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LACTOPEROXIDASE-CATALYZED IODINATION OF CHLOROPLAST MEMBRANES

I. ANALYSIS OF SURFACE-LOCALIZED PROTEINS

C. J. ARNTZEN, P. A. ARMOND, C. S. ZETTINGER, C. VERNOTTE and J. M. BRIANTAIS

Department of Botany, University of Illinois, Urbana, Ill. (U.S.A.) and Laboratoire de Photosynthèse, du C.N.R.S., 91190 Gif-sur-Yvette (France)

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SUMMARY

Lactoperoxidase-catalyzed iodination of chloroplast membranes has been employed to characterize the vectorial distribution of lamellar proteins. The enzymatic reaction is highly specific for only the outermost membrane components (Phillips, D. R. and Morrison, M. (1971) Biochemistry 10, 1766–1771); we have determined the distribution of ^{125}I label and changes in photochemical activities after iodination in an effort to identify these components. Three major conclusions are evident:

1. The coupling factor for photophosphorylation is highly exposed and is selectively and rapidly inhibited by the iodination reaction.

2. A loss of Photosystem I activity (NADP reduction) resulted from iodination. Partial reactions indicated the effect was on electron-transport components on the reducing side of Photosystem I. There was also a limited inhibition of methyl viologen reduction.

3. Iodination of intact membranes caused a reduction in rates of Photosystem II-dependent Hill reaction activity. This inhibition could not be explained solely on the basis of iodination effects on electron-transport components involved in the oxidation of water. The implications of these data with respect to previous chloroplast-membrane models are discussed.

INTRODUCTION

It is widely accepted that a specific pattern of organization of enzyme complexes within the chloroplast membrane must be of importance for the functioning of these systems. Certain processes such as light-induced proton transport and the generation of a membrane potential are directional [2, 3] and therefore imply a

Abbreviations: DABS, *p*-(diazonium)-benzene sulfonic acid; DCMU, 3-(3-4-dichlorophenyl)-1, 1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DPC, diphenyl carbazide; PMS, phenazine methosulfate; Tricine, *N*-Tris-(hydroxymethyl)-methylglycine.

structural asymmetry of at least some redox components of the system. The nature of the spatial distribution of macromolecules across the membrane is still incompletely understood, although several techniques have been developed in recent years which offer the opportunity to probe this aspect of membrane structure.

Arntzen et al. [4, 5], on the basis of analysis of digitonin-derived submembrane fragments, suggested that Photosystems I and II are asymmetrically distributed in such a way that Photosystem I is more externally localized and Photosystem II is more internally localized in a binary membrane. A similar conclusion was drawn by Briantais [6] based on successive extraction of chloroplasts with Triton X-100. Immunological techniques have demonstrated that certain components of Photosystem I (NADP reductase, ferredoxin-reducing substance, ferredoxin, and the P700 complex) are surface exposed (see ref. 7). In contrast, antibodies reactive against isolated Photosystem II submembrane preparations are largely inactive against intact chloroplast lamellae [8, 9], thus supporting the view that Photosystem II is more internally localized in the membrane.

Dilley et al. [10] have adopted the use of non-permeant membrane-reactive reagents to analyze the vectorial distribution of chloroplast-membrane components. Their data suggest that Photosystem II has very limited surface exposure in dark-treated chloroplasts. If membranes were reacted with the non-permeant probe *p*-(diazonium)-benzene sulfonic acid (DABS) in the light, however, Photosystem II-mediated O_2 evolution was inhibited [11]. It was suggested that components of the electron transport chain on the donor side of Photosystem II become sensitive to the action of DABS in light-activated chloroplasts.

Protease digestion of chloroplast membranes has also been used to selectively modify the surface proteins of the plastid lamellae. These procedures have resulted in the loss of Photosystem II but not Photosystem I activity, thus implying that Photosystem II may be externally localized [12–14]. An alternative explanation, however, is that protease treatment simply alters the structure of the membrane and thereby indirectly affects Photosystem II [7, 14].

We will describe in this paper a new approach to the analysis of the vectorial distribution of chloroplast-membrane proteins which is highly specific for identification of surface-exposed components. The procedure is an adaptation of that used for erythrocyte membranes by Phillips and Morrison [1, 15]. In this technique the enzyme lactoperoxidase, in the presence of H_2O_2, catalyzes the iodination of exposed tyrosine and histidine groups of membrane protein. Since the iodination involves formation of an amino acid (substrate)–enzyme complex [16] and since the enzyme is relatively large (mol. wt = 78 000), the reaction is limited to the outermost proteins of the membrane [1, 15].

MATERIALS AND METHODS

Chloroplasts were isolated either from peas (*Pisum sativum*) grown under fluorescent lights, in vermiculite moistened with 1/2-strength Hoagland's solution or from lettuce (*Lactuca sativa*) obtained at a local market. The plastids from peas were used for all experiments employing radioactive iodine. Most experiments reported in this paper which analyze the effects of iodination on photochemical activities were determined with plastids from lettuce; however, comparative experiments have also

been conducted with pea chloroplasts with essentially identical results.

Pea chloroplasts were prepared by grinding leaves in 0.5 M sucrose containing 0.05 M K_2HPO_4 (pH 7.4) and 0.01 M KCl as was previously described [5]. The pellet obtained by centrifugation was resuspended in 0.05 M K_2HPO_4 (pH 7.4) containing 0.15 M KCl. Chlorophyll and protein assays were determined by the procedures of Arnon [17] and Lowry et al. [18]. Iodination in the presence of carrier-free ^{125}I was conducted in a solution containing 0.05 M K_2HPO_4 (pH 7.4) $1.5 \cdot 10^{-8}$ M lactoperoxidase, $1.2 \cdot 10^{-4}$ M [^{125}I]KI and 0.45 mg chlorophyll in a total volume of 27 ml. Aliquots of H_2O_2 (0.92 μmoles) were added in small volumes at 2-min intervals. The control for all experiments utilizing ^{125}I was a similar reaction from which lactoperoxidase was omitted.

Chloroplasts to be used for analysis of photochemical activities were isolated by grinding 75 g of lettuce or pea leaves in 100 ml of a 0.4 M sorbitol solution containing 0.1 M sodium *N*-tris-(hydroxymethyl)-methylglycine (sodiumTricine), pH 7.8, 0.05 M sodium ascorbate and 2.5 mg/ml bovine serum albumin. The homogenate was passed through four and then twelve layers of cheesecloth and centrifuged at $1000 \times g$ for 10 min. The pellet was resuspended in 25 ml of 0.01 M NaCl and then centrifuged for 10 min at $5000 \times g$. The pellet from this final centrifugation was resuspended in 0.4 M sorbitol containing 0.01 M sodium Tricine (pH 7.8) and 0.01 M NaCl to a final chlorophyll concentration of 1.5–2.5 mg chlorophyll/ml. Unless otherwise indicated, chloroplasts used for enzymatic analysis were iodinated by first mixing 400 μg chlorophyll with 0.2 mg lactoperoxidase (purchased from Sigma Chemical Co.; a stock solution of 1 mg/ml was prepared fresh daily in 0.05 M sodium phosphate buffer, pH 7.8); the chlorophyll–enzyme mixture was then diluted with 4 ml of a solution containing 0.1 M sodium phosphate, pH 7.8; $4 \cdot 10^{-4}$ M KI; and varying amounts of H_2O_2. After 2 min incubation at room temperature, the iodination of chloroplasts was terminated by addition of 10 mg bovine serum albumin in a volume of 0.2 ml. (It was found in preliminary assays that bovine serum albumin added at the onset of the iodination negated virtually all effects on chloroplast photoreactions. It therefore is a very suitable alternative substrate for lactoperoxidase and is effective in stopping the plastid iodination.)

Electron-transport rates of the isolated chloroplasts were determined either by monitoring the rate of light-induced O_2 uptake in the presence of methyl viologen or by measuring the rate of NADP or 2,6-dichlorophenolindophenol (DCIP) reduction in a recording spectrophotometer adapted for direct sample illumination. These experiments were conducted as previously described [4, 5]. Phosphorylation and proton uptake by chloroplast thylakoids were determined titratimetrically as described previously [19, 20]. All iodination reactions were conducted at room temperature Photochemical assays were at 20 °C and at saturating light intensities.

RESULTS

Initial experiments were conducted to determine whether the procedure for iodination of erythrocyte membranes previously described by Phillips and Morrison [15] was adaptable to chloroplast membranes. As is shown in Fig. 1, lactoperoxidase will indeed catalyze the addition of ^{125}I to chloroplast preparations. It should be noted that these plastids were not osmotically shocked and therefore contained both

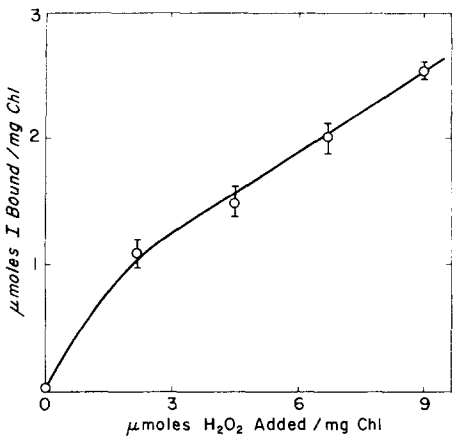

Fig. 1. Lactoperoxidase-catalyzed iodination of chloroplast lamellae. The incorporation of ^{125}I was conducted as described in Methods, using sequential additions of H_2O_2. Immediately before each H_2O_2 addition, aliquots of chloroplasts were removed. These were trapped and washed on a millipore filter. Each filter was analyzed by liquid scintillation counting for the presence of radioactive ^{125}I. A corresponding control experiment was conducted in which lactoperoxidase was omitted from the reaction mixture to determine the amount of non-enzyme-catalyzed ^{125}I binding. These control values were constant over the range of H_2O_2 concentrations employed; the values were subtracted out in this figure to give the true enzyme-catalyzed ^{125}I binding. Calculations of binding were based on the specific activity of iodine in the solution and the amount of chlorophyll and ^{125}I trapped on each filter. Vertical lines for each point indicate the standard error determined from three replications. Specific activity of the ^{125}I used for iodination was $3.4 \cdot 10^6$ cpm/μmole I.

stroma and membrane proteins. Samples prepared by this procedure were then analyzed to determine the general distribution of the radioactive label among membrane components. The first approach was to wash the iodinated chloroplasts to remove stroma proteins and then treat the lamellae with dilute EDTA. EDTA washing has previously been shown to release surface-bound membrane proteins [22, 23]. As a control, a separate sample was washed with a KCl–phosphate buffer solution after removal of soluble proteins. Essentially all protein in the salt-washed chloroplast fraction was recovered in the pellet after centrifugation (Table I). In contrast, a significant amount of solubilized protein was recovered in the colorless supernatant of the EDTA-washed lamellae. This protein had a 10-fold higher ^{125}I content than did the chlorophyll-containing pellet (on a protein basis). There was also a corresponding decrease in the ^{125}I content of the EDTA-washed membranes as compared to the controls, when expressed either on a protein or a chlorophyll basis.

Since high levels of ^{125}I were still bound to the lamellar membranes even after removal of most of the surface-bound enzymes by EDTA washing (Table I), it was of interest to determine the effects of iodination on photochemical activities of the plastids. It was first necessary to determine the time course of any iodination effects to establish reaction conditions for further experiments. This was accomplished by stopping the plastid iodination reaction at various intervals by adding excess quantities of an alternative protein substrate (bovine serum albumin) to the reaction mixture. The results are shown in Fig. 2. Two major points are evident. First, with methyl viologen as the electron acceptor, both whole-chain electron-transport and Photo-

TABLE I

REMOVAL OF ^{125}I-LABELED MEMBRANE PROTEINS BY EDTA WASHING

Chloroplasts were iodinated as described in Fig. 1 to give approx. 2.5 μmoles ^{125}I/mg chlorophyll. After centrifugation and washing once in 0.05 M K_2HPO_4 (pH 7.4) containing 0.01 M KCl, the plastids were then divided into two samples of 0.5 mg chlorophyll each and incubated in 5 ml of the indicated solution. Pellets were obtained by centrifugation at 10 000 $\times g$ for 15 min. The slight reduction in chlorophyll recovery in Sample 2 was the result of a loosely packed pellet; care was taken to avoid contamination of the supernatant sample with pigmented material, however. Samples of the supernatant solution and of the resuspended pellets were dried in glass vials prior to determination of ^{125}I content by liquid scintillation analysis.

Sample	Washing treatment	Fraction	mg Chlorophyll recovered	mg Protein recovered	Protein/ chlorophyll	cpm/mg protein	cpm/mg chlorophyll
1	50 mM potassium phosphate, 10 mM KCl	Pellet	0.50	1.55	3.1	16 221	50 200
		Supernatant	—	—	—	—	—
2	1 mM EDTA	Pellet	0.47	1.25	2.7	11 318	36 000
		Supernatant	—	0.14	—	114 350	—

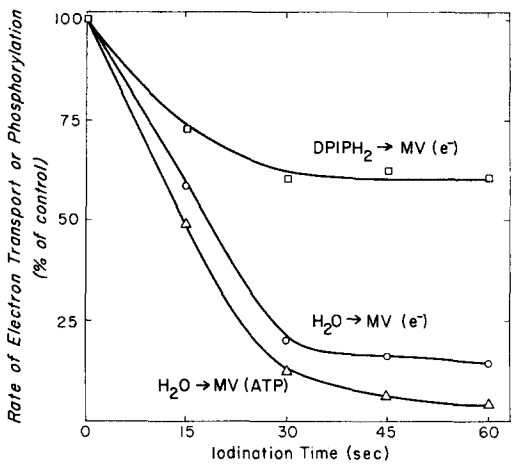

Fig. 2. Inhibition of chloroplast photoreactions as a function of the time of incubation under conditions of iodination. In a volume of 2 ml, 50 μg chlorophyll were mixed with 20 μg lactoperoxidase, 10 μmoles Na_2HPO_4 (pH 7.8) and 0.48 μmoles KI at the start of each determination. The iodination reaction was initiated by addition of 0.66 μmoles H_2O_2. At the times indicated, the plastid iodination was stopped by adding 1 mg bovine serum albumin to act as an alternate substrate for iodination. Additions were then made to bring the solution to a total volume of 6 ml containing (in μmoles): methyl viologen, 5; NaN_3, 5; $MgCl_2$, 17; KCl, 10; Na_2HPO_4 (pH 7.8), 10; and ADP, 10. The reaction was then illuminated and the rate of electron flow from H_2O to methyl viologen, and the rate of the concomitant phosphorylation, were recorded by measurement of O_2 uptake and change in pH in the medium. After a 1-min illumination, DCIP (1.6 μmoles), DCMU (0.24 μmole) sodium ascorbate (10 μmoles), NH_4Cl (30 μmoles) and sodium Tricine, pH 7.8 (100 μmoles) were added in small volumes to the reaction mixture which was then illuminated again for 1 min. The rate of electron flow from reduced DCIP to methyl viologen was determined from the rate of O_2 uptake. Control rates of the reactions were: $H_2O \rightarrow$ methyl viologen electron flow, 295 μmoles methyl viologen reduced/mg chlorophyll per h; reduced DCIP $\rightarrow$ methyl viologen electron flow, 980 μmoles methyl viologen reduced/mg chlorophyll per h; $H_2O \rightarrow$ methyl viologen phosphorylation, 160 μmoles ATP/mg chlorophyll per h.

system I activity are modified by treatment with whole-chain transport being more sensitive to inhibition. In addition, non-cyclic phosphorylation coupled to methyl viologen reduction was inhibited. Second, the iodination was nearly completed within about 1 min; the effect of 2-min iodination was only slightly greater than that at 1 min (data not shown). In subsequent experiments, we have therefore terminated all reactions after 2 min of incubation with the assumption that the iodination is complete.

For further comparative studies on the effects of iodination on various partial reactions, it was necessary to control the reaction more precisely than was possible by adding bovine serum albumin at various time intervals. This was accomplished by varying the amount of H_2O_2 added during the iodination. The results from several experiments are shown in Table II. Over a concentration range which strongly inhibited cyclic photophosphorylation, light-induced proton transport was first only marginally affected at low H_2O_2 concentrations and then strongly inhibited at high H_2O_2 levels. Whole-chain electron transport from water to either methyl viologen or NADP was also inhibited, with the latter showing a stronger effect at all H_2O_2 concentrations.

TABLE II

THE EFFECT OF IODINATION ON VARIOUS PHOTOREACTIONS OF CHLOROPLAST LAMELLAE

Techniques for iodination and enzyme assays were as described in Methods. Solutions for assays were as follows. For phenazine methosulfate (PMS)-catalyzed phosphorylation, a 3-ml reaction mixture contained 50 μg chlorophyll and (in μmoles): PMS, 0.15; NaCl, 50; $MgCl_2$, 10; sodium phosphate, 10; ADP, 5; DTE, 3.6. This was assayed at an initial pH of 7.8. The solution for assay of proton transport was identical except that sodium phosphate and ADP were omitted and the initial pH was 6.0. The reaction mixture for monitoring electron transport from H_2O to methyl viologen contained (in μmoles): sodium phosphate (pH 7.8), 100; methyl viologen, 1.25; NaN_3, 1.5 and 50 μg chlorophyll in a 2-ml volume. For measuring electron flow from reduced DCIP to methyl viologen, the $H_2O \rightarrow$ methyl viologen mixture was supplemented with (in μmoles/2ml): DCMU, 0.01; DCIP, 0.8 and sodium ascorbate, 10. Rates of NADP reduction were determined in solutions identical to those used for methyl viologen reduction except that the pH was adjusted to 6.7 and methyl viologen and NaN_3 were replaced by NADP (0.5 μmole/2 ml) and saturating amounts of a crude ferredoxin mixture [28]. Uncoupling was achieved in the experiments indicated by adding gramicidin D (0.04 μmole/2 ml).

Photochemical assay	Control rate of reaction	H_2O_2 concentration during iodination ($\times 10^4$ M)				
		0	0.46	0.69	0.92	4.6
		Rate of reaction after iodination (% of control)				
(1) Cyclic phosphorylation (with PMS)	449 μmoles ATP formed/mg chlorophyll per h	100	31	20	9	0
(2) "Proton pump" (with PMS)						
(a) Initial rate	320 μmoles H^+ taken up/mg chlorophyll per h	100	94	84	70	30
(b) Extent	0.34 μmoles H^+ taken up/mg chlorophyll per h	100	110	87	82	12
(3) Whole-chain electron transport						
(a) $H_2O \rightarrow$ (methyl viologen)	320 μmoles methyl viologen reduced/mg chlorophyll per h	100	85	60	41	20
(b) $H_2O \rightarrow$ NADP	60 μmoles NADP reduced/mg chlorophyll per h	100	67	48	36	13
(4) Photosystem I						
(a) Reduced DCIP $\rightarrow$ MV (coupled)	600 μmoles MV reduced/mg chlorophyll per h	100	102	110	88	86
(b) Reduced DCIP $\rightarrow$ MV (uncoupled)	1500 μmoles MV reduced/mg chlorophyll per h	100	93	86	73	66
(c) Reduced DCIP $\rightarrow$ NADP (coupled)	58 μmoles NADP reduced/mg chlorophyll per h	100	71	36	25	20
(d) Reduced DCIP $\rightarrow$ NADP (uncoupled)	100 μmoles NADP reduced/mg chlorophyll per h	100	75	40	20	20

TABLE III

INHIBITION OF DCIP PHOTOREDUCTION BY CHLOROPLAST IODINATION AND LIMITED RESTORATION OF ACTIVITY BY AN ELECTRON DONOR FOR PHOTOSYSTEM II

Chloroplasts were iodinated as described in Fig. 2 for 3 min at $3.3 \cdot 10^{-4}$ M H_2O_2. For the control experiment (Expt 1), lactoperoxidase was omitted from the reaction mixture. Heat-treated chloroplasts (Expt 3) were incubated at 1 mg chlorophyll/ml at 50 °C for 3 min prior to assay. For spectrophotometric measurement of DCIP reduction, aliquots of either the chloroplasts in the iodination solution or heat-treated chloroplasts were diluted to a volume of 2 ml containing (in μmoles): sodium phosphate (pH 6.7), 200; DCIP, 0.04; NH_4Cl, 10; and 40 μg chlorophyll. This suspension was subjected to three short illumination periods with red light (Corning filters Nos 2403 and 1-69) during which changes in absorbance of the suspension were monitored at 580 nm. Following the first illumination, 5 μmoles DPC were added to the cuvette. Following the second illumination 0.06 μmole of DCMU was added.

Expt	Sample	μmoles DCIP reduced/mg chlorophyll per h		
		No additions	+DPC	+DPC +DCMU
1	Chloroplast+iodination solution (−lactoperoxidase)	280	280	17
2	Chloroplasts+iodination solution (+lactoperoxidase)	31	112	28
3	Heat-treated chloroplasts	56	197	10

Photosystem I activities (Table II) were also modified by treatment. Transport of electrons from reduced DCIP to methyl viologen was stimulated at low H_2O_2 concentration in coupled chloroplasts. When the uncoupler gramicidin was added to the reaction mixture, however, iodination caused an immediate reduction in the observed rate of electron flow. A similar, but more pronounced inhibition of Photosystem Imediated NADP reduction (with reduced DCIP as electron donor) is also indicated in Table II.

In other assays not shown here, it was determined that iodination of chloroplasts inhibited Photosystem II-mediated O_2 evolution in the presence of ferricyanide or DCIP. The extent of inhibition of the photoreduction of DCIP is demonstrated in Table III; we have also indicated the rate of DCIP reduction after addition of diphenyl carbazide (DPC), an electron donor for Photosystem II. The restoration of electron transport in the presence of DPC in the iodinated chloroplasts (Expt 2) was low compared to that in heat-treated chloroplasts (Expt 3). Iodination was also found to decrease the sensitivity of the DPC → DCIP electron transport to inhibition by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

DISCUSSION

The data presented have indicated that lactoperoxidase can catalyze the iodination of amino acids in chloroplast-membrane proteins (Fig. 1, Table I). Since this protein iodination requires the direct interaction between the amino acid

substrate and the relatively large lactoperoxidase molecule [1, 15], we can safely assume that only those membrane components which were surface exposed in the thylakoid membrane were iodinated [15, 21]. We have used two general approaches in an attempt to determine the identity of these surface-localized proteins. First, radioactive ^{125}I was used to label the substrates for monitoring in a subsequent washing or fractionation experiment; second, we have analyzed the photochemical activities of various partial reactions of photosynthetic energy-coupling processes. In the latter approach, we have assumed that the modification of constituent amino acids of various proteins might alter their biochemical functioning.

From the data of Table I it is evident that a protein fraction removed from chloroplast thylakoids by EDTA washing is heavily labeled with ^{125}I. This EDTA-removable fraction has been shown to consist primarily of an ATPase, or coupling factor, which is thought to mediate the terminal steps of photophosphorylation [22, 23]. This suggestion is in agreement with the finding that phosphorylation was rapidly and strongly inhibited in the iodinated membranes (Fig. 2 and Table II) while proton-transport activities were much more moderately effected (Table II). If we assume that proton uptake by thylakoids is a measure of the ability of the membranes to maintain an energized state [2], the more rapid inhibition on phosphorylation points to a modification of the coupling factor that blocks the utilization of high-energy intermediate(s).

Chloroplasts iodinated with ^{125}I and then washed with EDTA to remove surface-localized proteins still retained high levels of radioactivity (Table I), thus indicating that some portions of the photosystems which form an integral part of the membrane were exposed to lactoperoxidase action. This is consistent with the observation that iodination caused a reduction in the rates of photochemical electron transport (Fig. 2). Comparison of the rates of partial reactions of Photosystem I over a range of H_2O_2 concentrations (Table II) showed that NADP reduction was more strongly inhibited than methyl viologen reduction. These data suggest that iodination strongly affects some electron carrier on the reducing side of Photosystem I. Since these chloroplasts had been previously osmotically shocked and had therefore lost their endogeneous ferredoxin, the carrier affected presumably was a component in the chain before ferredoxin reduction. Under certain conditions it has previously been demonstrated that NADP reduction but not methyl viologen reduction could be inhibited by an antibody active against a "ferredoxin-reducing substance" [24, 25]. It seems reasonable that this component may also be the site of iodination effects. Dilley and coworkers (personal communication) have also found that DABS labeling of the external membrane surface more strongly inhibits NADP reduction than methyl viologen reduction.

Transfer of electrons from reduced DCIP to methyl viologen was initially stimulated and then inhibited by iodination of coupled chloroplasts (Table II). The initial stimulation is very likely due to an uncoupling action of the treatment since at the same H_2O_2 concentration at which maximum stimulation was observed, phosphorylation and "proton pumping" were already inhibited. In the presence of an uncoupler, reduced DCIP → methyl viologen electron flow was inhibited even at the lowest level of iodination. It has previously been demonstrated that "ferredoxin-reducing substance" is required for methyl viologen reduction [25]. The suggested effect of iodination on this electron-transport carrier (described above) may be

related to the observed reduction in rates of methyl viologen reduction in the uncoupled chloroplasts (Table II).

Photosystem II-dependent whole-chain electron transport was also inhibited by iodination (Table II). The strong inhibition of methyl violet reduction when water was the electron donor as compared to more moderate effects on the Photosystem I-mediated methyl viologen reaction suggest an additional effect of treatment near Photosystem II. This suggestion was strengthened by the finding that both ferricyanide and DCIP-catalyzed Hill reactions were also strongly inhibited by iodination. To determine whether the altered electron carriers were confined to the oxidizing side of Photosystem II, we have attempted to restore the rate of DCIP reduction by addition of DPC, an electron donor to Photosystem II. As was previously reported [26, 27], DPC (or similar compounds) strongly stimulate DCIP reduction in chloroplasts which are incapable of oxidizing water (heat-treated plastids, Table III). DPC was less effective in restoring activity in iodinated chloroplasts, however (Table IV). These data indicate that iodination has a limited effect on the enzymatic steps involved in the oxidation of water, but these effects cannot totally explain the inhibitory action of the treatment on Photosystem II. We will show in a forthcoming publication (manuscript in preparation) that the primary effect of lactoperoxidase-catalyzed iodination on Photosystem II is at the level of the reaction center. This conclusion will be supported by analysis of the fluorescence characteristics of treated chloroplasts and by analysis of the photochemical electron-transport activities in normal and glutaraldehyde-fixed lamellae after iodination. We will demonstrate that damage to the water-splitting reactions of Photosystem II, which can be partially overcome by addition of an artificial electron donor to Photosystem II (Table III), are only associated with high levels of membrane iodination (i.e. high levels of H_2O_2 as was used in Table III), and are the result of secondary membrane-structural changes.

Our earlier analysis of chloroplast membrane structure suggests that the membrane is asymmetric with Photosystem II more internally localized, and Photosystem I more externally localized [4, 6, 7]. The data we have presented in this publication, and the DABS labeling studies of Dilley et al. [10] and Giaguinta et al. [11], indicate that in fact, a portion of Photosystem II is exposed atthe external surface of the chloroplast membrane. Thus it is necessary to modify the proposed chloroplast membrane models to emphasize this fact. The data of Dilley and coworkers do indicate, however, that a greater amount of Photosystem I than Photosystem II is surface exposed (based upon relative reactivity to radioactive labeling on surface specific reactions). We may therefore conclude that the basic hypothesis suggesting an asymmetric localization of the bulk of the two photosystems within the chloroplast membrane is still reasonable.

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