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STUDIES ON ELECTRON TRANSPORT ASSOCIATED WITH PHOTOSYSTEM I

II. ROLE OF PLASTOCYANIN IN METHYL VIOLOGEN PHOTOREDUCTION IN FRENCH PRESS-TREATED CHLOROPLASTS

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SUMMARY

1. Disruption of chloroplast structure by passing spinach chloroplasts through a French press resulted in a marked decrease in the activities of methyl viologen photoreduction with water, ascorbate or reduced 2,6-dichlorophenolindophenol (DCIP) as electron donor. Addition of plastocyanin to the treated chloroplasts markedly restored the activity of methyl viologen photoreduction. The plastocyanin–ascorbate couple could donate electrons to Photosystem I more efficiently than DCIP–ascorbate in the treated chloroplasts.

2. French press treatment of chloroplasts gave rise to a solubilization of most of plastocyanin from the chloroplast membrane.

3. $HgCl_2$ at a concentration similar to that of chlorophyll strongly inhibited the activity retained in the French press-treated chloroplasts or in the light and heavy particles fractionated from the treated chloroplasts.

4. Reduced plastocyanin could support a high rate of methyl viologen photoreduction in Photosystem I particles prepared by detergent or mechanical treatment of chloroplasts. Reduced DCIP or ascorbate was much less effective than reduced plastocyanin in supporting the photoreduction.

INTRODUCTION

In the last few years evidence has been presented from several laboratories which indicates the presence of Photosystem I in two different parts of the thylakoid membrane system, *i.e.* stroma lamellae and grana stacks of chloroplasts in higher plants[1–3]. Jacobi and Lehmann[1] and Sane *et al.*[2] showed that small chloroplast fragments which were enriched in Photosystem I could be released from stroma lamellae by mechanical disruption of chloroplasts. As far as the photochemical activities such as NADP photoreduction and cytochrome *f* photooxidation are concerned, the reported results are unequivocal in that the Photosystem I is well preserved in the membrane fragments prepared by French press treatment of chloroplasts[2–7]. There are, however,

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

marked discrepancies among the published views concerning the role of plastocyanin in the activity of Photosystem I in the preparation because of the differences in the plastocyanin contents in the chloroplast fragments used as well as the effects of added plastocyanin on the activities of the preparations. Arnon *et al.*[4] described that their preparation of Photosystem I particles was free of plastocyanin. Murata and Fork[7] could not detect any significant amount of plastocyanin in the stroma lamellae and the grana stacks which had been prepared by French press treatment. On the contrary, Baszynski *et al.*[6] and Arntzen *et al.*[3] found that chloroplast fragments prepared by a similar technique contained substantial amounts of plastocyanin. One of the major causes for this discrepancy might be due to the technical difficulty in determining plastocyanin in the extract of the chloroplast fragments, especially of the Photosystem I particles which could be isolated from chloroplasts with only a poor yield[2].

It was indicated in the preceding paper[8] that $HgCl_2$ could be used as a specific inhibitor of plastocyanin *in situ* and that in intact chloroplasts $HgCl_2$ blocked the electron transfer from cytochrome *f* to P700, thus indicating that plastocyanin functions between cytochrome *f* and the reaction centre of Photosystem I. In view of the conflicting results described above, the present work was designed to determine the role of plastocyanin in Photosystem I in the different parts of thylakoid membrane, by testing the effects of $HgCl_2$ on photochemical activities in the chloroplast fragments prepared by the French press technique. It will be shown that plastocyanin is an essential electron carrier in Photosystem I located in both stroma and grana lamellae. It will be also indicated that the effectiveness of externally added electron donors including plastocyanin on the activities of Photosystem I in chloroplasts depends upon the structure of thylakoid membrane.

MATERIALS AND METHODS

Chloroplasts were prepared from fresh leaves of commercial spinach as described previously[9] and finally suspended in 0.15 M NaCl containing 50 mM phosphate (pH 7.8). Chloroplasts were disrupted by passing the suspension through a French press (Ohtake Co.,) at a flow rate of about 20 ml/min and at the indicated pressure. Chloroplast fragments enriched in Photosystem I were prepared by digitonin treatment of chloroplasts as described by Ohki and Takamiya[10] or sonic treatment according to the method described by Jacobi and Lehmann[1] with slight modifications. Where indicated, chloroplasts or chloroplast fragments were incubated with $HgCl_2$ at 0 °C for 30 min at a molar ratio of $HgCl_2$ to chlorophyll of about unity.

Chlorophyll was determined by the method of Arnon[11]. Isolation, purification and determination of plastocyanin were carried out as described previously[12].

Activity of methyl viologen photoreduction was determined by following the absorption of oxygen with a Clark-type oxygen electrode as described previously[13]. The basal reaction mixture for the Hill reaction contained, in a final volume of 2.0 ml, in mM: phosphate of indicated pH, 50; NaCl, 10; sodium azide, 0.5; methyl viologen, 0.1; methylamine·HCl, 14; ascorbate, 0.5; and chloroplasts or chloroplast fragments. For the photoreduction mediated by Photosystem I, 50 μM 2,6-dichlorophenolindophenol (DCIP) and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were added. Hill activity with DCIP or ferricyanide as electron acceptor was determined spectrophotometrically as described previously[13].

RESULTS

Disruption of chloroplast structure by passage through the French press caused a marked change in the activity of methyl viologen photoreduction with various electron donors as shown in Table I. In the absence of any addition, *i.e.* with water as electron donor, the rate of methyl viologen photoreduction was decreased markedly by the treatment. Addition of plastocyanin had no or only a slight effect on the activity in the untreated chloroplasts, but gave rise to a significant restoration of the activity in the treated chloroplasts. Ascorbate induced an enhancement in rate of methyl viologen photoreduction in the treated and untreated chloroplasts. Since DCMU almost completely eliminated the photoreduction in the presence of ascorbate, the stimulating effect of ascorbate observed may be ascribed to its capacity for serving as electron donor of Photosystem II[14,15]. This ascorbate-supported and DCMU-sensitive photoreduction in the French press-treated chloroplasts indicates that the treatment induces an inhibition of electron transport somewhere between water and Photosystem II. An observation that is pertinent to this was that the activity of the DCIP Hill reaction in the treated chloroplasts was significantly accelerated by diphenylcarbazide, a specific electron donor for Photosystem II[16].

Addition of plastocyanin in combination with ascorbate induced a more marked stimulation of methyl viologen photoreduction with chloroplasts treated by French press than ascorbate or plastocyanin alone. The exact extent of restoration of the entire electron transport system including Photosystems I and II by the couple could not, however, be determined, since the photoreduction supported by the plastocyanin–ascorbate couple became largely insensitive towards DCMU after the

TABLE I

EFFECT OF FRENCH PRESS TREATMENT ON METHYL VIOLOGEN PHOTOREDUCTION IN THE PRESENCE OF VARIOUS ELECTRON DONORS

Oxygen uptake was determined with a Clark-type oxygen electrode at 27 °C and under illumination with red light (600–850 nm) of $1 \cdot 10^5$ ergs/cm^2 per s. The basal reaction mixture contained, in a final volume of 2 ml, in mM: phosphate, 50; sodium azide, 0.5; methylamine·HCl, 14; methyl viologen, 0.1; and chloroplasts or chloroplast homogenate containing 26 μg chlorophyll. The activity was determined at the respective optimum pH: all the reactions in untreated chloroplasts at pH 7.8, the Hill reaction with or without addition of ascorbate in treated chloroplasts at pH 6.2, and the photoreduction with reduced plastocyanin or DCIP as electron donor at pH 6.8, respectively. Where indicated, 11 μM spinach plastocyanin, 0.5 mM ascorbate, 50 μM DCIP and 10 μM DCMU were added.

Additions	*Rate of O_2 uptake (μmoles O_2 absorbed/mg chlorophyll per h) by chloroplasts*		
	Untreated	*Treated at 150 kg/cm^2*	*Treated at 600 kg/cm^2*
—	290	33	9
Plastocyanin	284	92	94
Ascorbate	380	118	71
Ascorbate, DCMU	10	10	15
Ascorbate, plastocyanin	410	376	620
Ascorbate, plastocyanin, DCMU	70	226	500
Ascorbate, DCIP, DCMU	380	226	123
Ascorbate, plastocyanin, DCIP, DCMU	586	450	686

treatment. Obviously, reduced plastocyanin could feed electrons directly to Photosystem I in the treated chloroplasts. It is noted that the DCIP–ascorbate couple was much more effective than the plastocyanin–ascorbate couple in restoring the activity in the untreated chloroplasts, but became less effective when the chloroplast structure had been destroyed. A similar reversible inactivation of electron transport reactions including Photosystems I and II or Photosystem I alone, and the inverse effectiveness of plastocyanin and the dye as electron donor for Photosystem I in the treated and untreated chloroplasts have been previously observed in the sonic treatment of chloroplast[17-21].

A similarity in the effects of the two mechanical treatments of chloroplasts was noted also in other respects. As was the case with sonic treatment[19-21], French press treatment resulted in a decrease in activity of the Hill reaction with DCIP or ferricyanide as electron acceptor, but much less in extent than that of methyl viologen photoreduction. This is interpreted to indicate that the French press treatment caused, besides a partial inactivation of Photosystem II, an extensive inhibition of the electron transport chain connecting the two photosystems.

A shift in optimum pH for these activities to acidic pH was also observed in the treated chloroplasts[19-21]. Murata and Brown[5] reported that the pH profile of ferricyanide Hill reaction showed two peaks at pH 6 and 8 in the chloroplast fragments prepared by French press treatment. We confirmed their observation in that the activity of ferricyanide Hill reaction in the French press-treated chloroplasts did not drop at a pH region higher than 7, provided the reduction of ferricyanide was determined by measuring absorbance decrease at 420 nm. When the activity was determined polarographically by following oxygen evolution, however, there was only one peak at pH 6. It was further found that the apparent high activity at the alkaline pH observed in the spectrophotometric determination was due to a photobleaching of carotenoids which became significant in the treated chloroplasts in the presence of ferricyanide[22,23]. This is another indication that the treatment affected the electron transport system between water and Photosystem II.

Sonic treatment of chloroplasts was shown previously to give rise to a solubilization of plastocyanin[20]. Table II shows that French press treatment of chloroplasts at 150 kg/cm^2 resulted in a release of plastocyanin corresponding to a ratio of chlorophyll *a* to the protein of about 800. At higher pressures of the treatment, more plastocyanin was released to give a ratio of about 700. Since spinach chloroplasts contain the protein at a ratio of 600 (ref. 24), it is inferred that most of, but not all of, plastocyanin was released from chloroplasts. This finding, together with the above observed restoration of the activity by plastocyanin, strongly indicates that the inactivation of methyl viologen photoreduction induced by French press treatment is due to a release of plastocyanin from the chloroplast membrane.

A question arises, in this context, whether the activities persisting in the treated chloroplasts depends upon plastocyanin remaining in the membrane-bound state. It was clearly demonstrated in the preceding paper[8] that $HgCl_2$ could be used as a specific inhibitor of plastocyanin in the chloroplasts. The effect of $HgCl_2$ on the activity of methyl viologen photoreduction in chloroplasts treated in the French press was, therefore, studied. The chloroplasts or chloroplast fragments were incubated for 30 min with $HgCl_2$ at a molar ratio of $HgCl_2$ to chlorophyll of unity. This treatment has been shown to completely inactivate plastocyanin with only a slight effect on the

TABLE II

AMOUNTS OF PLASTOCYANIN SOLUBILIZED BY FRENCH PRESS TREATMENT OF CHLOROPLASTS

Chloroplasts were passed through a French press at indicated pressure and the homogenate was centrifuged at 130000 × *g* for 60 min. The amount of plastocyanin in the supernatant was determined spectrophotometrically.

Exp No.	*Pressure of treatment (kg/cm²)*	*Chlorophyll content (μmoles)*	*Plastocyanin solubilized (μmoles)*	*Chlorophyll / Plastocyanin*
1	150	87	0.102	852
	500	87	0.120	725
2	150	50	0.062	810
	500	50	0.077	650
	800	50	0.072	690

TABLE III

EFFECT OF $HgCl_2$ ON METHYL VIOLOGEN PHOTOREDUCTION IN FRENCH PRESS-TREATED CHLOROPLASTS

Experimental conditions were as described in Table I. Chloroplasts or chloroplast homogenate containing 13 μg chlorophyll were added.

Pressure of treatment (kg/cm²)	*Addition*	*Rate of O_2 uptake (μmoles O_2/mg chlorophyll per h)*			
		Electron donor: H_2O		*DCIP–ascorbate*	
		Untreated	*Treated with $HgCl_2$*	*Untreated*	*Treated with $HgCl_2$*
0	—	56	0	126	6
150	—	29	0	107	11
400	—	13	0	82	9
800	—	4	0	45	9
0	Plastocyanin	56	7	150	29
400	Plastocyanin	39	19	188	129

photooxidation of P700 (ref. 8). Table III shows that the activity of the methyl viologen Hill reaction was completely abolished by incubation of chloroplasts with $HgCl_2$. The photoreduction supported by the DCIP–ascorbate couple was also severely, but not completely, inhibited by $HgCl_2$. Addition of plastocyanin slightly reversed the $HgCl_2$-induced inhibition of the Hill reaction and the donor-supported photoreduction in the intact chloroplasts. A more marked effect of plastocyanin in reversing the inhibition was observed in the treated chloroplasts. Amounts of plastocyanin which had been solubilized by the treatment and brought into the reaction mixture with chloroplast fragments were negligibly small. It is inferred from these observations that plastocyanin associated with the membrane structure is responsible for the activity remaining in the chloroplast homogenate. It is also apparent that the effectiveness of plastocyanin in reversing the $HgCl_2$-induced inhibition is highly dependent on the structure of chloroplast membrane used.

Effect of $HgCl_2$ was further studied on the photochemical activity of the chloroplast fragments prepared by French press treatment followed by differential centrifugation as described by Sane *et al.*[2]. Table IV shows that chloroplast fragments precipitated by centrifugation at 130000 × *g* had a higher chlorophyll *a* to *b* ratio than that of the original chloroplasts, indicating an enrichment of Photosystem I in this fraction. Correspondingly, in this fraction the Hill activity was negligible whereas the activity of methyl viologen photoreduction with reduced DCIP was highest among the fractions tested.

$HgCl_2$ strongly inhibited the residual activity of the donor supported photoreduction with the heavier fractions, thereby indicating that the activity of these fragments is dependent on plastocyanin retained in the membrane structure. The light particles precipitated at 130000 × *g* were relatively insensitive towards $HgCl_2$. This may be a reflection of a situation that in this fraction plastocyanin was mostly absent and the reaction center of Photosystem I was largely exposed to the added

TABLE IV

EFFECT OF $HgCl_2$ ON METHYL VIOLOGEN PHOTOREDUCTION IN CHLOROPLAST FRAGMENTS PREPARED BY FRENCH PRESS TREATMENT

Chloroplasts were treated with French press at 400 kg/cm² and chloroplast fragments were precipitated by differential centrifugation. The activity was determined at pH 7.2 with 500 μM ascorbate and 50 μM DCIP as electron donors in the presence of 10 μM DCMU. Other experimental conditions were as described in Table I.

Preparations	*Chlorophyll a / Chlorophyll b*	*Rate of O_2 uptake (μmoles O_2/mg chlorophyll per h)*	
		Untreated	*Treated with $HgCl_2$*
Original homogenate	3.1	22	4
Fragments precipitated			
at 6000 × *g*	3.1	20	4
at 20000 × *g*	3.5	23	4
at 50000 × *g*	4.8	49	15
at 130000 × *g*	6.0	59	25

TABLE V

EFFECTS OF VARIOUS ELECTRON DONORS ON METHYL VIOLOGEN PHOTOREDUCTION IN PHOTOSYSTEM I PARTICLES

Experimental conditions were as described in Table I. Photosystem I particles containing 18–32 μg chlorophyll were added.

Electron donors	*Rate of O_2 uptake (μmoles O_2/mg chlorophyll per h)*		
	Photosystem I particles prepared by:		
	French press	*Sonication*	*Digitonin*
Ascorbate	11	26	13
Ascorbate, DCIP	86	67	119
Ascorbate, plastocyanin	370	138	500
Ascorbate, DCIP, plastocyanin	413	176	630

electron donors. However, in this fraction the extent of the inhibition exceeded 50 % of the original activity. This is significantly higher than the extent of inhibition of P700 or cytochrome *c* photooxidation which was determined to be only 10 to 20 % at the same ratio of $HgCl_2$ to chlorophyll (see Fig. 7, ref. 8). It appears, therefore, that a small amount of plastocyanin remaining in the membrane-bound state is still contributing to the activity of this fraction.

An observation that is in accord with the above assumption is shown in Table V. Addition of plastocyanin *plus* ascorbate induced a remarkable stimulation of methyl viologen photoreduction in the Photosystem I particles; the level of the activity thus attained was several folds higher than that observed on addition of the DCIP–ascorbate couple. Similar high efficiency of plastocyanin in supporting the methyl viologen photoreductions was observed in the Photosystem I particles prepared by sonic and detergent treatment of chloroplasts, in which, because of the drastic procedures used for the disruption of chloroplasts, plastocyanin must have been mostly solubilized and the reaction centre of Photosystem I exposed to the external electron donors.

DISCUSSION

The results obtained in the present work indicate that plastocyanin is an essential component for methyl viologen photoreduction with either water, ascorbate or reduced DCIP as electron donor. Treatment of chloroplasts with a French press caused a release of most of plastocyanin from the membrane structure which was accompanied by a marked loss of the activity. Addition of plastocyanin was effective in restoring the activity in the treated chloroplasts. These observations indicate that the drop in the activity was caused by the solubilization of plastocyanin from the chloroplast membrane.

In addition, the residual activity in the treated chloroplasts was found to be highly sensitive toward $HgCl_2$. Results presented in the preceding paper[8] showed that $HgCl_2$ under the experimental conditions employed here inactivates plastocyanin but not the photooxidation of P700. It is, therefore, plausible to infer that plastocyanin in a state bound to the membrane structure is essential for the methyl viologen photoreduction surviving in the French press-treated chloroplasts. This can be held to be the case with all the Photosystem I irrespective of its localization either on the stroma lamellae or the grana stacks, since $HgCl_2$ was inhibitory towards the activity in the light and the heavy particles prepared from French press-treated chloroplasts. Baszynski *et al.*[6] and Arntzen *et al.*[3] have arrived at a similar conclusion from a different line of evidence.

There is contradiction among the published reports concerning the effects of externally added plastocyanin on the activity of Photosystem I particles prepared by French press treatment. Murata and Brown[5] and Arnon *et al.*[4] showed that plastocyanin was only slightly stimulative on the NADP photoreduction whereas Arntzen *et al.*[3] found that the protein was required to sustain a high rate of the photoreduction in the particles. Plastocyanin was also found to induce a marked stimulation in methyl viologen photoreduction in our Photosystem I particles.

The results obtained in the present work indicate that the effectiveness of added electron donors in the Photosystem I reactions of chloroplasts or chloroplast

fragments depends highly on the structure of the thylakoid membrane. Ascorbate, and ascorbate *plus* plastocyanin were found to be inefficient as electron donor for Photosystem I in intact chloroplasts. On the other hand, the ascorbate–plastocyanin couple could support a high rate of methyl viologen photoreduction when chloroplast membrane was disrupted by mechanical or detergent treatments. It appears, therefore, that in intact chloroplasts plastocyanin and P700 are located inside the thylakoid membrane so that they are not accessible to ascorbate or plastocyanin in the reaction medium. Similar localization of plastocyanin in the chloroplast membrane has been suggested by Hauska *et al.*[25] from the results of experiments with the antiserum prepared against plastocyanin. The role of DCIP in promoting the photoreduction of methyl viologen in intact chloroplasts can be explained by assuming that the dye can readily penetrate into the membrane, thereby acting as electron carrier from ascorbate to the bound plastocyanin in the membrane. In $HgCl_2$-treated chloroplasts, reduced DCIP could not sustain a high rate of methyl viologen photoreduction since P700 was blocked by the presence of inactivated plastocyanin in the membrane-bound state.

When the structure of the thylakoid membrane is disrupted, plastocyanin leaks out and P700 is exposed to the electron donors in the external medium. Efficiencies of the electron donors added are now mainly determined by the specificity of P700 for the donor. Results shown in Table V indicate that plastocyanin is the highest in reactivity with P700 among the donors tested, followed by DCIP with much diminished level; ascorbate is only a poor electron donor for P700. In this respect, we could not distinguish Photosystem I particles prepared by mechanical disintegration of chloroplasts from those prepared with digitonin. Ohki and Takamiya[10] showed that by their digitonin procedure 40 % of chlorophyll and 86 % of P700 were recovered in the Photosystem I fraction. Arntzen *et al.*[3] estimated that about 15 % of chlorophyll in chloroplasts occurred in the stroma lamellae whereas 40 % of chlorophyll in grana belonged to Photosystem I. By assuming that a ratio of P700 to chlorophyll is constant in Photosystem I in stroma and grana lamellae, it is calculated that, at least, more than two-thirds of P700 in the Photosystem I fraction prepared with digitonin were derived from grana lamellae. It appears, therefore, that there is no difference in the donor specificity of P700 between Photosystem I in stroma and grana lamellae (Table V).

It can be inferred from the foregoing discussion that difference in effectiveness of added plastocyanin in promoting the activities of Photosystem I is a reflection of difference in extent of disintegration of the thylakoid membrane and of solubilization of plastocyanin in the preparations employed.

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