ANTIBODIES AGAINST AN INTERMEDIATE ON THE WATER SIDE OF PHOTOSYSTEM II OF PHOTOSYNTHESIS

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1. Introduction

The chemical intermediates of the dark reactions of Photosystem II (PS II)* associated with the evolution of oxygen from water are unknown. Work with chemical inhibition [1, 2], UV light inactivation [3], and heating effects [4] seem to indicate that at least one chemical component, probably proteinaceous in nature, is involved, the isolation and characterization of which have not been possible due to its extreme lability. Honeycutt and Krogman [5] have shown an additional site of inhibition by phenylmercuric acetate, located between the Tris and 3-(3,4-dichlorophenyl)-1, 1 dimethylurea (DCMU) blocks. We have found that antibodies made against PS II particles specifically inhibit oxygen evolution at a site between $MnCl_2$ donation [6] and diphenylcarbazide (DPC) donation [7], that is probably against the same component inactivated (or extracted) by high concentrations of Tris buffer. The fact that this component is antigenic suggests that it is most likely either a protein of significant molecular weight or is complexed to a carrier molecule.

The immunological technique has been well exploited as far as Photosystem I (PS I) intermediates are concerned. Antibodies have been directed against ferredoxin [8], ferredoxin-NADP reductase [9], ferredoxin reducing substance (FRS) [10], P700 [11] and the coupling factor [12]; the accessibility of these antibodies to the photosynthetic components in the chloroplast has been taken as evidence for their exterior localization on the membrane. Conversely, antibodies against plastocyanin [13] and cytochrome *f* [11] do not inhibit photosynthetic electron transport, indicating that these two intermediates are buried in the membrane. Recently, Radunz et al. [14] have shown that antibodies made against chlorophyll (Chl) partially inhibit PS II reactions, presumably specific for the PS II reaction center chlorophyll. The low level of inhibition (maximum of 22%) which they obtained, is most easily understood as a limited accessibility of the reaction center for the antibody in their chloroplast preparation. This is in agreement with the data of Briantais [15], Briantais and Picaud [16] (who also have antibodies agaisnt system II but theirs is a structural study of the localization of the two photosystems utilizing the immunological approach) and Arntzen et al. [17] which support an asymmetric distribution of the photosystems with Photosystem I (PS I) localized closest to the outside of the membrane. To date, Radunz et al.'s work is the only immunochemical work on PS II intermediates.

2. Methods

Photosystem II particles were isolated from market spinach according to Huzisige et al. [18]. The Chl *a:b* ratio of the preparations varied from 1.9 to 2.4.

* Abbreviations: PS II: photosystem II; DCMU: 3-(3,4-dichlorophenyl)-1,1 dimethylurea); DPC: diphenylcarbazide; FRS: ferredoxin reducing substance; Chl: chlorophyll; PS I: photosystem I; DCPIP: 2,6-dichlorophenol-indophenol; $NADP^+$: nicotinamide adenine dinucleotide phosphate; PPNR: photosynthetic pyridine nucleotide reductase; $DCPIPH_2$: DCPIP-Ascorbate (1:300).

Table 1
Inhibition of Hill reaction by antibodies made against Photosystem II particles.

Additions		μmoles $DCPIPH_2$ (A) (or NADPH (B))/mg Chl/hr	% Inhibition of control serum
(A)	None	290 ± 5	
	0.04 ml Control serum	241 ± 5	
	0.04 ml Antiserum	205 ± 4	14.9 ± 2
(B)	None	48.2 ± 0.8	
	0.16 ml Control serum	44.2 ± 0.9	
	0.16 ml Antiserum	38.8 ± 0.8	12.2 ± 2
	DCMU, $DCPIPH_2$	15.7 ± 0.1	
	DCMU, $DCPIPH_2$ plus 0.16 ml control serum	14.4 ± 0.1	0.0 ± 0
	DCMU, $DCPIPH_2$ plus 0.16 ml antiserum	14.4 ± 0.0	

(A) Three ml sample, containing 15 μg Chl in 0.05 M Tris-HCl buffer, pH 6.8, 0.01 M NaCl, and 4.5×10^{-5} M DCPIP, was illuminated for 45 sec in saturating white light filtered with 4½ inch water filter and 1 inch clear plastic. Change in absorbance of DCPIP was measured at 603 nm. These values represent an average of 20 measurements.

(B) Three ml sample containing 60 μg Chl in 0.05 M phosphate buffer, pH 7.2, 70 μmoles NaCl, 10 μmoles $MgCl_2$, 1 μmole $NADP^+$ and excess amounts of PPNR; where indicated, 0.03 μmoles DCMU, 0.02 μmoles DCPIP, and 6.0 μmoles Na ascorbate were added. $NADP^+$ photoreduction was measured at 340 nm after 2 min illumination. These values represent an average of 5 measurements.

The particles were stored at −15° in 0.05 M Tris-HCl buffer, pH 7.2, containing 0.4 M sucrose, and were injected into New Zealand white rabbits within three days after preparation. The immunization was started by injecting the particles containing 2 mg Chl (∼ 10 mg protein) emulsified with complete Freund's adjuvant into the hind food pads and intramuscularly into the back. After five weeks, the rabbit was boostered in the back muscles with the particles (1.5 mg Chl) in incomplete Freund's adjuvant; this was repeated at two month intervals for three more injections. Bleedings were taken from the marginal ear vein weekly after the third injection, as well as intermittently earlier in immunization; sera were stored at −15°. Control sera were collected prior to the first injection as well as weekly from two non-immunized rabbits to compare with immune sera of equal age.

Chloroplasts were prepared by homogenizing depetiolated spinach leaves in 0.05 M Tris-HCl buffer, pH 7.2, containing 0.4 M sucrose, 10 mM NaCl, and 1 mM $MgCl_2$. The homogenate was filtered through 8 layers of cheesecloth, centrifuged at 200 *g* for 2 min, and then at 1000 *g* for 10 min. The pellet was suspended in 0.05 M Tris-HCl buffer and centrifuged at 1000 *g* for 10 min; this final pellet was resuspended in a small amount of the homogenizing medium and frozen with dry-ice and acetone. Chl concentrations were determined by using MacKinney's equations [19].

Tris-washed chloroplasts were prepared according to Yamashita and Butler [1], with the time of incubation in the Tris extended to 30 min.

Forty microliters of (anti)serum were pre-incubated with broken chloroplasts ([Chl], 15 μg) to allow for the antibody–antigen binding.

Hill activity was assayed by measuring the photoreduction of 2,6-dichlorophenolindophenol (DCPIP) in saturating white light (see Mohanty et al. [20]). Nicotinamide adenine dinucleotide phosphate ($NADP^+$) photoreduction was monitored in a Cary 14 spectrophotometer equipped with actinic light source to allow simultaneous illumination and measurement; the chloroplasts were illuminated with saturating red light (Schott RG 645); the photomultiplier was protected from the exciting light by two Corning C.S. 7-60 filters.

Photosynthetic pyridine nucleotide reductase (PPNR) was prepared according to San Pietro and Lang [21]. Other details are given in the legends of the tables.

Table 2
Effect of antibodies on DCPIP photoreduction by Tris-washed chloroplasts.

Chloroplast treatment	Additions	μmoles $DCPIPH_2$/mg Chl/hr	% Inhibition of control serum
Normal	None	198.0	
Tris-washed	None	8.7	
Tris-washed	$MnCl_2$	10.5	
Tris-washed	DPC	87.5	
Tris-washed	DPC + 0.04 ml control serum	60.2 ± 1.9	
Tris-washed	DPC + 0.04 ml antiserum	59.3 ± 1.8	1.5 ± 3

Three ml of sample containing 18 μg Chl. Reaction mixture and measurement are as in table 1A; where indicated, 0.3 μmoles of $MnCl_2$ or 1.5 μmoles of DPC was added. These values are an average of 15 measurements.

Table 3
Inhibition of DCPIP photoreduction by antibodies in the presence and absence of $MnCl_2$.

Additions	μmoles $DCPIPH_2$/mg Chl/hr	% Inhibition of control serum
None	289 ± 5	
0.04 ml Control serum	251 ± 5	
0.04 ml Antiserum	217 ± 4	13.5 ± 2
0.04 ml Control serum + $MnCl_2$	226 ± 3	
0.04 ml Antiserum + $MnCl_2$	197 ± 3	12.8 ± 1.5

Three ml of sample containing 15 μg Chl. Reaction mixture and measurement are as indicated in table 1. Where indicated, 0.3 μmoles of $MnCl_2$ are added. These results represent an average of five measurements.

3. Results and discussion

Photosystem II particles rather than chloroplasts were used for injections for two reasons: 1) to considerably lessen the contaminating amounts of PS I antigenic components present, and 2) to concentrate the antigenic PS II intermediates. In all previous work in which chloroplasts had been used, no inhibition of PS II partial reactions could be observed. That we can see inhibition is probably due to the fact that antibodies are preferentially made against the PS II components and not against the perhaps more highly immunogenic and/or certainly more prevalent PS I intermediates.

Table 1 (A) shows that the addition of antiserum against PS II particles to chloroplasts results in a partial inhibition of Hill reaction as measured by DCPIP photoreduction. This value represents an average of twenty measurements, with the degree of inhibition varying somewhat with the activity of the chloroplasts before serum addition. There is a non-specific inhibition of chloroplast reactions with the control serum, but this is not a problem as the Hill reaction rates with antiserum addition are compared with those rates with control serum. The low levels of inhibition by the antibody can be explained by the probable limited accessibility of the antigenic site to the antibodies. (How the chloroplast can be manipulated to better expose the antigen is under investigation.)

Measurements of $NADP^+$ reduction with water as a donor indicate that the antiserum causes an inhibition comparable to that seen with DCPIP as the Hill oxidant, as seen in table 1 (B). However, the antiserum has no effect on $NADP^+$ photoreduction when DCMU and DCPIP-Ascorbate ($DCPIPH_2$) are added. This indicates that the site of antibody action is between water and the site of $DCPIPH_2$ donation, that is, before P700.

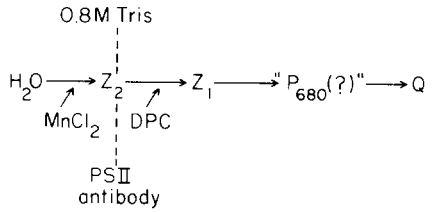

Fig. 1. Partial electron transfer chain of photosynthesis. Tris buffer, 0.8 M, pH 8.0, blocks oxygen evolution; electron flow can be restored by DPC but not by $MnCl_2$. The antibody against PD II particles also inhibits in the same manner; antibody-induced inhibition is observable with $MnCl_2$ or water donating but not with electron donation from DPC. Z_1 the primary electron donor of photosystem II; Z_2, the proteinaceous component necessary for oxygen evolution; P680 (?), hypothetical energy trap of pigment system II; and Q, the primary electron donor of PS II.

To further locate the site of action of the antibody, the chloroplasts were washed in 0.8 M Tris, pH 8.0, to inactivate oxygen evolution. Electrons were artificially fed into PS II through DPC, and DCPIP photoreduction was monitored in the presence of the antiserum and control serum. Table 2 shows that there is no inhibition with the antiserum, which signifies that the antibody must be acting on some component before the site of DPC donation; as DPC has been shown to donate only between the Tris block and reaction center II [7], either the antibody is working at the site of Tris inactivation (or extraction) or at another site between water and the Tris block.

To test which of these two conditions is more likely, we measured DCPIP photoreduction of chloroplasts pre-incubated with serum, in the presence and absence of $MnCl_2$. According to Ben-Hayyim and Avron [6], in normal chloroplasts Mn^{2+} competes with water as a donor to PS II, donating electrons at a site between the Tris block and water. Table 3 shows that the levels of inhibition of DCPIP photoreduction by the antiserum are approximately the same with or without $MnCl_2$. This experiment combined with other experiments done can be taken as evidence that the antibody made in response to PS II particles is probably inhibiting that component that is either inactivated (or extracted) by 0.8 M Tris buffer, pH 8.0. The site of antibody action and how it was determined is represented in the scheme in fig. 1. Preliminary experiments have been done with antibodies made against extracts from chloroplasts that appear to inhibit oxygen evolution in the same manner as those antibodies directed against PS II particles (paper in preparation).

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