CYTOCHEMICAL STUDY OF DIAMINO BENZIDINE OXIDATION BY ISOLATED VICIA CHLOROPLASTS UNDER ILLUMINATION

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Received for publication August 28, 1981

A concurrent microchemical and topochemical study of DAB* oxidation by isolated chloroplasts of *Vicia faba* under illumination was carried out. A new method for the quantification of the oxidation product of DAB was explored. The microchemical analysis by this method revealed that the photosystem responsible for the DAB oxidation was the PS-I. It also proved that endogenous active oxygen in the chloroplasts played only a little role in the oxidation. The topochemical analysis revealed that both the grana and the stroma membrane were positive in DAB photooxidation. The densitometric traces across the grana membranes in both surface and side views proved effective in locating the precise reaction sites. The partition layer showed a high degree of positive reaction. The thylakoid membrane proper also contained particles of positive reaction. The positive site extended to the thylakoid locus to form protrusions. These results are in harmony with the localization of the PS-I complex particles on the PF face side of the thylakoid membrane as revealed by the freeze fracture technique.

The concurrent microchemical and topochemical study of tetrazolium reduction by isolated *Vicia* chloroplasts under illumination has demonstrated that the photosystem responsible for the reduction is the PS-II*. It also revealed that the electron transfer activity mediated by this system is located in the thylakoid membrane proper, and extends to the surface of the locus (12). Similar cytochemical study along this line was undertaken of the DAB oxidation by isolated *Vicia* chloroplasts under illumination in order to get some insight into the following three points: first, the photosystem responsible for the oxidation reaction under illumination, secondly, the possible role of endogenous active oxygen in the reaction, and thirdly, the localization of the photosystem concerned in the thylakoid membrane. This paper deals with the results obtained.

* Abbreviations: PS-I, photosystem I; PS-II, photosystem II; DAB, 3,3'-diaminobenzidine tetrahydrochloride; DC, diphenyl carbazide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPA, 3',4'-dichloropropionanilide; MCC, methyl 3,4-dichlorocarbanilate; CIPC, isopropyl *m*-chlorocarbanilate; CAT, 2-chloro-4,6-bis(ethylamino)-*s*-triazine; DPIP, 2,6-dichlorophenol indophenol; ASC, Na ascorbate; CATL, catalase; SOD, superoxide dismutase; MV, methylviologen; EF face, exoplasmic fracture face; PF face, protoplasmic fracture face; ES, exoplasmic surface.

MATERIALS AND METHODS

Preparation of isolated chloroplasts: Two different procedures of chloroplast preparation were introduced in the present study. For microchemical purposes, the procedure as described in the previous paper was followed to prepare the so-called "once washed" chloroplast fraction (12). For topochemical purposes, the basal medium consisting of 0.25 M sucrose, 0.05 M tris-HCl, 2.5% ficoll, 0.01% serum albumin, 6 mM mercaptoethanol, 1 mM each of MgCl_2 and MnCl_2 , and 1.5 mM CaCl_2 (pH 7.8) was used. The so-called "once washed" chloroplast fraction was prepared, and further purified on a discontinuous density gradient in a cold room. The gradient consisted of four kinds of modified basal medium, which differed in final sucrose concentrations: 1.0, 1.5, 2.0 and 2.5 M. The "once washed" fraction, resuspended in the basal medium, was overlaid on the top of the gradient and spun down at 42,000 g for 30 min. A purified chloroplast fraction formed a green band on the interface between the 1.0 and the 1.5 M medium. This fraction was collected, gradually diluted with the basal medium, and was recovered by centrifugation. Both the "once washed" fraction for the microchemical use and the "purified" fraction for the topochemical use were then fixed with 1% glutaraldehyde dissolved in the basal medium concerned for 5 min, and washed three times with the basal medium prior to all the cytochemical reactions (11). These fractions will be referred as the "washed" and the "purified" chloroplast fraction respectively.

The chlorophyll determination procedure after Arnon (4) was followed with an aliquot of the "washed" and the "purified" fractions to control the chloroplast amount in individual cytochemical determination.

Microchemical methods: An amount of the "washed" chloroplasts, corresponding to 0.25 mg chlorophyll *a* and *b*, was used in each determination. The chloroplasts were suspended in 1.0 ml volume of the blank or one of the reaction mixtures, and were subjected to illumination of 1.7×10^4 erg/cm²-sec for 30 min at 30°C. In order to analyze the photosystem responsible for the DAB photooxidation by isolated chloroplasts, some chemicals which were expected to affect the PS-II specifically were selected for use. They were DC, DCMU, DCPA, MCC, CIPC and CAT. The effects of these chemicals on the photooxidation by the chloroplasts were studied. In addition, supplementary effects of both DPIP and ASC in the presence of DCMU or DCPA on the photooxidation were also tested. In order to analyze the possible role of endogenous active oxygen on the photooxidation by isolated chloroplasts, the effects of CATL (Sigma), SOD (Sigma) or both CATL and SOD on the photooxidation were examined. All these experiments were carried out in the absence and the presence of MV. The compositions of the blank and the reaction mixtures used will be described in detail in appropriate places.

A new method for quantitative determination of the DAB photooxidation product produced by isolated chloroplasts was developed, because the method proposed by Fahimi and Herzog (7) did not give a satisfactory result. The chloroplasts, once subjected to the DAB reaction under illumination, were washed twice with the basal medium, collected by centrifugation at 1,800 g for 5 min, and suspended in 2 ml of monoethanolamine by strong buzzing. To this suspension, 3 ml of distilled water were added and mixed well. This procedure extracted the chlorophyll pigments contained in the chloroplasts, without affecting the DAB oxidation product

at all. The chloroplasts were packed by centrifugation at 2,000 g for 10 min, and subjected to the extraction with monoethanolamine again. They were recovered by centrifugation, and finally suspended in 4 ml of monoethanolamine by strong buzzing. The chloroplasts positive in the reaction gave a brown, viscous, and stable suspension, which gave an absorption curve as shown in Fig. 1, when measured against the blank run with a spectrophotometer (Type 239, Hitachi Ltd., Tokyo). The determinations of the oxidation product were carried out at 427.5 nm.

Topochemical methods: The "purified" chloroplasts were pretreated with 1×10^{-3} M MV dissolved in the basal medium for 30 min under illumination of 1.7×10^4 erg/cm²·sec at 30°C. They were recovered from the pretreatment medium by centrifugation at 1,100 g for 5 min, and resuspended in the basal medium, whereupon the suspension was divided into two equal volumes. The chloroplasts in each aliquot were collected, washed with the basal medium again, and spun down by centrifugation. The packed chloroplasts were suspended in 0.5 mg/ml DAB dissolved in the basal medium or in the basal medium alone, and irradiated under the same conditions. The former will hereafter be denoted as the "treated", and the latter as the "blank" sample. The chloroplasts were recovered from the reaction media by centrifugation, and washed twice with the basal medium. The procedures of double fixation and of mounting in agarose gel as described previously were followed (12). The samples were dehydrated with a graduated series of acetone, and embedded in an epon mixture. The procedures of resin polymerization, ultrathin

FIG. 1. The absorption spectrum of DAB photooxidation product produced by isolated *Vicia* chloroplasts. The product was produced by treating the isolated chloroplasts with DAB-MV medium under illumination. After removal of chlorophyll pigments, the chloroplasts loaded with the product were suspended in monoethanolamine, and the absorbance due to the product was measured against the blank run.

sectioning, section mounting on copper grids, electron staining, examination under an electron microscope, and densitometric analysis of selected electron micrograph transparencies were carried out as described previously (12).

RESULTS

Photosystem responsible for DAB photooxidation by isolated chloroplasts: The "washed" chloroplasts turned brownish black within a minute when they were irradiated in the DAB medium, and attained black after 30 min of illumination. In contrast, they remained green when they were incubated in the blank medium under the same illumination. The addition of DC, DCMU, DCPA, MCC, CIPC or CAT to the DAB medium at low concentrations did not cause any recognizable change in degree of the chloroplast blackening so far as the visual examination was concerned. The chloroplasts turned faint black after 30 min of illumination when incubated in the DAB-DCMU or the DAB-DCPA medium supplemented with both DPIP and ASC. The quantitative determination of the DAB oxidation product, developed

TABLE 1. *Effects of some chemicals on DAB oxidation by isolated Vicia chloroplasts under illumination*

Reaction media*	Relative activity, as expressed by percentage amounts of DAB oxidation product produced		
	Mean	Standard deviation	
Blank	0.0		
DAB	100.0	±	3.4
DAB-DC	93.9	±	5.6
DAB-DCMU ₁	155.9	±	2.0
DAB-DCMU ₂	163.6	±	4.6
DAB-DCPA	120.8	±	3.4
DAB-MCC	150.2	±	4.6
DAB-CIPC	113.3	±	7.8
DAB-CAT	128.0	±	5.8
DAB-DCMU-DPIP-ASC	26.6	±	1.9
DAB-DCPA-DPIP-ASC	24.8	±	2.0

* The basal medium used (BM) was as follows: 0.35 M sucrose, 0.01 M tricine-NaOH buffer (pH 7.4) and 1×10^{-4} M MgCl_2 . The final concentrations of the ingredients contained in the reaction media were as follows: Blank, BM; DAB, 0.5 mg/ml DAB in BM; DAB-DC, 0.5 mg/ml DAB, 1×10^{-4} M DC in BM; DAB-DCMU₁, 0.5 mg/ml DAB, 1×10^{-5} M DCMU in BM; DAB-DCMU₂, 0.5 mg/ml DAB, 1×10^{-4} M DCMU in BM; DAB-DCPA, 0.5 mg/ml DAB, 1×10^{-5} M DCPA in BM; DAB-MCC, 0.5 mg/ml DAB, 1×10^{-5} M MCC in BM; DAB-CIPC, 0.5 mg/ml DAB, 1×10^{-4} M CIPC in BM; DAB-CAT, 0.5 mg/ml DAB, 1×10^{-5} M CAT in BM; DAB-DCMU-DPIP-ASC, 0.5 mg/ml DAB, 1×10^{-4} M DCMU, 1×10^{-4} M DPIP, 2×10^{-4} M ASC in BM; DAB-DCPA-DPIP-ASC, 0.5 mg/ml DAB, 1×10^{-5} M DCPA, 1×10^{-4} M DPIP, 2×10^{-4} M ASC in BM; All the reactions were carried out under the illumination of 1.7×10^4 erg/cm²-sec for 30 min at 30°C. The amounts of DAB oxidation product were determined at 427.5 nm by the method developed by the present authors. The values given were the means and the standard deviations of at least two experiments, each consisting of duplicate sets.

TABLE 2. *Effects of some chemicals on DAB oxidation by isolated Vicia chloroplasts under illumination in the presence of MV*

Reaction media*	Relative activity, as expressed by percentage amounts of DAB oxidation product produced		
	Mean	Standard deviation	
Blank	0.0		
DAB	49.2	±	2.5
Blank-MV	0.0		
DAB-MV	100.0	±	2.2
DAB-DC-MV	90.6	±	0.9
DAB-DCMU ₁ -MV	128.0	±	2.8
DAB-DCMU ₂ -MV	147.8	±	3.7
DAB-DCPA-MV	128.8	±	4.7
DAB-MCC-MV	131.3	±	4.3
DAB-CIPC-MV	123.2	±	4.3
DAB-CAT-MV	134.0	±	7.3
DAB-DCMU-DPIP-ASC-MV	21.4	±	2.8
DAB-DCPA-DPIP-ASC-MV	21.5	±	2.1

* The abbreviations of the ingredients contained in the reaction media, except MV, appear in Table 1. In the reaction media denoted by MV, MV was further added at the final concentration of 1×10^{-3} M. The conditions for reaction, illumination, and determination were the same as those given in Table 1.

TABLE 3. *Effects of CATL and SOD on DAB oxidation by isolated Vicia chloroplasts under illumination*

Reaction media*	Relative activity, as expressed by percentage amounts of DAB oxidation product produced		
	Mean	Standard deviation	
Blank	0.0		
DAB	100.0	±	3.4
DAB-CATL	81.6	±	4.0
DAB-SOD	91.6	±	5.4
DAB-CATL-SOD	82.6	±	4.7
Blank-MV	0.0		
DAB-MV	100.0	±	2.2
DAB-CATL-MV	83.0	±	1.7
DAB-SOD-MV	87.0	±	6.7
DAB-CATL-SOD-MV	83.2	±	3.5

* The final concentrations of the ingredients contained in the reaction media were as follows: Blank, BM; DAB, 0.5 mg/ml DAB in BM; DAB-CATL, 0.5 mg/ml DAB, 0.1 mg/ml CATL in BM; DAB-SOD, 0.5 mg/ml DAB, 0.1 mg/ml SOD in BM; DAB-CATL-SOD, 0.5 mg/ml DAB, 0.1 mg/ml CATL, 0.1 mg/ml SOD in BM; Blank-MV, 1×10^{-3} M MV in BM; DAB-MV, 0.5 mg/ml DAB, 1×10^{-3} M MV in BM; DAB-CATL-MV, 0.5 mg/ml DAB, 0.1 mg/ml CATL, 1×10^{-3} M MV in BM; DAB-SOD-MV, 0.5 mg/ml DAB, 0.1 mg/ml SOD, 1×10^{-3} M MV in BM; DAB-CATL-SOD-MV, 0.5 mg/ml DAB, 0.1 mg/ml CATL, 0.1 mg/ml SOD, 1×10^{-3} M MV in BM. The conditions for reaction, and for determination were the same as those given in Table 1.

Figs. 2, 4, 6 and 8 are electron micrographs of isolated chloroplasts of *Vicia faba*, and Figs. 3, 5, 7 and 9 densitometric traces across the grana membranes shown above. The axis of abscissae denotes the distance scanned, and the axis of ordinates the optical density. Scanning beam was 0.1 mm (Figs. 3, 5) or 0.05 mm (Figs. 7, 9) in width and 1.0 mm in height on the transparencies scanned. In all the figures, M_1 and M_2 are the start and the finish marks of scanning, and all the bars correspond to $0.1\ \mu\text{m}$.

FIG. 2. Electron micrograph of an isolated *Vicia* chloroplast, showing surface views of the grana and the stroma membranes in blank sample. G, electron lucent particles in the grana membrane; S, similar particles in the stroma membrane; E, edge of the grana stack. $\times 85,000$

FIG. 3. Densitometric trace across the grana membrane in surface view, shown in Fig. 2. The trace in the central part of the membrane shows a series of mono- and multi-peak waves. E_1 and E_2 , edges of the grana membrane.

FIG. 4. Electron micrograph of an isolated *Vicia* chloroplast, showing surface views of the grana and the stroma membranes in treated sample. G, electron dense deposits in the grana membrane; S, similar deposits in the stroma membrane; E, electron dense deposits on the edge of the grana stack. 85,000 $\times$

FIG. 5. Densitometric trace across the grana membrane in surface view, shown in Fig. 4. Note that multi-peak waves in the central part of the membrane are higher in optical density and wider in width in this figure than in Fig. 3. G, electron dense deposits in the grana membrane; E₁ and E₂, electron dense edge of the grana membrane.

by us, permitted the reaction intensity to be evaluated exactly. Table 1 shows the relative value obtained.

The chloroplasts turned deep black after 30 min of illumination when they were incubated in the DAB medium supplemented with MV, or with both MV and one

FIG. 6. Electron micrograph of an isolated *Vicia* chloroplast, showing side views of the grana and the stroma membranes in blank sample. P, partition layer; G, somewhat electron dense particles in the grana membrane; S, similar particles in the stroma membrane. $\times 119,000$

FIG. 7. Densitometric trace, running normally to the stack, across the grana membranes shown in Fig. 6. The trace reveals a multi-peak wave (i), and tri-peak waves (a-h).

of the selected chemicals. They turned faint black when irradiated in the DAB-DCMU or DAB-DCPA medium containing MV, DPIP and ASC simultaneously. In contrast, they remained green after the same period of illumination in the blank medium containing MV. The quantitative data obtained are given in Table 2.

Possible role of endogenous active oxygen on DAB photooxidation by isolated chloroplasts: The chloroplasts turned black when they were irradiated in the DAB medium and the medium supplemented with CATL and SOD, separately and simultaneously. They turned deep black under illumination when incubated in the DAB medium containing MV, and in the medium supplemented with MV and one or both of these enzymes. Table 3 gives the results quantitatively.

Topochemical localization of DAB oxidation product in the thylakoid membrane: The "purified" chloroplast fraction consisted exclusively of the chloroplasts of class II. In the surface view of the grana membranes in the treated sample, the DAB photooxidation product was visible as electron dense particulate deposits in the thylakoid membrane. Some of them were solitary and measured about 40–60 Å in diameter. Others were aggregated to form large deposits of 120–240 Å diameter (G in Fig. 4). In contrast, electron lucent particles, solitary and aggregated, were visible in the grana membrane of the blank sample, the former measuring 40–60 Å and the latter about 120 Å in diameter (G in Fig. 2). Besides these deposits in the stacked membranes, the edge was lined with a row of electron dense deposits of 180–240 Å width in the treated sample, while it remained less dense in the blank one (E in Figs. 2 and 4). In the surface view of the stroma membranes, electron dense particles, solitary or aggregated, were also observable in the thylakoid membrane of the treated sample, while similar less electron dense particles appeared in the thylakoid membrane of the blank one (S in Figs. 2 and 4). The solitary particles measured about 40–60 Å in diameter, and the aggregated ones 120–160 Å.

The densitometric trace across the central part of the grana membrane in surface view consisted of a series of mono- and multi-peak waves in the blank sample (Fig. 3), and a series of multi-peak waves in the treated one (Fig. 5). The average peak height as expressed by optical density was 0.266 ± 0.093 in the treated sample and 0.080 ± 0.022 in the blank one, showing clearly that the waves in the former sample were the positive sites. Besides the increase in peak height, an increase in wave width was also recognizable. The mean width was 132.3 ± 67.7 Å in the treated sample (Fig. 5) and 64.7 ± 19.7 Å in the blank one (Fig. 3). In addition, there was an increase in optical density at the bottom of the wave valley, that is, the average density in the former sample was 0.111 ± 0.050 , and that in the latter 0.045 ± 0.023 . Similarly an increase in optical density was also observed along the edge of the grana stack, the average value being 0.65 (Fig. 5) and 0.40 (Fig. 3) respectively.

In the side view of the grana membranes, the partition layer revealed itself as an electron dense line in the treated sample, while it remained less dense in the blank one (P in Figs. 6 and 8). The thylakoid membrane was found electron denser in the treated sample (Fig. 8) than in the blank one (Fig. 6), and, in favourable cases, revealed a row of electron dense deposits of ellipsoidal shape along its length.

FIG. 8. Electron micrograph of an isolated *Vicia* chloroplast, showing side views of the grana and the stroma membranes in treated sample. P, electron dense partition layer; G, electron dense deposits in the grana membrane; S, similar deposits in the stroma membrane; E, electron dense deposits on the edge of the grana stack. $\times 119,000$

FIG. 9. Densitometric trace, running normally to the stack, across the grana membranes shown in Fig. 8. The trace shows tri-peak waves (b–j) and a multi-peak one (a). All the waves are high in peak height. Note that they are wider in width than those in Fig. 7, as the result of fusion of waves, b and c, d–f, and g–i.

They measured about 100–120 Å in long axis, and 40–60 Å in short one, and lay with their long axis parallel with the membrane length. Some of them protruded into the thylakoid locus, and, in extreme cases, partly filled it up (G in Fig. 8). In addition, electron dense deposits were found here and there on the edge of the stacked membranes in the treated sample (E in Fig. 8). In the side view of the stroma membranes, electron dense deposits in the membrane were observed in the treated sample (S in Fig. 8). They were of ellipsoidal shape, and measured about 60–120 Å in long axis and 40–60 Å in short one. In contrast, similar less electron dense particles were visible in the blank sample (S in Fig. 6).

The densitometric traces, which run perpendicular to the stack across the grana membranes in side view, revealed multi-peak waves. In typical cases, these waves were tri-peak waves, showing a median high peak interposed between two low side peaks or shoulders (a–h in Fig. 7 and b–j in Fig. 9). Besides these tri-peak waves, multi-peak waves, each equipped with a median high peak, and an inner medium and an outer low side peak or shoulder in either limb, were met with (i in Fig. 7, a in Fig. 9). The comparison of the traces, shown in Figs. 7 and 9, revealed that the mean optical density of the median high peaks was 0.711 ± 0.121 in the treated sample, and 0.418 ± 0.068 in the blank one; that of the side peaks or shoulders, 0.403 ± 0.120 and 0.234 ± 0.116 ; and that of the valleys, 0.181 ± 0.120 and 0.016 ± 0.010 , respectively. Besides these changes in optical density of the waves, there occurred some change in width of the waves as well. The average width of the waves was found to be 258.3 ± 121.8 Å in the treated sample and 116.0 ± 15.9 Å in the blank one.

DISCUSSION

The photosystem responsible for DAB photooxidation by isolated chloroplasts: DC has been known to be an effective electron donor to the PS-II (27). The addition of DC to the DAB medium brought about a slight suppression of the DAB photooxidation. The addition of one of the PS-II inhibitors (26) to the medium did not cause any inhibitory effect on the photooxidation at all, but showed a slight stimulatory one. Supplementing the PS-II activity to the medium by adding DPIP and ASC in the presence of DCMU or DCPA (13) resulted in a marked inhibition of the photooxidation. All these facts point to the conclusion that a high level of the PS-II activity is detrimental to the DAB photooxidation by isolated chloroplasts (Table 1). There seems to exist some competition with respect to the electron donation to the PS-I between DAB and the endogenous electron donor produced by the preceding photosystem or the exogenous donor added (19). MV has been known as an electron acceptor of the PS-I (9), and the addition of MV to the medium brought about a remarkable enhancement of the photooxidation. But in spite of this high level of reaction, the addition of DC and one of the PS-II inhibitors to the medium containing MV caused effects quite parallel with those mentioned above (Table 2). These facts lead us to the conclusion that the PS-II does not play a role in the photooxidation, but the PS-I is the photosystem concerned.

The nature of the DAB photooxidation by chloroplasts has been studied by some investigators. According to Nir and Seligman (19), the photooxidation by spinach chloroplasts is not affected by the presence of DCMU. Marty (16) has

obtained similar results with *Coleus* chloroplasts. Contrary to these, Lauritus *et al.* (15) have found that the photooxidation of DAB by photosynthetic lamellae in *Microcystis*, *Anacystis* and *Nostoc* is sensitive to DCMU. Though there seems to exist some discrepancy among these results, clean-cut evidence has been obtained from the study of some mutant strains of *Chlamydomonas* defective in the PS-I or II activity by Chua (6), who has found that the former strain fails to conduct the DAB photooxidation, while the latter does not. This means that the photosystem responsible for the photooxidation is the PS-I. Our present results add some additional quantitative evidence to support this view.

Possible role of endogenous active oxygen on DAB photooxidation by isolated chloroplasts: According to Asada (5), endogenous active oxygen is produced by chloroplasts under illumination. He also has pointed out a possibility that when the chloroplasts treated with MV are irradiated, MV is reduced to a radical by the action of the PS-I, and the radical produced, in turn, reduces oxygen to superoxide radical (5). In view of the facts that hydrogen peroxide enhances the DAB photooxidation by photosynthetic lamellae in *Microcystis* and others (15), and that DAB is oxidized by peroxidase in the presence of hydrogen peroxide (10), it seems necessary to check the possible role of endogenous active oxygen on the DAB photooxidation by isolated chloroplasts. Attempts to scavenge endogenous active oxygen, if any, by the addition of CATL and SOD, solitary or combined, were carried out. As shown in Table 3, the photooxidation of DAB was only slightly suppressed by the addition of these enzymes to the reaction medium. The results obtained lead us to the conclusion that endogenous active oxygen does not play a major role in the DAB photooxidation here. This view is supported by the fact that isolated spinach chloroplasts scavenge hydrogen peroxide rapidly under illumination (18).

Localization of PS-I in the thylakoid membrane: The densitometric analysis of the electron micrograph transparencies obtained proved effective not only in elucidating the reaction intensity, but also in determining the precise localization of the positive reaction sites. The densitometric analysis was carried out only on the grana membranes, though both the grana and the stroma membranes were positive in the reaction.

As a first approach, the topographic correspondence between the densitometric trace and the structural architecture was checked in the blank sample. The trace across the side view of the grana membranes showed a series of multi-peak waves, mainly tri-peak ones (waves a-h in Fig. 7) when the scanning was made perpendicular to the membrane length. As the interstitial grana membrane consists of two layers of the thylakoid membrane lying side by side, and holding the partition layer in between them, the median high peak of each tri-peak wave is no other than the partition layer. The side peaks, interposing the median one, denote the thylakoid membranes themselves. Besides these typical tri-peak waves, a multi-peak wave was also observed (wave i in Fig. 7), showing that there was some complexity in structure in the membrane. The densitometric trace across the surface view of the grana membrane (Fig. 3) exhibited a series of low mono- and multi-peak waves in its central part. These waves correspond to some particulate structure in the grana membrane. Besides these waves, there were high multi-peak waves on both sides of the trace, which showed the edges of the grana membrane.

The partition layer is one of the positive reaction sites, as it revealed in side view an enhanced electron density in the DAB treated sample (Fig. 9), though it also

exhibited some degree of density in the blank one (Fig. 7) under the present experimental conditions (cf. 20). The grana membrane proper is also a positive site, based on the following two facts. First, the trace across the central area of the grana membrane in surface view revealed multi-peak waves of high electron density in the treated sample (Fig. 5), while only mono- and multi-peak waves of low density in the blank one (Fig. 3). The former waves correspond to the particulate structures of the thylakoid membrane, which are loaded with the DAB oxidation product. Secondly, the mean electron density of the thylakoid membranes in side view was enhanced in the treated sample as compared with that in the blank one (cf. Figs. 7 and 9). The positive site of the grana membrane extends to the thylakoid locus, and protruded into it. This was proved by the facts that: (1), the trace across the grana membranes in side view showed clearly that the membranes became thicker in width in the treated sample than in the blank one, and, at the same time, fused tri-peak waves revealed themselves in the former sample; and (2), coinciding with the protrusions seen in the side view, the trace across the central part of the grana membrane in surface view showed that the mean width of the waves was larger in the treated sample than in the blank one. Besides these positive sites located in the central part of the grana membrane, another positive site was found on the edge of the grana stack (E_1 and E_2 in Figs. 3 and 5).

The localization of the PS-I activity in the chloroplasts has been studied topographically by means of DAB photooxidation by some investigators. Nir and Seligman (19) have found that in the chloroplasts of *Elodea* and *Spinacia* the oxidation product is formed on the thylakoid membrane. Nir and Pease (20) have also reported that in *Spinacia* chloroplasts the deposits are located on the thylakoid membrane, and with longer incubation, are accumulated in the locus. Marty (16) has observed in *Coleus* chloroplasts that the DAB photooxidation product is localized within the grana and the stroma membranes. The appearance of positive DAB reaction in primary thylakoids has been studied during the process of greening in *Phaseolus* (28). Porat *et al.* (22) have done research on the localization of DAB photooxidation deposits by the freeze fracture technique, and have found that they are located on ES of the thylakoid membrane. They are of the opinion that this surface is the electron donor side of the PS-I. The results obtained by these authors do not always completely coincide with one another.

Recently, the freeze fracture technique has been introduced to the study of the particulate structure of the thylakoid membrane (1, 2, 4, 17, 23, 24). The small particles, presumably the PS-I complexes, are embedded in the thylakoid membrane in a manner that they are localized more externally to protrude partially into the partition side, that is, the outer surface side (1, 2, 4, 17), or to have a wider frontage on this side (23, 24). The topochemical results we obtained are in accord with these views of the spatial localization of the PS-I complex particles in the thylakoid membrane.

In view of the fact that an "overstaining" with DAB oxidation product does occur in *Oryza* mitochondria (21), it would be reasonable to assume the resolution of our topochemical study to be at the level of the particulate architecture of the thylakoid membrane, and not at the level of the molecular one. If so, we may draw a tentative conclusion that the PS-I activity is localized in the partition layer, the PS-I complex particles in the thylakoid membrane, and extends to the locus. In

contrast, the PS-II activity is located in the thylakoid membrane proper, and extends to the inner surface of the locus (12). These findings certainly contribute something to the better understanding of the functional polarity of the thylakoid membrane (8, 14, 25).

ACKNOWLEDGEMENTS

The present authors would like to express their sincere appreciation to late Dr. Hideo Takamatsu, Professor Emeritus of Kyoto University, for his encouragement and financial support during this investigation. Thanks are also due to Miss. Toshiko Hiraoka, a member of Analytical Application Laboratory, Shimadzu Corp., Kyoto, for her assistance in densitometry.

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