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PHOTOOXIDATION OF 3,3'-DIAMINOBENZIDINE BY BLUE-GREEN ALGAE AND *Chlamydomonas reinhardii*

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SUMMARY

1. The photooxidation of 3,3'-diaminobenzidine was investigated in whole cells of the wild-type and two mutant strains of *Chlamydomonas reinhardii* and in four species of blue-green algae.

2. In the wild-type strain, this reaction is insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea and is dependent on the presence of methyl viologen. The photooxidation can be demonstrated in a mutant strain lacking Photosystem II activity. However, it is absent from a mutant strain deficient in Photosystem I activity.

3. Experiments performed with chloroplast fragments isolated from the wild-type and mutant strains yielded results similar to those obtained with whole cells. These results established that the photooxidation of 3,3'-diaminobenzidine is mediated by Photosystem I only.

4. In contrast to *Chlamydomonas reinhardii*, blue-green algae are able to photooxidize 3-3'-diaminobenzidine at high rates in the absence of methyl viologen. This property is attributed to the ability of the primary electron acceptor of Photosystem I to undergo autooxidation *in vivo*.

5. The possible applications of this reaction in the studies of photosynthetic electron transport reactions in intact algae and in the investigations of chloroplast substructures are discussed.

INTRODUCTION

Investigations on the various partial reactions of the photosynthetic electron transport pathway in algae have been hindered by the difficulty of preparing active chloroplast fragments from these organisms. Chloroplast preparations which still retain their photochemical capacities have been obtained from only green[1,2] and blue-green algae[3,4], *Euglena*[5] and a species of *Hymenomonas*[6]. So far, no one has yet reported on the isolation of active chloroplasts from the other groups of algae.

Among the former groups of algae the isolation of chloroplasts usually requires some form of mechanical treatment of the cells. This often results in extensive loss

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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of phycobilins[3,4] and occasionally components of the photosynthetic electron transport chain[5] from the photosynthetic lamellae. Therefore, it seems desirable to develop methods of measuring electron transport activities of Photosystem I and Photosystem II, using intact algal cells. The main problem encountered in determining these activities is the impermeability of the cell wall and cell membrane to chemicals that are used in experiments with chloroplasts. Warburg and Luttgens[7] overcame this difficulty by using *p*-benzoquinone which penetrates cell membranes. This quinone has been widely used as a Hill oxidant in intact algae. Hallier and Park[8], on the other hand, fixed unicellular algae with formaldehyde or glutaraldehyde which render the cell membrane permeable to chemicals.

Recently, we reported that cell membranes of blue-green algae and *Chlamydomonas reinhardii* are permeable to methyl viologen which could be used as an electron acceptor of Photosystem I (ref. 9). Under appropriate conditions, the Hill reaction with methyl viologen as the oxidant could be demonstrated in intact cells.

This paper describes a method of measuring non-cyclic electron transport through Photosystem I in whole cells of blue-green algae, and in the wild-type and mutant strains of *C. reinhardii*. The procedure entails the use of 3,3′-diaminobenzidine as an electron donor to Photosystem I. The photooxidation of 3,3′-diaminobenzidine by Photosystem I was also observed in chloroplast fragments prepared from *C. reinhardii* and the results obtained were compared with those of whole cells. The possible site(s) of electron donation by 3,3′-diaminobenzidine will be discussed.

MATERIALS AND METHODS

Four species of blue-green algae and the wild-type (137 c) and two mutant strains of *C. reinhardii* were used in these experiments. The blue-green algae were *Anabaena flos-aquae* (1444), *Phormidium luridum var. olivacae* (426), *Plectonema boryanum* (594) and *Plectonema calothricoides* (598). They were obtained from the culture collection at Indiana University. The two mutant strains (F-34 and F-14) of *C. reinhardii* were obtained by treating the wild-type strain with a chemical mutagen, methylmethane sulfonate. They were isolated by Dr. Pierre Bennoun as high fluorescence mutants using a special technique[10]. F-34 has been characterized by Chua and Levine[11] and some photosynthetic properties of this strain have been described[11–13]. Photosynthetic properties of F-14 are presented here for the first time.

Cells of the blue-green algae were grown in liquid culture medium[14] under conditions previously described[9]. Both the wild-type and mutant strains of *C. reinhardii* were cultured in liquid Tris–acetate–phosphate medium according to Gorman and Levine[1]. Cells of all these algae were harvested during the late exponential phase of growth. The cell pellets were washed once in distilled water and finally resuspended in 0.1 M potassium phosphate buffer (pH 7.0).

Photochemically active chloroplast fragments of *C. reinhardii* were prepared by grinding cell paste with purified sand according to Gorman and Levine[1]. They were finally suspended in a medium containing 10 mM potassium phosphate buffer (pH 7.0), 20 mM KCl, and 2.5 mM $MgCl_2$.

Chlorophyll concentration of *C. reinhardii* was estimated in 80% acetone extract as described by Arnon[15]. Chlorophyll content of the blue-green algae was determined with 90% methonal extract according to Ogawa and Shibata[16].

Activities of the various photochemical reactions in whole cells or chloroplast fragments were measured with a Clark-type O_2 electrode (Yellow Springs Co. Inc., Model 5331) as reported recently[9]. The reaction chamber (1.50 ml) was illuminated from one side with a Leitz projector fitted with an incandescent lamp (Philips, 500 W). The light beam was first passed through a cut-off filter before it was focused onto the entrance face of the reaction chamber. A red perspex filter ($\lambda > 590$ nm) was used for experiments with *C. reinhardii* whereas an orange perspex filter ($\lambda > 540$ nm) was used for those with blue-green algae. The light intensities of the red and the orange actinic light as measured with a Radiometer (Yellow Springs Co. Inc., Model 65) were $3.5 \cdot 10^5$ and $4.8 \cdot 10^5$ ergs/cm^2, respectively. These light intensities were saturating for all the photochemical reactions observed. The temperature during the experiments was about 28 °C.

Activities of the Hill reaction with either ferricyanide or *p*-benzoquinone as the oxidant were recorded using the rate of O_2 evolution as a measurement. The Hill reaction with methyl viologen as the electron acceptor was observed through the rate of O_2 consumption due to the Mehler reaction[9]. Rates of photooxidations of 2,6-dichlorophenolindophenol (DCIP) and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) were determined according to Izawa *et al.*[17]. Photooxidation of 3,3'-diaminobenzidine or 3,3'-diaminobenzidine/ascorbate couple was determined with a similar procedure.

3,3'-Diaminobenzidine, methyl viologen and sodium ascorbate were purchased from Sigma; *p*-benzoquinone (analar) from Koch-Light and DCIP from Merck. TMPD is a product of British Drug House and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was a gift from E.I. du Pont de Nemours and Co., Inc.

RESULTS

The (3,3'-diaminobenzidine→ methyl viologen) reaction in whole cells of the wild-type strain

Fig. 1, Curve a illustrates the time course of the methyl viologen Hill reaction in whole cells of the wild-type strain. Fig. 1, Curve b shows that the O_2 consumption is sensitive to DCMU, indicating the dependence of this reaction on Photosystem II activity. Addition of 3,3'-diaminobenzidine to the DCMU-inhibited cells, however, restores the light-induced O_2 consumption (Fig. 1, Curve c).

Control experiments demonstrate that there is no light-stimulated O_2 uptake when the cells are omitted from the reaction mixture (Fig. 1, Curve d). In the absence of methyl viologen, 3,3'-diaminobenzidine alone elicits only a small amount of O_2 uptake in DCMU-treated cells (Fig. 1, curve e).

It is well known that DCMU blocks electron flow at a site close to Photosystem II (ref. 18). In our experiments, 3,3'-diaminobenzidine is able to bypass this site of inhibition and serves as an electron donor to Photosystem I. Under these conditions, the rate of O_2 uptake indicates the rate of non-cyclic electron flow from 3,3'-diaminobenzidine through Photosystem I to methyl viologen. This reaction will be referred to hereafter as the (3,3'-diaminobenzidine→ methyl viologen) reaction. If the above interpretation is correct, then the (3,3'-diaminobenzidine→ methyl viologen) reaction should be present in a mutant strain defective in Photosystem II but absent from a mutant strain deficient in Photosystem I activity.

The effects of varying concentrations of 3,3'-diaminobenzidine on the rate of

light-induced O_2 consumption in the wild-type strain were investigated. It was found that the reaction saturates at about 6 mM and higher concentrations of the electron donor become inhibitory.

The (3,3'-diaminobenzidine→ methyl viologen) reaction in whole cells of F-34

The mutant strain, F-34, is deficient in cytochrome 559 (ref. 11), a component of the photosynthetic electron transport chain close to Photosystem II (ref. 19). Chloroplast fragments isolated from this strain are unable to do the Hill reaction but they possess normal Photosystem I activity[11,13]. Electron transport capacities of F-34 are given in Table I.

TABLE I

PHOTOCHEMICAL REACTIONS IN CHLOROPLAST FRAGMENTS PREPARED FROM THE WILD-TYPE STRAIN, F-34 AND F-14

Columns 1 and 2 are the Hill reactions with methyl viologen and ferricyanide as the respective electron acceptors. Columns 3 and 4 are the photooxidations of $DCIPH_2$ and $TMPDH_2$, respectively. For the ferricyanide Hill reaction the mixture contained chloroplast fragments (15 μg chlorophyll/ml) and the following components: potassium phosphate buffer (pH 7.0), 25 mM; KCl, 20 mM; $MgCl_2$, 2.5 mM; NH_4Cl, 2 mM; and potassium ferricyanide, 1 mM. The reaction mixture for the methyl viologen Hill reaction contained chloroplast fragments (15 μg chlorophyll/ml) and the following components: potassium phosphate buffer (pH 7.0), 25 mM; KCl, 20 mM; $MgCl_2$, 2.5 mM; NH_4Cl, 2 mM; methyl viologen, 0.1 mM; and KCN, 1 mM. Photooxidations of $DCIPH_2$ or $TMPDH_2$ was performed with a reaction mixture which contained chloroplast fragments (5 μg chlorophyll/ml) and the following components: potassium phosphate buffer (pH 7.0), 25 mM; KCl, 20 mM; $MgCl_2$, 2.5 mM; NH_4Cl, 2 mM; methyl viologen, 0.1 mM; DCIP or TMPD, 0.1 mM; sodium ascorbate, 3 mM; and DCMU, 10 μM. Other experimental conditions have been described under Materials and Methods.

Strain	*Rate (μmoles O_2 evolved or consumed/mg chlorophyll per h)*			
	Methyl viologen Hill reaction	*Ferricyanide Hill reaction*	*$DPIPH_2$*	*$TMPDH_2$*
Wild-type	85	99	656	584
F-34	0	0	1059	919
F-14	<7	30	50	54

Fig. 2, Curve a shows that whole cells of F-34 are unable to photoreduce methyl viologen. This agrees with results obtained with chloroplast fragments, and together, they support the fact that electron flow in F-34 is blocked at a site near Photosystem II. Fig. 2, Curve b shows the (3,3'-diaminobenzidine→ methyl viologen) reaction in this mutant strain. The kinetics of O_2 uptake due to the photooxidation of 3,3'-diaminobenzidine are similar to those obtained with the wild-type strain and are not affected by DCMU. These results support the contention that the (3,3'-diaminobenzidine → methyl viologen) reaction in *C. reinhardii* can operate in the absence of Photosystem II activity and therefore is presumably a Photosystem I-mediated process.

The (3,3'-diaminobenzidine→ methyl viologen) reaction in whole cells of F-14

The activities of the various photosynthetic electron transport reactions in F-14 are shown in Table I.

Chloroplast fragments prepared from this mutant strain are able to photo-

reduce ferricyanide at a rate 30 % that of the wild-type strain. However, the rates of the methyl viologen Hill reaction and $DCIPH_2$ and $TMPDH_2$ photooxidations in the same chloroplast preparation are only about 7 % of the wild-type values.

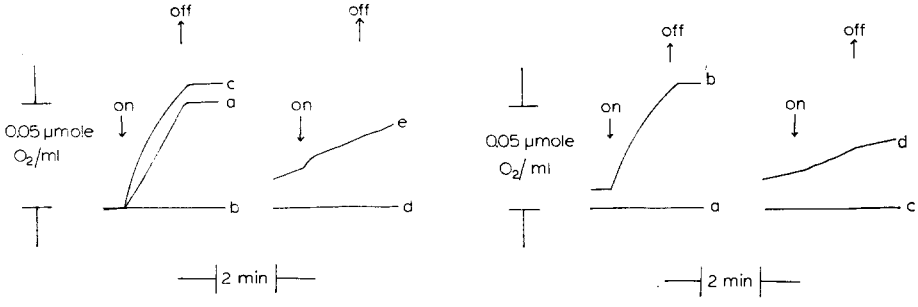

Fig. 1. Light-induced O_2 uptake by whole cells of the wild-type strain of *C. reinhardii*. Chlorophyll, 15 µg/ml; potassium phosphate buffer (pH 7.0), 50 mM; methyl viologen, 2 mM; and KCN, 1 mM. Cells were incubated with methyl viologen in the dark for approx. 20 min. For experiments using 3,3′-diaminobenzidine the compound was added to the mixture 2–3 min before the experiment was started. Other experimental conditions can be found under Materials and Methods. In the tracing shown an upward deflection indicates O_2 consumption by the cells. (a) No further addition; (b) *plus* 10 µM DCMU; (c) *plus* 10 µM DCMU and 6 mM 3,3′-diaminobenzidine; (d) same as (c) except no cells; (e) same as (c) except no methyl viologen.

Fig. 2. Light-induced O_2 uptake by whole cells of F-34 and F-14. Chlorophyll, 15 µg/ml; potassium phosphate buffer (pH 7.0), 50 mM; methyl viologen, 2 mM, and KCN, 1 mM. All other conditions as in Fig. 1. (a) F-34, no further additions; (b) F-34 *plus* 6 mM 3,3′-diaminobenzidine; (c) F-14, no further addition; (d) F-14, *plus* 6 mM 3,3′-diaminobenzidine. Addition of 10 µM DCMU has no effects on (b) and (d).

Furthermore, experiments on light-induced absorbance change at 700 nm reveal that there is little or no P700 signal in this mutant strain (N.H. Chua and R. P. Levine, unpublished observations). Therefore, the photosynthetic properties of F-14 are similar to those of ac-80a[20–21] and F-1[11–13], described earlier. These properties indicate that F-14 is a Photosystem I mutant deficient in P700. However, the block in electron transport is not complete as reflected by the residual Photosystem I activity (Table I).

Fig. 2, Curve c shows that in whole cells of F-14 there is almost no light-induced O_2 uptake due to the methyl viologen Hill reaction. Similarly, there is only a very slow rate of 3,3′-diaminobenzidine photooxidation which is about 4 % of that detected in the wild-type strain (Fig. 2, Curve d). This slow rate could be attributed to the residual electron flow through Photosystem I. The rate of the *p*-benzoquinone Hill reaction, on the other hand, is 40 % that of the wild-type value, suggesting an active Photosystem II (see Table II). These findings confirm the contention that the (3,3′-diaminobenzidine → methyl viologen) reaction is dependent on the normal functioning of Photosystem I.

Table II gives the rates of the (3,3′-diaminobenzidine → methyl viologen) reaction in whole cells of the wild-type and mutant strains. The rates of the *p*-benzoquinone Hill and the methyl viologen Hill reactions are also given for the purpose of comparison.

The (3,3'-diaminobenzidine/ascorbate→ methyl viologen) reaction in chloroplast fragments of the wild-type strain, F-34 and F-14

As in whole cells, the (3,3'-diaminobenzidine→ methyl viologen) reaction could be demonstrated with chloroplast fragments prepared from the wild-type strain (Fig. 3, Curve a). Fig. 3, Curve b shows that the addition of ascorbate to the sample stimulates the initial rate of this reaction by 2-fold and maintains this rate linear for about 2 min.

TABLE II

PHOTOCHEMICAL REACTIONS IN WHOLE CELLS OF THE WILD-TYPE STRAIN, F-34 AND F-14

Columns 1 and 2 refer to the Hill reactions with methyl viologen and *p*-benzoquinone as the respective electron acceptors. Column 3 is the photooxidation of 3,3'-diaminobenzidine with methyl viologen as the electron acceptor. The methyl viologen Hill reaction was carried out as described previously[9]. The reaction mixture contained cells (15 μg chlorophyll/ml) and the following components: potassium phosphate buffer (pH 7.0), 50 mM; methyl viologen, 2 mM; and KCN, 1 mM. Cells were incubated with methyl viologen in the dark for approx. 20 min before illumination. The mixture for the *p*-benzoquinone Hill reaction contained cells (15 μg chlorophyll/ml) and the following components: potassium phosphate buffer (pH 7.0), 50 mM; and *p*-benzoquinone, 2 mM. The *p*-benzoquinone was added just before the experiment was started. The photooxidation of 3,3'-diaminobenzidine was carried out with a reaction mixture containing cells (15 μg chlorophyll/ml) and the following components: potassium phosphate buffer (pH 7.0), 50 mM; methyl viologen, 2 mM; 3,3'-diaminobenzidine, 6 mM; KCN, 1 mM; and DCMU, 10 μM. Cells were incubated with methyl viologen and DCMU in the dark for about 20 min. The 3,3'-diaminobenzidine and KCN were added 2–3 min before the experiment was started. For other experimental details see Materials and Methods.

Strain	*Rate (μmoles of O_2 evolved or consumed/mg chlorophyll per h)*		
	Methyl viologen Hill reaction	*p-benzoquinone Hill reaction*	*(3,3'-diaminobenzidine → methyl viologen) reaction*
Wild-type	106	242	273
F-34	0	0	250
F-14	<7	99	<10

The effects of increasing concentrations of 3,3'-diaminobenzidine on the rate of the (3,3'-diaminobenzidine/ascorbate→ methyl viologen) reaction were examined in wild-type chloroplast fragments. The optimal concentration for the reaction is about 2 mM.

Table III lists the requirements for the (3,3'-diaminobenzidine/ascorbate→ methyl viologen) reaction in wild-type chloroplast fragments. The rates of the same reaction in the mutant strains are also given. These results are consistent with those obtained with whole cells in that the reaction is present in F-34 but almost absent from F-14.

The (3,3'-diaminobenzidine→ methyl viologen) reaction in whole cells of blue-green algae

Fig. 4, Curves a and b depict the kinetics of the methyl viologen Hill reaction in whole cells of *P. luridum* and the sensitivity of this reaction toward DCMU. Fig. 4, Curve c shows that 3,3'-diaminobenzidine is able to restore the light-induced O_2 uptake in DCMU-inhibited cells. These results are similar to those described for *C. reinhardii* and they support the proposal that 3,3'-diaminobenzidine bypasses the

site of DCMU inhibition and serves as an electron donor to Photosystem I *in vivo*. In contrast to results obtained with *C. reinhardii*, however, there is a substantial rate of O_2 uptake in the absence of the exogenous, autooxidizable compound, methyl viologen (Fig. 4, Curve d). This observation can be easily explained if one or more electron carriers on the reducing side of Photosystem I are capable of interacting with O_2. Among these carriers, X, the unidentified primary electron acceptor of

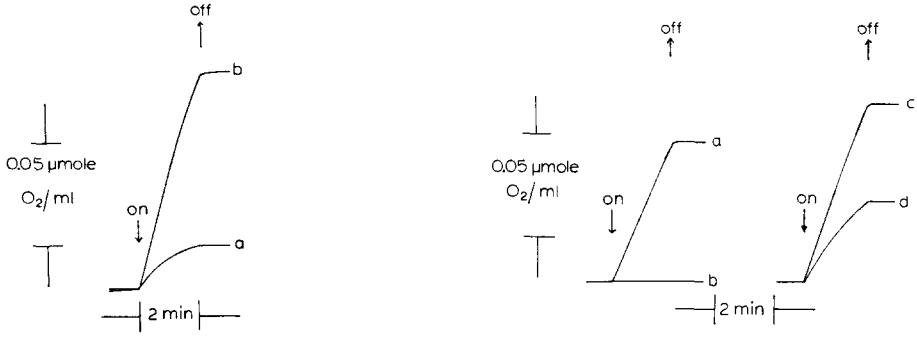

Fig. 3. The effects of ascorbate on the (3,3′-diaminobenzidine → methyl viologen) reaction in chloroplast fragments of the wild-type strain. The reaction mixture contained chloroplast fragments (7.5 μg chlorophyll/ml) and the following components: potassium phosphate buffer (pH 7.0), 25 mM; KCl, 20 mM; $MgCl_2$, 2.5 mM; NH_4Cl, 2 mM; 3,3′-diaminobenzidine, 2 mM; methyl viologen, 0.1 mM; KCN, 1 mM; and DCMU, 10 μM. (a) No further additions; (b) *plus* 3 mM sodium ascorbate.

Fig. 4. Light-induced O_2 uptake by whole cells of *P. luridum*. Chlorophyll, 5 μg/ml for (a) and (b) and 2.5 μg/ml for (c) and (d); potassium phosphate buffer (pH 7.0), 50 mM; methyl viologen, 2 mM; and KCN, 1 mM. Other experimental conditions same as Fig. 1. (a) No further addition; (b) *plus* 10 μM DCMU; (c) *plus* 10 μM DCMU and 6 mM 3,3′-diaminobenzidine; (d) same as (c) except no methyl viologen.

TABLE III

REQUIREMENTS AND RATES OF THE (3,3′-DIAMINOBENZIDINE → METHYL VIOLOGEN) REACTION IN CHLOROPLAST FRAGMENTS OF THE WILD-TYPE STRAIN, F-34 AND F-14

The (3,3′-diaminobenzidine/ascorbate → methyl viologen) reaction was carried out with chloroplast fragments (15 μg chlorophyll/ml). The complete reaction mixture contained, in addition to chloroplasts, the following components: potassium phosphate buffer (pH 7.0), 25 mM; KCl, 20 mM; $MgCl_2$, 2.5 mM; NH_4Cl, 2 mM; 3,3′-diaminobenzidine, 2 mM; sodium ascorbate, 3 mM; methyl viologen, 0.1 mM; KCN, 1 mM; and DCMU, 10 μM. Other experimental details can be found under Materials and Methods.

Strain	*Reaction*	*Rate of O_2 uptake (μmoles/mg chlorophyll per h)*
Wild-type	Complete	540
	—Ascorbate	202
	—3,3′-diaminobenzidine	24
	—Methyl viologen	82
	—Methyl viologen, ascorbate	19
	—3,3′-diaminobenzidine, ascorbate	0
	—3,3′-diaminobenzidine, methyl viologen	0
	—Chloroplasts	0
F-34	Complete	840
F-14	Complete	31

Photosystem I seems to be a likely candidate. Honeycutt and Krogmann[22] presented evidence that in photosynthetic lamellae of *Anabaena variabilis* X is able to reduce O_2 directly. Their results have been confirmed recently by Neumann *et al.*[23]. The data presented here are consistent with those of Honeycutt and Krogmann[22] and Neumann *et al.*[23], and they further suggest that X is capable of reducing O_2 even in the intact cell. The photooxidation of 3,3′-diaminobenzidine by whole cells of blue-green algae in the absence of MV will be referred to hereafter as the (3,3′-diaminobenzidine → X) reaction.

Table IV presents the rates of O_2 consumption attributed to the (3,3′-diaminobenzidine→ X) and the (3,3′-diaminobenzidine → methyl viologen) reactions in four species of blue-green algae.

TABLE IV

RATES OF PHOTOOXIDATION OF 3,3′-DIAMINOBENZIDINE WITH AND WITHOUT METHYL VIOLOGEN IN INTACT CELLS OF FOUR SPECIES OF BLUE-GREEN ALGAE

Experimental conditions as in Fig. 4.

Organism	*Rate of O_2 consumption (μmoles/mg chlorophyll per h)*	
	−Methyl viologen	*+Methyl viologen*
P. luridum	500	1040
P. boryanum	550	1125
P. calothricoides	346	865
A. flos-aquae	390	1325

DISCUSSION

3,3′-Diaminobenzidine was first introduced by Graham and Karnovsky[24] in 1966 to demonstrate peroxidase activity in mouse kidney. Since then, it has been employed extensively for the cytochemical localization of catalase activity in microbodies of both plant[25,26] and animal tissues[27–29]. Seligman *et al.*[30] observed that 3,3′-diaminobenzidine could be readily oxidized by cytochrome c in the mitochondria. Upon oxidation it forms an osmiophilic polymer which localizes the site of cytochrome oxidase activity. Recently, Nir and Seligman[31] reported that 3,3′-diaminobenzidine could be photooxidized by chloroplast lamellae and the process is insensitive to DCMU.

This paper presents results which confirm the findings of Nir and Seligman[31]. These results further provide evidence that in blue-green algae and *C. reinhardii* the photooxidation of 3,3′-diaminobenzidine is a Photosystem I process and can occur independently of Photosystem II activity. In the presence of an autooxidizable electron acceptor such as methyl viologen and KCN, the photooxidation of 3,3′-diaminobenzidine involves an open chain electron flow from 3,3′-diaminobenzidine through Photosystem I to methyl viologen. The reoxidation of reduced methyl viologen by a Mehler-type reaction leads to the O_2 consumption[9]. Under these conditions the uptake of 1 O_2 corresponds to the transfer of a pair of electrons through Photosystem I. The stoichiometry is the same as that during the photooxidations of

$DCIPH_2$ and $TMPDH_2$ in chloroplasts[17]. In contrast, the methyl viologen Hill reaction involves the uptake of 1 O_2 per 4 electrons transversing the entire electron transport pathway.

The photooxidation of 3,3′-diaminobenzidine in the four species of blue-green algae investigated differs from that in *C. reinhardii* in one important aspect. The reaction in the blue-green algae is not strictly dependent upon methyl viologen although the addition of the latter increases the rate of O_2 consumption by 2- to 3-fold. Similar observations have been reported for the photooxidation of reduced indophenol dyes by photosynthetic lamellae prepared from *A. variabilis*[22,23]. These results, together with the data presented in this paper, suggest that the primary electron acceptor of Photosystem I in blue-green algae is capable of reducing O_2 *in vitro* as well as *in vivo*. This property of the primary electron acceptor has relevance to the photosynthesis of blue-green algae under high light intensity and low CO_2 tension and may account for the photorespiration of *Anacystis nidulans* reported by Hoch *et al.*[32].

In chloroplast fragments of *C. reinhardii*, the rate of the (3,3′-diaminobenzidine → methyl viologen) reaction decreases rapidly with time (Fig. 3, Curve a). It is possible that the formation of the polymer by oxidized 3,3′-diaminobenzidine blocks the further transfer of electrons. One effect of ascorbate is to keep the 3,3′-diaminobenzidine in the reduced state, thus preventing this rate decline. Similar explanations could be used for the (3,3′-diaminobenzidine → methyl viologen) reaction in whole cells. However, in this case, there is no effect of ascorbate presumably due to the impermeability of the cell membrane to this compound.

Izawa *et al.*[17] reported that photooxidations of $DCIPH_2$, $TMPDH_2$, reduced diaminodiurene, and trichlorohydroquinone in chloroplasts are mediated by Photosystem I. The photooxidations of 3,3′-diaminobenzidine and the 3,3′-diaminobenzidine/ascorbate couple add to this list. The rate of the (3,3′-diaminobenzidine/ascorbate → methyl viologen) reaction in chloroplast fragments of *C. reinhardii* is comparable to that of $DCIPH_2$ or $TMPDH_2$ photooxidation (*cf.* Tables I and III).

The site of the electron donation by 3,3′-diaminobenzidine is not clarified by our present studies. Results obtained with F-34 indicate that 3,3′-diaminobenzidine donates electrons to the photosynthetic electron transport chain of *C. reinhardii*[19] at a site between cytochrome 559 and Photosystem I. In blue-green algae, cytochrome 559 is replaced by cytochrome 557, a similar *b*-type cytochrome[33]. There is a host of artificial electron donors capable of donating electrons to the photosynthetic electron transport pathway in spinach chloroplasts[17,34–38] as well as blue-green algae[22]. It is generally agreed that electrons from these donors enter the pathway at more than one site depending on the concentration of the donor (ref. 22). Similar concentration effects on the site of electron donation by 3,3′-diaminobenzidine could conceivably exist in blue-green algae and *C. reinhardii*.

In whole cells of blue-green algae and *C. reinhardii*, the (3,3′-diaminobenzidine → methylviologen) reaction together with the *p*-benzoquine Hill reaction and the methyl viologen Hill reaction can be used to measure electron transport through Photosystem I, Photosystem II, and Photosystems II and I. In contrast to the procedure of Hallier and Park[8], algae used for these reactions do not have to be prefixed with chemicals. This eliminates whatever damaging effects the chemicals may have on the photosynthetic reactions. Furthermore, the rates of the Photosystem I and Photosystem II reactions reported here are higher than those obtained

with chemically fixed algae[8]. We anticipate that these reactions will be useful in the investigations of photosynthetic electron transport in other groups of algae from which active chloroplast preparations have not been obtained.

The photooxidation of 3,3'-diaminobenzidine by chloroplasts results in a polymer which is electron opaque. Nir and Seligman[31] noted that the deposition of the polymer on the chloroplast lamellae is not uniform. Our results suggest that the sites of deposition delineate the localizations of the electron carrier which accepts electrons from 3,3'-diaminobenzidine. Since it has been established in this paper that photooxidation of 3,3'-diaminobenzidine is mediated by Photosystem I this electron carrier is probably close to or may even be part of the Photosystem I particle[39]. If the latter is true, then the oxidative polymerization of 3,3'-diaminobenzidine will have important applications in the elucidation of the arrangements of Photosystem I and Photosystem II units on the photosynthetic lamellae[40,41] as well as in the studies of chloroplast morphogenesis[42,43].

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