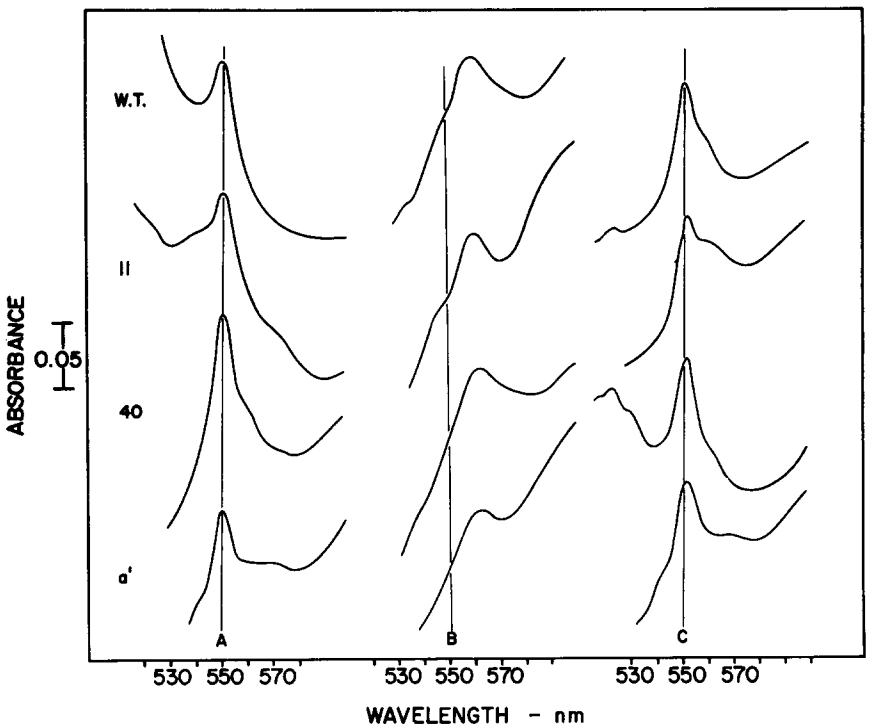

Fig. 1. Absorption difference spectra of lamellar protein extracts of wild-type, mutant 11, mutant 40, and mutant a'. 300 mg of acetone powder of insoluble lamellar fraction solubilized in 15 ml of 3% sodium dodecyl sulphate. Section A. Difference spectra of untreated sample *versus* ferricyanide treated. Section B. Difference spectra of dithionite treated sample *versus* normal sample. Section C. Difference spectra of dithionite *versus* ferricyanide treated sample. Solubilization of the acetone powder with 1% Triton X-100 (in 0.2 M Tris, pH 7.5) or 0.5% sodium deoxycholate did not alter the general features of the difference spectra obtained from that noted for the sodium dodecyl sulphate fractions.

Conclusions

As an approach to understanding the mechanism of action of Photosystem II, the application of the classic technique of biochemical genetics appeared to be a particularly useful approach when coupled with other experimental techniques employed in photosynthesis. In our studies on Scenedesmus, the methods employed for the selection of Photosystem II mutants have resulted in the isolation of nine mutants, some of whose characteristics are described in this paper. One of the more intriguing aspects of this study is the nearly identical behavior and characteristics observed in these strains. The features of lowered plastoquinone A, lowered total chlorophyll, and altered complementation of the carotenoids suggest that the change produced by the mutation, *i.e.* the loss of Photosystem II activity, is not necessarily the result of the loss of the ability of the alga to synthesize a single factor, such as plastoquinone, but perhaps rather to the loss of a discrete part of the Photosystem II unit within the chloroplast. The results of the *in vivo* and *in vitro* analyses for the activity of the separate photosystems (Table I and II) show clearly that the Photosystem II mutants lack any demonstrable partial reaction related to the reductive

portion of this photosystem. The inability of chloroplasts of these mutants to photoreduce DCIP with an exogenous electron donor (diphenylcarbazide) correlates with our observations on the lowered plastoquinone content of these mutants. The absence of PQ-A in Photosystem II should cause the loss of the reductive activity of this system. Since other electron donor systems, such as the DCIP–ascorbate couple, can by-pass the plastoquinone site in the photoreduction $NADP^+$, isolated chloroplasts of the Photosystem II mutants should have the capacity for this reaction as was observed.

The ability of whole cells of the Photosystem II mutants to photoreduce carbon dioxide similarly indicates the non-essentiality of this portion of Photosystem II in photoreduction, a conclusion which is also apparent from the lack of sensitivity of photoreduction to DCMU.

Evidence has been obtained by a number of investigators that plastoquinone may also function in Photosystem I or in cyclic photophosphorylation[13]. The finding that plastoquinone synthesis is not completely blocked in the various mutants perhaps reveals that some portion of the plastoquinone pool is functional in Photosystem I. However, the conclusion that plastoquinone functions in two sites is difficult to comprehend since in our analysis only plastoquinone A was found (in healthy young cells) and the separation of this pool into two separate physical pools, each differing grossly in physicochemical properties, does not appear feasible at this time. If plastoquinone functions at only one site, then the presence of limited amounts of PQ-A may be representative of either that contained in nonphotosynthetic inclusions of the cell or of the photosynthetically incompetent portions of Photosystem II. More definitive studies with various mutant phenotypes are in progress in order to test the hypothesis of multiple sites of action of the plastoquinones.

Additional evidence gained from fluorescence studies will be presented in a subsequent paper which supports the interpretation that the Photosystem II mutants lack the reducing portion of this photosystem. A more comprehensive interpretation of the results of this paper will be presented after documentation of the fluorescence properties of the Photosystem II mutants has been presented.

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REFERENCES

1 S. GRANICK, *Ann. Rev. Plant Physiol.*, 2 (1951) 115.
2 H. CLAES, *Z. Naturforsch.*, 13b (1958) 222.
3 S. BENDIX AND M. B. ALLEN, *Arkiv Mikrobiol.*, 41 (1962) 115.
4 D. VON WETTSTEIN, *The Photochemical Apparatus, Its Structure and Function, Brookhaven Symp. Biol.*, 11 (1958) 138.
5 R. SAGER, *The Photochemical Apparatus, Its Structure and Function, Brookhaven Symp. in Biol.*, 11 (1958) 101.
6 L. BOGORAD, F. V. MERCER AND R. MULLENS, *Photosynthetic Mechanisms in Green Plants, Natl. Acad. Sci. Natl. Res. Council Publ.*, 1145 (1963) 560.

7 R. SAGER AND M. ZALOKAR, *Nature*, 182 (1958) 98.
8 S. VOLK AND N. I. BISHOP, *Photochem. Photobiol.*, 8 (1968) 213.
9 N. K. BOARDMAN AND H. R. HIGHKIN, *Biochim. Biophys. Acta*, 126 (1966) 189.
10 I. C. ANDERSON AND D. S. ROBERTSON, *Plant Physiol.*, 35 (1960) 531.
11 R. M. SMILLIE AND R. P. LEVINE, *J. Biol. Chem.*, 238 (1963) 4058.
12 J. LAVOREL AND R. P. LEVINE, *Plant Physiol.*, 43 (1968) 1049.
13 R. P. LEVINE, *Ann. Rev. Plant Physiol.*, 20 (1969) 523.
14 N. I. BISHOP, *Record Chem. Progr.*, 25 (1964) 181.
15 N. I. BISHOP, *Nature*, 195 (1962) 55.
16 L. H. PRATT AND N. I. BISHOP, *Biochim. Biophys. Acta*, 153 (1968) 664.
17 E. C. WEAVER AND N. I. BISHOP, *Science*, 140 (1963) 1095.
18 E. C. WEAVER AND N. I. BISHOP, *Photosynthetic Mechanisms in Green Plants, Natl. Acad. Sci. Natl. Res. Council Publ.*, 1145 (1963) 138.
19 W. BERTSCH, J. R. AZZI AND J. B. DAVIDSON, *Biochim. Biophys. Acta*, 142 (1967) 129.
20 W. L. BUTLER AND N. I. BISHOP, *Photosynthetic Mechanisms in Green Plants, Natl. Acad. Sci. Natl. Res. Council Publ.*, 1145 (1963) 91.
21 H. SENGER AND N. I. BISHOP, *Plant Cell Physiol.*, 7 (1966) 441.
22 M. HOLDEN, in T. W. GOODWIN, *Chemistry and Biochemistry of Plant Pigments*, Academic Press, New York, 1965, p. 466.
23 G. MACKINNEY, *J. Biol. Chem.*, 140 (1941) 314.
24 F. L. CRANE, *Ciba Found. Symp. Quinones in Electron Transport*, 1961, p. 68.
25 R. POWLS, J. WONG AND N. I. BISHOP, *Biochim. Biophys. Acta*, 180 (1969) 490.
26 D. S. GORMAN AND R. P. LEVINE, *Plant Physiol.*, 41 (1966) 1637.
27 D. S. GORMAN AND R. P. LEVINE, *Plant Physiol.*, 41 (1966) 1643.
28 H. MATSUBARA, *J. Biol. Chem.*, 243 (1968) 370.
29 T. YAMASHITA AND W. L. BUTLER, *Plant Physiol.*, 43 (1968) 1978.
30 L. P. VERNON AND E. R. SHAW, *Biochem. Biophys. Res. Commun.*, 36 (1969) 878.
31 R. H. NIEMAN, H. NAKAMURA AND B. VENNESLAND, *Plant Physiol.*, 34 (1959) 262.
32 J. ERWIN AND K. BLOCH, *Biochem. Biophys. Res. Commun.*, 9 (1962) 103.
33 J. ERWIN AND K. BLOCH, *Biochem. Z.*, 338 (1963) 496.
34 S. KATOH, I. SHIRATORI AND A. TAKAMIYA, *J. Biochem. Tokyo*, 51 (1962) 32.