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LACTOPEROXIDASE-CATALYZED IODINATION OF CHLOROPLAST MEMBRANES

II. EVIDENCE FOR SURFACE LOCALIZATION OF PHOTOSYSTEM II REACTION CENTERS

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SUMMARY

The enzyme lactoperoxidase was used to specifically iodinate the surface-exposed proteins of chloroplast lamellae. This treatment had two effects on Photosystem II activity. The first, occurring at low levels of iodination, resulted in a partial loss of the ability to reduce 2,6-dichlorophenolindophenol (DCIP), even in the presence of an electron donor for Photosystem II. There was a parallel loss of Photosystem II mediated variable yield fluorescence which could not be restored by dithionite treatment under anaerobic conditions. The same pattern of inhibition was observed in either glutaraldehyde-fixed or unfixed membranes. Analysis of the lifetime of fluorescence indicated that iodination changes the rate of deactivation of the excited state chlorophyll. We have concluded that iodination results in the introduction of iodine into the Photosystem II reaction center pigment–protein complex and thereby introduces a new quenching. The data indicate that the reaction center II is surface exposed.

At higher levels of iodination, an inhibition of the electron transport reactions on the oxidizing side of Photosystem II was observed. That portion of the total rate of photoreduction of DCIP which was inhibited by this action could be restored by addition of an electron donor to Photosystem II. Loss of activity of the oxidizing side enzymes also resulted in a light-induced bleaching of chlorophyll a_{680} and carotenoid pigments and a dampening of the sequence of O_2 evolution observed during flash irradiation of treated chloroplasts. All effects on electron transport on the oxidizing side of Photosystem II could be eliminated by glutaraldehyde fixation of the chloroplast lamellae prior to lactoperoxidase treatment. It is concluded that the electron carriers on the oxidizing side of Photosystem II are not surface localized; the functioning of these components is impaired by structural disorganization of the membrane occurring at high levels of iodination.

Abbreviations: DABS, *p*-Diazoniumbenzenesulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide, F_M, maximal yield of fluorescence; F_0, instantaneous level of fluorescence (dead fluorescence); Tricine, *N*-tris(hydroxymethyl)methylglycine.

Our data are in agreement with previously published schemes which suggest that Photosystem II mediated electron transport traverses the membrane.

INTRODUCTION

The enzyme lactoperoxidase, in the presence of H_2O_2, has been shown to catalyze the iodination of the amino acids tyrosine, and to a lesser extent, histidine in membrane proteins. This iodination reaction requires the formation of a complex between the amino-acid substrate and the enzyme; it is, therefore, limited only to those portions of the membrane proteins which are surface exposed [1, 2]. We have previously used this procedure in an attempt to identify the externally localized proteins of chloroplast lamellae [3]. Based on incorporation of ^{125}I and/or effects on the activities of various partial photoreactions, we concluded that the coupling factor (CF_1, or ATPase [4]) and components of the electron transport chain on the reducing side of Photosystem I are exposed to the lactoperoxidase treatment. We also found that Photosystem II activity was markedly inhibited by iodination, but were unable to offer any definite suggestion as to the nature of the electron transport component was affected. This paper will be devoted to a more thorough evaluation of the Photosystem II effects.

There have been numerous previous studies of membrane treatments which have modified Photosystem II activity; these have included analysis of chloroplast aging, high pH treatment, washing with high concentrations of Tris or chaotropic agents, heat or ultraviolet radiation treatments, and digestion of membranes with lipases or proteolytic enzymes [5–17]. It has been generally interpreted that each of these treatments induces some degree of structural damage to the membrane which results in inhibition of the activities of certain electron transport components either on the oxidizing or reducing side of Photosystem II [10, 11, 16, 17]. In most of these same treatments Photosystem I activities have been only marginally affected. A reason for close correlation between a high degree of membrane structural integrity and Photosystem II activity (coupled to water oxidation), but not Photosystem I activity, has not been evident. It may be supposed that the unique enzymatic steps involved in the oxidation of water, which are most sensitive to the above mentioned treatments, have very restrictive structural requirements.

Hypotheses relating the structural organization of Photosystem II components to functional activities have been developed from both theoretical and experimental evaluation of phosphorylating membrane systems. Witt and coworkers [18] have found that Photosystem II electron flow, during a flash irradiation, gives rise to the rapid generation of a charge separation across the membrane. They have concluded that the electron donor and electron acceptor for the photoact must reside on opposite sides of the membrane. This is in agreement with the generalized model of electron transport "loops" hypothesized by Mitchell [19] in support of his chemiosmotic hypothesis for ATP formation. If these concepts are correct, it should be possible to selectively analyze the components at opposite sides of the membrane and demonstrate the nature of any possible asymmetry.

Immunochemistry has been used in an attempt to determine the nature of externally exposed chloroplast membrane proteins. In general, most antibodies active against chloroplasts have been selective against the externally exposed coupling factor

or against components of Photosystem I (see discussion in review [20]). In a few cases, however, Photosystem II activities have been modified by antibody reactions. A limited inhibition of a ferricyanide Hill reaction (but not ferricyanide-mediated photophosphorylation) was observed with antibodies specific for chlorophyll by Radunz et al. [21]. Later work by Radunz and Schmidt [22] demonstrated that an antiserum to lutein reacts with chloroplasts to give a low level of inhibition of electron flow from water to DCIP but not from diphenylcarbazide (an electron donor to Photosystem II) to DCIP. Braun and Govindjee [23] have reported that an antiserum to detergent-derived Photoserum II particles causes a partial (15 %) inhibition of the activity of a component on the oxidized side of Photosystem II in intact chloroplasts.

Dilley and workers [24, 25] have used non-permeant membrane-reactive reagents to analyze surface-exposed lamellar proteins and lipids. Their data demonstrate that the reactive probe *p*-diazoniumbenzenesulfonic acid (DABS) will inactivate Photosystem II in chloroplasts in the light but not in the dark. The effects were attributed to inactivation of an electron carrier on the donor side of Photosystem II.

Trypsin treatment of chloroplast lamellae, which presumably only affects the surface-exposed proteins during brief periods of incubation, also reduces Photosystem II activity [10, 12, 13]. Water oxidation capacity was found to be lost more rapidly than DCIP reduction in the presence of an electron donor; it was thus suggested that components on the oxidizing side of Photosystem II are surface exposed [12, 13]. Alternatively, it may be hypothesized that the observed changes in the presence of trypsin are secondary to structural disorganization of the lamellae [10, 20].

MATERIALS AND METHODS

Chloroplasts were isolated from lettuce (*Lactuca sativa*) obtained from a local market. Deveined leaves were ground in a sorbitol–Tricine–ascorbate–bovine serum albumin solution; the chloroplasts were centrifuged and washed in 0.01 M NaCl and resuspended in sorbitol–Tricine–NaCl as previously described [3]. A 2-min iodination of chloroplast lamellae (400 μg chlorophyll) was conducted in 4 ml of solution containing 0.2 mg lactoperoxidase, (Sigma Chemical Co., St. Louis), 0.01 M Na_2HPO_4 (pH 7.8), $4 \cdot 10^{-4}$ M KI and varying amounts of H_2O_2. This reaction and all electron transport reactions were conducted as previously described [3]. A control for all experiments was run using the various levels of H_2O_2 in the reaction mix but omitting lactoperoxidase. H_2O_2 and KI had no effect on control reactions under our conditions in the absence of enzyme. For fluorescence studies, chloroplasts after iodination were diluted with an equal volume of 0.4 M sorbitol containing 0.01 M Tricine (pH 7.8), 0.01 M NaCl and 0.01 M NH_4Cl. $MgCl_2$ was added to this suspension (unless otherwise indicated) to a final concentration of 10 mM. Fluorescence was monitored at room temperature using a chloroplast suspension held in a 1.5 mm diameter capillary tubing. The chlorophyll concentration was 50 μg/ml. For measurements at -196 °C, a chloroplast suspension absorbed on two layers of cheesecloth was held at the bottom of a dewar flask filled with liquid nitrogen. The excitation light for all fluorescence measurements was at 470 nm ($\Delta\lambda$, 10 nm); it served as both the actinic and analytic beam. Fluorescence was detected through a cut-off filter ($\lambda > 660$ nm), or through a Bausch and Lomb monochromator. The data obtained were either photographic recordings from a fast response oscilloscope or recordings

of spectra from a strip-chart recorder. Simultaneous measurements of life-time and intensity of fluorescence have been measured using a phase fluorimeter previously described [29, 30].

Decay of luminescence was measured with an apparatus previously described by Lavorel [26], for both the μs and ms range after flash irradiation. Each point of the decay was determined as the summed average of the luminescence intensity measured for 10000 flashes (wavelength, 632.8 nm) of 120 μs duration; flashes were separated by a 50 ms dark period. The sample was changed after each 200 flashes.

O_2 evolution during flash irradiation (white saturating flashes separated by 900 ms darkness) were measured with a Haxo-Blinks type electrode. Each measured O_2 burst was recorded on an oscilloscope.

RESULTS

Our previous study demonstrated that Photosystem II mediated Hill reactions were inhibited by high levels of iodination. We have now extended this observation by analyzing the effect of iodination over a range of substrate (H_2O_2) concentrations. Inhibition of electron flow from water to DCIP was found to be inhibited to a maximum of approximately 20 % of the control rate under our experimental conditions (Fig. 1). In the same samples, electron flow from diphenylcarbazide (an electron donor to Photosystem II) to DCIP was also inhibited (Fig. 1). Approximately equal inhibition of both photoreactions was observed at low H_2O_2 levels. In contrast, at high H_2O_2 levels electron flow with water as the donor was more strongly affected. These data suggest that iodination has two effects on Photosystem II.

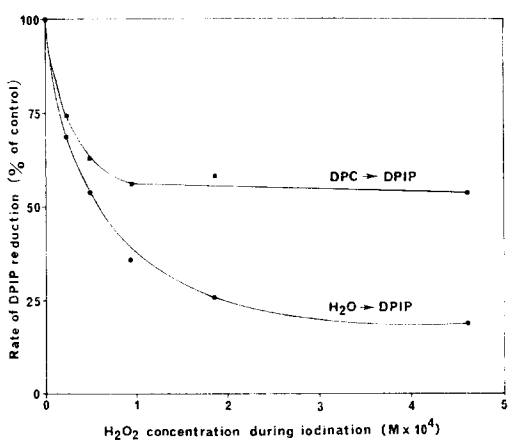

Fig. 1. Rate of DCIP (DPIP in figure) photoreduction by iodinated chloroplasts in the presence (■) and absence (●) of an artificial electron donor for Photosystem II. Varying amounts of H_2O_2 during iodination were used to control the extent of the reaction. Assays were run in a Cary-14 spectrophotometer adapted for sample illumination at saturating intensities. Each reaction contained (in μmoles): DCIP, 0.04; Na_2HOP_4 (pH 6.7), 200; NH_4Cl, 10 and 50 μg chlorophyll in a volume of 2 ml. 2.5 μmoles diphenylcarbazide (DPC) was added as an electron donor for Photosystem II where indicated; only the DCMU ($2 \cdot 10^{-5}$ M) sensitive portion of the diphenylcarbazide to DCIP electron flow was considered to be Photosystem II mediated.

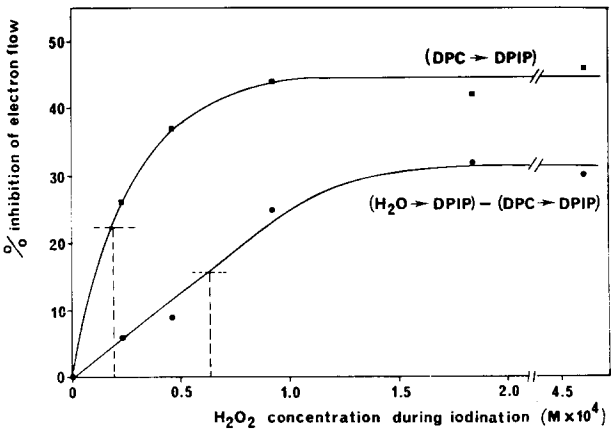

Fig. 2. Inhibition of DCIP (DPIP in figure) photoreduction in iodinated chloroplasts. The portion of electron flow which could not be restored by an electron donor to Photosystem II (■) and that portion which could be restored by an electron donor (●) are indicated. The plots are derived from Fig. 1. Vertical dotted lines indicate H_2O_2 concentrations required during iodination to get a half maximal inhibition for each effect. DCP, diphenylcarbazide.

To further emphasize the dual action of iodination, the two effects on Photosystem II are quantitatively separated in the plot of Fig. 2. For the first curve (electron flow from diphenylcarbazide to DCIP) we have simply inverted that portion of Fig. 1 which deliniated this inhibition. The data show that the presence of $0.2 \cdot 10^{-4}$ M H_2O_2 during the iodination reactions results in half maximal inhibition of Photosystem II activity which cannot be restored by an electron donor. Also in Fig. 2 is plotted that portion of the Photosystem II mediated Hill reaction inhibition which can be restored by an electron donor (water to DPIP electron flow inhibition minus the inhibition measured in diphenylcarbazide to DPIP electron flow). This is the area between the two curves of Fig. 1. It represents damage to that portion of the Photosystem II electron transport chain which is prior to the site of electron donation by diphenylcarbazide; the concentration of H_2O_2 required to give half maximal inhibition for this effect is approximately $0.65 \cdot 10^{-4}$ M.

To obtain further evidence for the existence of two different sites of iodination action on Photosystem II and for a better understanding of the mechanisms of these actions, we have conducted an analysis of the fluorescence characteristics of iodinated membranes. Fig. 3 shows the pattern of fluorescence induction measured in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) for control and iodinated membranes. The component of the fluorescence rise which shows an instantaneous initial increase at the onset of illumination (dead fluorescence; F_0) did not differ in control and treated samples. The well-known sigmoidal pattern of increase in fluorescence yield during illumination [27] was also observed in both samples; the only difference in the treated chloroplasts was that maximal fluorescence intensity (F_M) and therefore, the amplitude of variable fluorescence ($F_M - F_0$), was reduced. It should be noted that the decrease in F_M was not due to a shift in fluorescence emission spectra; the spectra of control and iodinated chloroplasts were identical at both 20 °C and −196 °C with the exception that the relative stationary fluorescence intensity (F_M) was altered (on a chlorophyll basis) in the treated samples.

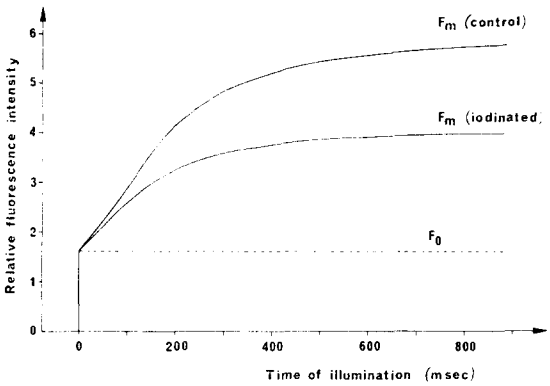

Fig. 3. Fluorescence induction curves for control chloroplasts and chloroplasts iodinated in $0.92 \cdot 10^{-4}$ M H_2O_2. Chlorophyll concentration was 25 μg/ml; $5 \cdot 10^{-3}$ M DCMU and 10^{-2} M $MgCl_2$ were present in the sample. The sample was illuminated with an exciting light at 470 nm; fluorescence was monitored through a cut-off filter at wavelengths longer than 660 nm. The data shown are enlargement tracings of the actual recordings photographed from a fast response oscilloscope.

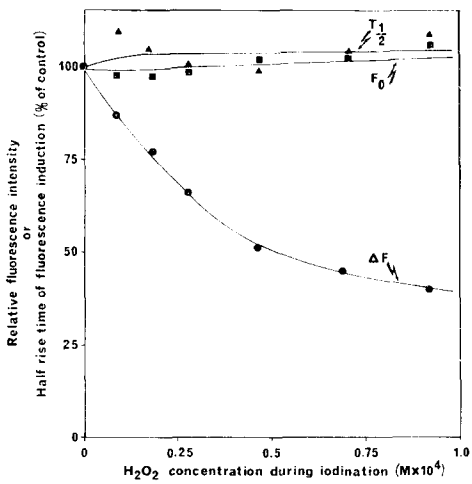

Fig. 4. Fluorescence characteristics of chloroplasts iodinated at different H_2O_2 concentrations. Data were measured from tracings similar to those shown in Fig. 3. F_0, instantaneous initial fluorescence (dead fluorescence); F, amplitude of variable fluorescence ($F_M - F_0$); $T_{\frac{1}{2}}$, half rise time of variable fluorescence. Control values for $T_{\frac{1}{2}}$ in this experiment were 142 ms.

Analysis of the various parameters of fluorescence induction are described in Fig. 4 for a range of H_2O_2 concentrations. The most striking effect was that variable fluorescence was decreased over the entire range of H_2O_2 concentrations. This inhibition closely followed the loss of Hill reaction activity measured under limiting light intensities (when both were expressed as percent of control values). In contrast, the F_0 fluorescence did not change significantly in any of the samples. There was also no significant change in the half rise time of the photochemical phase of residual variable fluorescence (measured in the presence of DCMU).

TABLE I

THE EFFECT OF VARIOUS LEVELS OF IODINATION ON ELECTRON FLOW AND FLUORESCENCE

Electron flow was determined by O_2 uptake in the presence of methylviologen as previously described [3]. The inhibition of fluorescence is indicated in parentheses as percentage of control values. For measurements of fluorescence in dithionite, chloroplast suspensions were made anaerobic by nitrogen bubbling for 10 min; dithionite was then added and fluorescence was assayed after 15 min of reductive treatment. The ΔF calculations for dithionite treated chloroplasts were made assuming that the F_0 level remained constant before and after reductive treatment. DCMU at $5 \cdot 10^{-5}$ M was present in each sample for fluorescence determinations.

Sample	H_2O_2 concentration during iodination ($M \times 10^4$)	Rate of electron flow (% of control)	Relative fluorescence intensity (arbitrary units)		
			F_0	ΔF	ΔF (with dithionite)
1	0	100	10	32 (100 %)	29 (100 %)
2	0.23	68	10	20 (62 %)	22 (76 %)
3	0.92	34	11	11 (35 %)	18 (62 %)
4	4.6	11	11	8 (25 %)	12 (41 %)

It is known that the redox state of Q (the primary electron acceptor or quencher of Photosystem II) influences F_M level. In the presence of dithionite, when Q is chemically reduced, F_0 fluorescence is very close if not equal to F_M level. As is shown in Table I, however, variable fluorescence in the iodinated membranes could not be fully restored to the control F_M level by dithionite treatment. In strongly iodinated chloroplast preparations there was a greater relative restoration of variable fluorescence by dithionite than in the plastids treated in low levels of H_2O_2 (comparing the percent of inhibition of ΔF in the absence and presence of dithionite).

It has been suggested by Butler et al. [28] that the redox state of both the primary electron donor and electron acceptor of Photosystem II regulate the maximal fluorescence level. We have attempted to trap both of these components in a fully reduced state (which should give the highest fluorescence) by pretreating chloroplasts with 10 mM NH_2OH and 10^{-5} M DCMU plus a short period of irradiation. Iodination of chloroplasts after this treatment still resulted in loss of variable fluorescence, however.

Further evaluation of the fluorescence characteristics of control and iodinated membranes was conducted by simultaneously measuring fluorescence intensity and life-time with a phase fluorometer during Photosystem II induction. (The terminology and description of fluorescence parameters to be discussed here have been described previously [29, 30].) There is a linear relationship between excitation life-time (τ) and fluorescence intensity in untreated chloroplasts (Fig. 5; [30]). It was found that the slope of this relationship was unchanged in iodinated chloroplasts although there was a simultaneous decrease of τ and fluorescence yield in the treated samples.

We have also compared the variable fluorescence yield of iodinated chloroplasts at room temperature and at −196 °C. The data (Table II) show that the fluorescence of iodinated samples was inhibited to approximately the same extent when measured

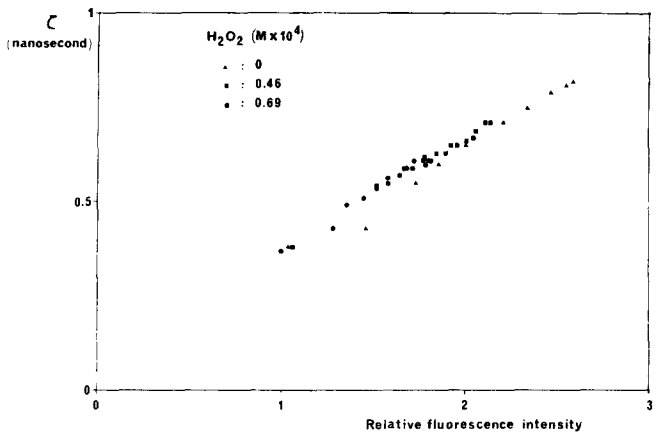

Fig. 5. Excitation life time (τ) and fluorescence yield relationships followed during fluorescence induction in control and iodinated chloroplasts. The amounts of H_2O_2 used during iodination are indicated. Chlorophyll concentration during assay was 40 μg/ml; DCMU at $5 \cdot 10^{-5}$ M was present in the samples.

TABLE II

AMPLITUDE OF VARIABLE FLUORESCENCE IN CONTROL AND IODINATED CHLOROPLASTS AT ROOM TEMPERATURE AND AT LIQUID N_2 TEMPERATURE

Measurements of F_0 and F_M from fast oscilloscope tracings were used to calculate the variable fluorescence (ΔF). DCMU at $5 \cdot 10^{-5}$ M was present in the samples used for fluorescence measurements.

H_2O_2 concentration during iodination ($M \times 10^4$)	Amplitude of variable fluorescence (% control)	
	20 °C	−196 °C
0	100	100
0.46	53	31
0.92	31	33

at either temperature. The quenching introduced by iodination is, therefore, not temperature dependent.

We have also investigated the chlorophyll absorption spectra of control and iodinated membranes. Initial studies showed no differences between the two samples, even when high levels of H_2O_2 were used during iodination. When the samples were subjected to irradiation (white or red light) after iodination, however, a marked change in the spectra was observed. Difference spectra (irradiated, iodinated membranes as the sample; non-irradiated, iodinated membranes or control chloroplasts as the reference) showed that there was a partial bleaching of chlorophyll (chlorophyll a_{680}) and carotenoid pigments, induced by the light treatment. These spectra were qualitatively identical to those described by Yamashita and Butler for irradiated Tris-washed chloroplasts (Fig. 1A of ref. 8). It was also found that the pigment bleaching was time dependent. It was, therefore, possible to compare the rate of

TABLE III

MEASUREMENTS OF THE RATE OF CAROTENOID PHOTOBLEACHING AND ELECTRON TRANSPORT IN IODINATED CHLOROPLASTS

Electron flow was measured by monitoring O_2 uptake in the presence of methylviologen as previously described [3]. Carotenoid photobleaching was monitored by irradiating samples in a Cary-14 spectrophotometer using a Corning 2-58 (red) filter for sample irradiation and a 4-96 (blue) filter to protect the phototube. The samples contained 20 μg chlorophyll/ml suspended in 0.4 M sorbitol–0.05 M Tricine–0.01 M NaCl. In Sample 5, 20 mM NH_2OH and $5 \cdot 10^{-5}$ M DCMU were added to the sample prior to irradiation.

Sample	H_2O_2 concentration during iodination ($M \times 10^4$)	% inhibition of electron flow	ΔA_{490nm} / 6 min
1	0	0	0.011
2	0.046	12	0.010
3	0.46	32	0.013
4	4.6	89	0.045
5	4.6 (+NH_2OH, DCMU during illumination)	—	0.006

photobleaching in chloroplast samples with different levels of iodination. Both carotenoid and chlorophyll bleaching followed the same kinetics [8]. We have, therefore, monitored the reaction at 490 nm. The data, shown in Table III, demonstrated that the rate of loss of pigment absorbance in samples treated with low levels of H_2O_2 was approximately the same as that of controls. High levels of H_2O_2 ($4.6 \cdot 10^{-4}$ M) during iodination predisposed the chloroplasts to bleaching. This bleaching could be overcome by the addition of DCMU and an electron donor (NH_2OH) to the sample prior to illumination (Table III, Sample 5).

Since the data of Fig. 1 had suggested an effect of treatment on electron transport components on the oxidizing side of Photosystem II, we have analyzed the pattern of O_2 evolution during flash irradiation of previously dark-adapted plastids. As has previously been demonstrated [31, 32], almost no O_2 is evolved until the third illuminating flash with control chloroplasts (Fig. 6). In subsequent flashes, there are gradually dampening oscillations of O_2 evolution which show maxima with a periodicity of approximately four flashes. In the chloroplast sample iodinated in $0.46 \cdot 10^{-4}$ M H_2O_2 the maximal rate of O_2 evolution was reduced, but the sequence of relative O_2 yield per flash was nearly identical to that of the control. In contrast, the third sample shown in Fig. 6 ($0.92 \cdot 10^{-4}$ M H_2O_2), which was more strongly inhibited in O_2 evolution, also showed a more pronounced damping of the O_2 evolution oscillations. This presumably indicates a change in the functioning of the "charge accumulator" [31, 32] or a modification of the connection between the charge accumulator and the Photosystem II reaction center.

We have also attempted to determine the functioning of Photosystem II electron transport components via measurement of luminescence as previously described by Lavorel [26, 33]. The amplitude of the initial level of luminescence (50 μs after the flash irradiation) and of the slow luminescence (in the ms range after flash irradiation)

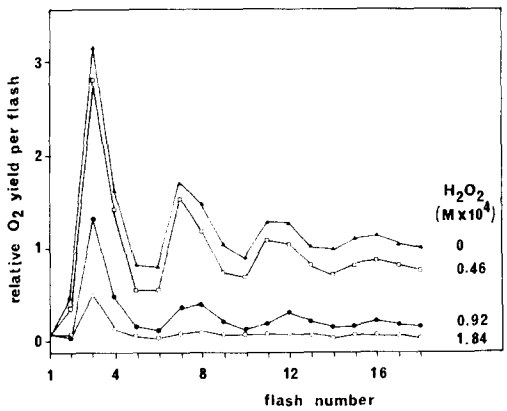

Fig. 6. Relative yield of O_2 evolution for each successive white saturating flash in control and iodinated chloroplasts. O_2 was measured amperometrically. Flash duration was 5 μs; the dark period between each flash was 900 ms.

was decreased in iodinated chloroplasts proportionally to the decrease in variable fluorescence determined with the same samples. In samples subjected to higher iodination, a change in the rate of the rapid phase of luminescence decay was observed; specifically, the life-time of the rapid phase [33] increased after iodination. Values of 66, 94 and 118 μs were observed for chloroplast samples iodinated in the presence of 0, $1.8 \cdot 10^{-4}$ and $3.7 \cdot 10^{-4}$ M H_2O_2, respectively. These data may be best interpreted in view of a simplified scheme based on the proposals of Butler et al. [28] and Lavorel [33] for the primary photoact of Photosystem II:

$$Z \cdot \text{chlorophyll} \cdot Q \underset{K_{-1}}{\overset{h\nu}{\rightleftharpoons}} Z \cdot \text{chlorophyll}^+ \cdot Q^- \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} Z^+ \cdot \text{chlorophyll} \cdot Q^-,$$

where Z and Q are the electron donor and acceptor, respectively, for the Photosystem II reaction center. In this scheme the back reaction K_{-1} is responsible for the first stage of luminescence. If the effect of iodination is an inactivation of Z or a blockage of the Z · chlorophyll interaction (reaction K_2), the back reaction K_{-1} would be stimulated. This was observed.

Since it is possible that some of the effects observed after iodination treatment may result from structural alterations to the membranes, we have analyzed the effect of iodination on glutaraldehyde-fixed chloroplasts. It is well known that glutaraldehyde-fixation blocks structural changes of the membrane but only partially reduces the photochemical activities of the plastids [34, 35]. In Fig. 7 we have plotted the effect of iodination at different H_2O_2 concentrations on Photosystem II mediated DCIP reduction. The data are presented in the same manner as was described for Fig. 2. It is evident that lactoperoxidase is still capable of catalyzing iodination in the stabilized membranes; the maximal extent of inhibition of DCIP reduction (in the presence of an electron donor, diphenylcarbazide) is nearly identical to that observed in Fig. 2 for unfixed membranes. (The slight increase in H_2O_2 concentration required to obtain half maximal inhibition may indicate some small structural restrictions to enzymatic attack.) The effect of iodination on DCIP reduction was identical when

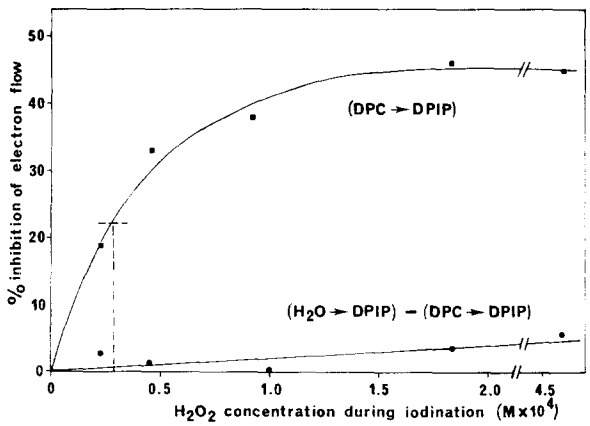

Fig. 7. Inhibition of DCIP (DPIP in figure) photoreduction in chloroplasts prefixed in glutaraldehyde prior to iodination. Varying concentrations of H_2O_2 during iodination were used to limit the iodination reaction. All data presented are in the same form used for Fig. 2. Reaction conditions for photochemical assays were as in Fig. 1. For glutaraldehyde fixation, chloroplasts were ground in the usual buffer and resuspended in sorbitol–Tricine–NaCl as described in Materials and Methods. 50 % glutaraldehyde was then added dropwise to the stirred sample to a final concentration of 0.5 %. After 2 min the sample was pelleted by centrifugation at $3000 \times g$ for 10 min. The fixed chloroplasts were resuspended and iodinated in the usual solutions.

either water or diphenylcarbazide was the electron donor in the fixed membranes. This can be seen in the curve (water → DCIP)—(diphenylcarbazide → DCIP) of Fig. 7; the maximal effect (7 % of control rate) indicates almost no effect of iodination on the oxidizing side of Photosystem II.

We have also analyzed the pattern of fluorescence induction in glutaraldehyde-fixed chloroplasts. As in unfixed lamellae, the F_0 was unchanged while F_M and ΔF were inhibited. The extent of reduction of variable fluorescence was parallel to the percent inhibition of DCIP photoreduction.

DISCUSSION

Lactoperoxidase-catalyzed chloroplast membrane iodination results in the inhibition of Photosystem II mediated electron transport and a reduction in the amplitude of variable fluorescence (Figs 1 and 3). These effects could conceivably be caused either by modification of components on the oxidizing or the reducing side of Photosystem II or by a direct effect on the reaction center itself. Since the iodination reaction is highly specific for surface-localized proteins [1, 2], the identification of the modified electron carrier protein(s) will reveal new information on the molecular organization of the photosynthetic membranes.

The inhibition of DCIP reduction shown in Figs 1 and 2 appears to be due to two different actions of the treatment. At low levels of iodination, electron transport was inhibited to approximately the same extent when either water or diphenylcarbazide were acting as the electron donor. In contrast, at high levels of iodination, DCIP reduction with water as the electron donor was more strongly affected than electron transport in the presence of diphenylcarbazide. The extent of diphenylcarbazide

restoration of DCIP reduction in iodinated chloroplasts (the area between the two curves of Fig. 1) was plotted in Fig. 2. A half maximal level of this effect was observed at $0.65 \cdot 10^{-4}$ M H_2O_2. It has previously been demonstrated that diphenylcarbazide or similar compounds can donate electrons to Photosystem II, and thus restore electron flow, when enzymes on the water-splitting side of Photosystem II are inhibited [6, 36]. This must also be true in our highly iodinated membranes.

Previous studies by Yamashita and Butler [5, 8] have shown that Tris washing or heat treatment of chloroplasts inactivates components on the oxidizing side of Photosystem II. In these preparations, maximal fluorescence yield was inhibited but could be restored by reducing agents, particularly by dithionite [5, 6]. Dithionite was partially able to restore maximal fluorescence in strongly iodinated chloroplasts but had little effect on chloroplasts iodinated in low levels of H_2O_2 (Table I). Yamashita and Butler [8] also found that the chloroplasts inactivated on the oxidizing side of Photosystem II were susceptible to a light-dependent, irreversible bleaching of chlorophyll a_{680} and carotenoid pigments. This same bleaching was observed in highly iodinated chloroplasts but not in samples treated with low levels of H_2O_2 (Table III). As was previously observed for Tris-washed chloroplasts [8], addition of an electron donor for Photosystem II in the presence of DCMU blocked the pigment bleaching (Table III). These analyses of the pigment bleaching and dithionite restoration of fluorescence are consistent with the concept that high, but not low levels of iodination cause inactivation of the water-splitting steps of Photosystem II electron transport.

It has been well documented that water oxidation occurs via the participation of a "charge-accumulating" electron carrier [31, 32]. When the pattern of O_2 evolution by chloroplasts during sequential flash irradiations was examined (Fig. 6), the previously described [31, 32] oscillations in O_2 yield, with a maximal yield at the third, seventh, eleventh flash were observed for control chloroplasts (Fig. 6). The same pattern of O_2 yield was observed for chloroplasts iodinated with $0.46 \cdot 10^{-4}$ M H_2O_2, even though the maximal rate of O_2 evolution was reduced. In contrast, higher levels of iodination not only inhibited O_2 evolution, but also dampened the oscillations of maximal O_2 yield. These data are consistent with the concept that high iodination levels alter the functioning of the water-oxidizing enzymes.

Analysis of highly iodinated chloroplasts showed an increase in the life-time of the fast phase of luminescence observed after flash irradition. As described in the results, these data are again consistent with the idea that components of the electron transport chain on the oxidizing side of Photosystem II are disconnected from the reaction center in the highly iodinated membranes.

We have emphasized above that lactoperoxidase-catalyzed iodination is specific for surface-localized membrane components. It could, therefore, be suggested that damage to components of the oxidizing side of Photosystem II is the result of surface localization of these enzymes. An alternative conclusion is apparent from the iodination effects in glutaraldehyde-fixed chloroplasts, however (Fig. 7). In these experiments, Photosystem II activity was partially inhibited by treatment but diphenylcarbazide was incapable of restoring activity. Since glutaraldehyde stabilizes membrane structural organization [34, 35] and since this stabilization blocks all iodination effects on the water-oxidizing enzymes, it seems highly probable that the electron transport components on the oxidizing side of Photosystem II are not surface-localized on the membrane. The loss of activity of the oxidizing enzymes in unfixed lamellae in highly

iodinated membranes is very likely due to structural modifications in the membrane which are secondary to the initial effects of iodination on other externally localized proteins.

This conclusion now leads back to the question of which components of Photosystem II are surface localized. The inhibition of electron transport from diphenylcarbazide to DCIP (Fig. 2) had a half maximal effect at low levels of H_2O_2. The action of iodination even in glutaraldehyde-fixed membranes indicates that structural modification of the lamellae is not involved. The protein(s) involved in this case must be surface exposed and must be modified by a direct action of iodination.

We have described above numerous tests for damage to the oxidizing side of Photosystem II. None of these assays has indicated an effect of low levels of iodination on these enzymes. The target site for iodination must, therefore, be either near the reaction center or at the acceptor side of the center (before the site of DCMU inhibition).

Butler and coworkers [14, 15] have shown that spectral changes at 550 nm (C_{550}; an indicator of the redox state of the primary electron acceptor for Photosystem II [37]) are destroyed by lipase or ultraviolet light treatment of chloroplasts. It was earlier shown that variable fluorescence yield was inhibited in these same preparations. A high fluorescence could be restored, however, by the addition of dithionite under anaerobic conditions. Dithionite treatment, under nitrogen, did not restore maximal fluorescence in the iodinated chloroplasts (Table I). The primary site of iodination action on Photosystem II is, therefore, not the same as for lipase or ultraviolet light treatment.

Under the conditions of our experiments, iodination induces a permanent quenching of about 50 % of the Photosystem II centers (Table I). This quenching is specific for Photosystem II variable fluorescence. It should be recalled, however, that the residual variable fluorescence is quite similar to that of control chloroplasts; the half rise time for the photochemical phase of the induction was constant and a sigmoidal pattern of induction remained (Figs 3 and 4). This indicates that we are not affecting the functioning of light-harvesting chlorophylls, which should modify $T_{\frac{1}{2}}$, or the transfer of excitation energy between Photosystem II units. It seems that the quenching action is of an "all or none" nature. Iodinated Photosystem II units are completely quenched while non-iodinated units function normally. If we assume that F_0 fluorescence arises from pigment species non-active in photochemistry, we can conclude that the new quenching induced by iodine is equally as efficient, if not more efficient, than photochemical quenching.

From the analysis of fluorescence life-time in iodinated chloroplasts (Fig. 5), it can be determined that iodination modifies the deactivation processes for the excited state fluorescent chlorophyll species. Since the slope τ_0 is unchanged and $\tau_0 = (1/k_f)$ (where k_f is the rate constant of fluorescence deactivation) we must conclude that k_f is unchanged. However, the fluorescence yield (ϕ) was reduced in iodinated samples. Since

$$\phi = \frac{k_f}{k_f + \Sigma K_d}$$

(where K_d represents the rate constants of deactivation processes other than fluorescence), the observed inhibition of ϕ, in the absence of any change in the absorption spectra, must indicate that iodination has introduced a new deactivation of the excited

state chlorophyll. This new rate of deactivation is not due to a change in thermal deactivation (Table II).

A mechanism for the increase in deactivation processes by iodination is suggested by an earlier observation that NaI acts as a quencher of chlorophyll fluorescence in an ethanol solution [38]. The concentration of NaI which gave half maximal quenching was very high: 2.6 M [38]. We may assume, however, that introduction of iodine into a specific site in the pigment-protein molecule which gives the variable fluorescence may greatly increase its effective concentration over the very low solution level used in our experiments.

Mechanisms of chlorophyll fluorescence quenching have been considered by Seely [38]. In all cases he concludes that quenching is evidence for interaction between the quencher and the excited state chlorophyll. Following the arguments for a quenching effect of iodine in treated chloroplasts stated above, and accepting the fact that iodination is restricted to only the outermost proteins of the lamellar membrane [1, 2], the most obvious conclusion is that the Photosystem II reaction center pigment–protein complex is surface localized in chloroplast membranes. The introduction of iodine into this complex can, therefore, be considered as creating a permanently "open trap".

It has been suggested that protons lost during the oxidation of water are released on the inside of the chloroplast thylakoid [39, 40]. This is consistent with the data described above which show that water oxidation enzymes are not surface localized. These conclusions, together with our demonstration that reaction center II is externally localized, support the concept that Photosystem II mediated electron flow must traverse the membrane [18, 19]. Since there is no evidence for a soluble, lipophilic electron carrier functioning on the oxidizing side of Photosystem II, we may suppose that the high degree of structural integrity required for this portion of the electron transport chain (see Introduction) may be related to the transmembrane functioning of these components.

In all of the experiments described above, we have observed a maximum inhibition of Photosystem II reaction centers (diphenylcarbazide to DCIP electron flow) of about 50 %. It should be noted, however, that our chloroplasts are pretreated in 10 mM NaCl prior to iodination; under these conditions the plastids have partially unstacked grana. We will demonstrate, in a subsequent publication, that iodination of Photosystem II is blocked in stacked membranes presumably because the reaction centers are localized in the partition region of stacked membranes [20]. Preliminary evidence from studies with unstacked chloroplast lamellae shows that essentially all Photosystem II reaction centers can be inactivated by iodination.

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