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ANTISERA AGAINST A COMPONENT ON THE OXYGEN-EVOLVING SIDE OF SYSTEM II REACTION: ANTISERA PREPARED AGAINST AN EXTRACT FROM FROZEN AND THAWED CHLOROPLASTS

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SUMMARY

Extracts of repeatedly frozen and thawed chloroplasts elicit production of antibodies that act specifically on the oxygen-evolving side of system II. Inhibition of electron flow from water to 2,6-dichlorophenolindophenol (DCPIP) (mainly a system II reaction) by this antiserum is about 15% with respect to broken chloroplasts with control serum, but this inhibition increases to 30% when photosystem II (PS II) particles are used as test systems. The inhibition of electron flow from water to $NADP^+$ (reactions involving both photosystems I (PS I) and II), by the same antiserum, is also 15%; however, no inhibition is observed for electron flow from reduced DCPIP to $NADP^+$ in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (a system I reaction). Likewise, no inhibition is observed in electron flow from diphenylcarbazide (DPC) to DCPIP (mainly a system II reaction) in Tris-washed (0.8 *M*, pH 8.0) chloroplasts that have lost the ability to evolve oxygen. The implications of the above results are discussed in terms of effects on the oxygen-evolving side of PS II.

INTRODUCTION

Despite continued efforts on the part of many research groups in photosynthesis, the chemical nature of the intermediates involved with O_2 evolution have not been elucidated. Manganese [1], chloride [1], and bicarbonate [2] ions have been implicated in oxygen evolution. Various experimental data

Abbreviations: Chl, chlorophyll; DABS, diazonium benzene sulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; $DCPIPH_2$, 2,6-dichlorophenolindophenol ascorbate (1:300); DPC, diphenylcarbazide; FRS, ferredoxin reducing substance; PPNR, photosynthetic pyridine nucleotide reductase, PS I, photosystem I; PS II, photosystem II.

[3—6] suggest quite strongly that, at least, one proteinaceous component is involved in these reactions, but no successful attempt of isolating it has been reported.

Our approach to this problem has been an immunochemical one. We have previously reported [7] antisera obtained against PS II particles that specifically (but only partially, *i.e.*, 15%) inhibit oxygen evolution at a site between electron donation by $MnCl_2$ [8] and by DPC [9], that is, probably against the identical (or close to) intermediate "inactivated" by 0.8 *M* Tris buffer at pH 8.0.

In this communication, we report that an antiserum against a soluble, non-membranous fraction obtained from repeatedly frozen and thawed chloroplasts also specifically but partially inhibits oxygen evolution, presumably at the same site attacked by the antiserum prepared against PS II particles. Our data suggest two possibilities: (*1*) the immunologically active site(s) of the new antigen is closely related to the corresponding site(s) of the native component involved in O_2 evolution, or (*2*) the antigenic site is immunologically similar to portions of certain structural proteins in the thylakoid membrane and that alteration of these proteins by antibody binding results in an inhibition of a membrane-sensitive component involved with H_2O oxidation.

MATERIALS AND METHODS

The antigen was prepared as follows. First, chloroplast fragments were isolated from market spinach as described previously [7]. The final suspension of chloroplasts ($\sim$ 500 μg Chl/ml) was lightly sonicated (sonication at 0° on position 4 with sonifier cell disruptor, model W1850 with 1/2″ tip, Heat Systems-Ultrasonics, Inc., Plainview, N.J.) for 30 sec and then centrifuged at 5000 × *g* for 20 min. (In such preparations, stroma proteins are lost.) The pellet was resuspended in a small amount of 50 m*M* Tris—Cl buffer, pH 7.2, containing 10 m*M* NaCl and 1 m*M* $MgCl_2$ and frozen quickly with dry ice and acetone. These fragments were then thawed at room temperature in the dark, followed by placement of the chloroplasts in −15° temperature and subsequent slow refreezing. After the thawing and freezing cycle was repeated five times (for a comparable method, see Black [10]), the chloroplast fragments were then centrifuged at 10 000 × *g* for 45 min, the pellet discarded, and the supernatant stored at −15°. This supernatant contains the new antigen(s) and will be referred to as "soluble extract"; its protein concentration was estimated by Warburg and Christian's [11] method as well as by a modified Folin—Ciocalteu test [12], arbitrarily using bovine serum albumin as a standard. Absorption spectra of the extract were measured with a Cary-14 spectrophotometer. Chl concentration of chloroplast preparations was estimated by MacKinney's method [13].

Injection of New Zealand white rabbits with the soluble extract from chloroplasts and subsequent bleedings proceeded as described earlier [7]. Supernatant containing 10 mg protein was used in the first injection, while following injections were of 5—7 mg protein.

Chloroplasts used in the various assays, to check antiserum inhibitory activity, were prepared from market spinach as described earlier [7]. 20 μl of (anti)serum was preincubated at 0° in the dark for 15 min with these broken chloroplasts ([Chl], 8 μg) to allow for the antibody—antigen interaction. Tris-washed chloroplasts were prepared according to Yamashita and Butler [3] with the incubation time in the 0.8 *M* Tris, pH 8.0, extended to 30 min. PS II particles were isolated following the method of Briantais [14] with minor modifications. PPNR was prepared according to San Pietro and Lang [15].

Hill activity was measured with a Cary-14 Spectrophotometer equipped with a side attachment that provided actinic illumination at right angles to the measuring beam. For monitoring DCPIP reduction (at 597 nm), the sample was illuminated simultaneously with saturating red light (GE 120V 650W DYV tungsten lamp; Corning glass filter, C.S. 2-59 (3 mm, plus a "heat" filter)). The photomultiplier was protected by a 597 nm Farrand interference filter (half-bandwidth, 10 nm). The intensity of the incident actinic light, measured by a Yellow Springs Instrument Radiometer, was $1.5 \cdot 10^6$ erg cm^{-2} sec^{-1}. $NADP^+$ reduction was measured at 340 nm, the photomultiplier protected by two C.S. 7-60 filters, with the sample simultaneously illuminated with saturating red light (Schott glass filter, RG 645). The intensity of the incident light was 10^6 erg cm^{-2} sec^{-1}. Samples were illuminated for 2 min and initial rates were recorded. (Some early measurements of Hill activity were done according to Braun and Govindjee [7], in which only steady state rates were observed.) For calculation of the rates, extinction coefficients of $20 \cdot 10^{-3} M^{-1}$ cm^{-1} at 597 nm (at pH 6.8) for DCPIP and of $6.22 \cdot 10^{-3} M^{-1}$ cm^{-1} at 340 nm (at pH 7.2) for $NADP^+$ were used. Mn^{2+} donation experiments were done following the experimental protocol of Ben-Hayyim and Avron [8], measuring O_2 uptake by methyl viologen with a Yellow Springs Clark (concentration) electrode. Other details are given in the legends of the tables.

RESULTS AND DISCUSSION

Supportive of a bilayer model of the chloroplast membranes with PS I localized close to the exterior face of the thylakoid membrane, immunological data show comparatively high levels of inhibition of photosynthesis when chloroplasts are incubated with various antibodies against PS I intermediates, *e.g.*, ferredoxin [16], ferredoxin-$NADP^+$-reductase [17], FRS [18], and P_{700} [19], while antibodies against two intersystem electron transport intermediates, plastocyanin [20] and cytochrome f [19], inhibit chloroplast reactions to the same extent only after the chloroplasts are sonicated in the presence of the antiserum. The data obtained using detergents that effectively split PS I and II from the chloroplast membranes [14, 21], too, have indicated that the PS I fraction is localized closest to the exterior of the thylakoid membrane, while PS II is more centrally located; this model is in agreement

with the structural information provided by Briantais and Picaud [22], using agglutinating properties of antisera directed against PS I and PS II particles and chloroplasts. The recent findings on the localization of some of the PS II intermediates, however, raise some questions over this general model of PS II being interiorly located in the membrane. The first suggestion to the contrary offered by previous proponents of an asymmetric bilayer membrane comes with the immunologic data of Radunz *et al.* [23] where they report an inhibition of PS II reactions (maximum of 22%) with an antiserum against Chl *a*. This was followed by papers on inhibition of H_2O oxidation activity by PS II (to a comparative extent) by antisera against PS II particles [7] and a pigment containing protein fraction from chloroplasts that exhibited PS II activity [24]. Generally, the limited accessibility of the antibodies to the antigenic site was suggested to be the most plausible factor accounting for the rather low levels of inhibition. Recent experiments complicate this straightforward thinking, however. First, use of a large, non-permeant protein modifier, DABS shows that a greatly increased inhibition of oxygen evolution can be observed under conditions where the chloroplasts are undergoing conformational change [25]. Second, the typical 15—20% inhibition of oxygen-evolving activity as seen this time by an antibody against lutein [26] can be increased to approx. 65% if the chloroplasts are extremely well coupled, a condition that again would be favorable to conformational change. These data suggest two possibilities: (*1*) that the conformational change results in better exposure of the oxygen-evolving site to the outside of the membrane and therefore more accessible to the inhibiting, non-permeant molecules, or (*2*) that these inhibitors are against some structural moiety of the membrane other than the oxygen-evolving site itself, and a perturbation of the membrane (secondarily effecting inhibition at the H_2O oxidation site, as this site appears to be extremely sensitive to membrane disturbance) may more noticeably affect oxygen-evolving activity when large conformational changes occur. (At the moment, these are simply speculations, and a paper will be forthcoming to argue these points; the main issue is to understand the mode of action of these inhibitors, and with the data presently available, one cannot eliminate any of the possibilities discussed.)

Table I indicates that the addition of the antiserum against a soluble factor from repeatedly frozen and thawed chloroplasts results in a 16% inhibition of DCPIP photoreduction by broken spinach chloroplasts. The 16% inhibition represents an average of 15 measurements of Hill reaction rates in the presence of the antiserum compared with rates in the presence of the control serum*. There is no doubt that this inhibition is real in view of the small errors and of

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*Comparison is made here and elsewhere with control serum data. The low level of inhibition of electron transport brought about by the addition of the control serum to the chloroplasts is not a major problem as it appears to be non-specific and is approximately of the same degree in all assays done (in contrast to the specific antiserum, reported in this paper).

TABLE I

INHIBITION OF HILL REACTIONS BY ANTISERA AGAINST A SOLUBLE CHLOROPLAST FACTOR

Additions	μmoles $DCPIPH_2$ or NADPH/mg Chl/h	% inhibition of control serum
DCPIP as acceptor [a]		
None	270 ± 5	
0.02 ml control serum	232 ± 4	
0.02 ml antiserum	195 ± 5	16
$NADP^+$ as acceptor [b]		
None	54.0 ± 0.9	
0.06 ml control serum	50.2 ± 0.8	
0.06 ml antiserum	43.1 ± 0.8	14
DCMU, $DCPIPH_2$	22.3 ± 0.1	
DCMU, $DCPIPH_2$ plus 0.06 ml control serum	20.9 ± 0.1	
DCMU, $DCPIPH_2$ plus 0.06 ml antiserum	20.7 ± 0.1	1

[a] DCPIP as acceptor. 1-ml sample contained 8 μg Chl in 50 m*M* phosphate buffer, pH 6.8, 0.01 *M* NaCl, and $4.5 \cdot 10^{-5}$ *M* DCPIP; it was simultaneously illuminated and measured for change in DCPIP absorbance at 597 nm as discussed in METHODS. These values represent an average of 15 measurements.

[b] $NADP^+$ as acceptor. 1-ml sample contained 24 μg Chl in 50 m*M* phosphate buffer, pH 7.2, 25 μmoles NaCl, 3 μmoles $MgCl_2$, 0.5 μmoles $NADP^+$ and excess amounts of PPNR; where indicated, 0.01 μmoles DCMU, 0.01 μmoles DCPIP, and 2.0 μmoles Na ascorbate were added. (Note that plastocyanin was not added, except what may have been present in PPNR.) $NADP^+$ reduction was measured at 340 nm simultaneously with illumination as described in METHODS. These values represent an average of 5 measurements.

negative results under other conditions. This level of inhibition is approximately the same as that reported for antisera against PS II particles [7]. As discussed above, the limited inhibition may be due to: (*1*) the limited accessibility of the antigen to the antibodies, (2) the weak affinity of the antibody—antigen interactions (assuming that the antigen has been somewhat altered from its native state during extraction, and, therefore, the antibody elicited against it will not strongly bind the native chloroplast component) or (3) the lack of significant conformational changes in these chloroplasts and the suggestions that can be drawn from that.

To eliminate the possibility that the inhibition seen is due to an antibody against a PS I component detectable because DCPIP might also accept electrons from PS I as suggested by several workers [27, 28], we measured $NADP^+$ photoreduction in the presence of the antiserum both with H_2O and $DCPIPH_2$ as electron donors (see Table I). No decrease in reaction rates was observed with the antiserum when the artificial electron donor $DCPIPH_2$ (with DCMU) was used in place of water (in all five experiments), indicating

TABLE II

EFFECT OF ANTISERA ON DCPIP PHOTOREDUCTION BY TRIS-WASHED CHLOROPLASTS[a]

Chloroplast treatment	Additions	μmoles $DCPIPH_2$/ mg Chl/h	% inhibition of control serum
Normal	None	225	
Tris-washed	None	7.5	
Tris-washed	DPC	78.5	
Tris-washed	DPC + 0.02 ml control serum	65.7 ± 2.0	
Tris-washed	DPC + 0.02 ml antiserum	64.2 ± 1.5	2.8 ± 2.5

[a] 1-ml of sample contained 8 μg Chl. Reaction mixture and measurement are as indicated in Table I; where indicated, 0.25 μmoles of DPC were added. These values represent an average of 10 measurements.

that the antibody inhibition must be before the site of $DCPIPH_2$ donation and thus not in PS I.

To determine whether the site of inhibition is on the oxidizing or reducing side of PS II, chloroplasts were washed with 0.8 *M* Tris, pH 8.0, to inactivate oxygen evolution. DCPIP reduction was measured in the presence of the antiserum and control serum using DPC as electron donor. Table II shows that with this system there is no longer any inhibition by the antiserum. As DPC has been shown only to donate between the Tris-block and reaction center II [9], the antibody against the soluble chloroplast fraction must attack (directly or indirectly) either at the site (or close to the site) of Tris inactivation or at some unknown component between the Tris-block and H_2O.

We then measured DCPIP reduction of chloroplasts preincubated with serum in the presence and absence of $MnCl_2$ to further localize the site of action of the antiserum. According to Ben-Hayyim and Avron [8] and confirmed as shown here in Fig.1 with the identical chloroplasts as used in the inhibition experiments, Mn^{2+} competes with H_2O as an electron donor to PS II in normal chloroplasts, donating electrons at a site between H_2O and the Tris-block. The degree of inhibition of Hill reaction by the antiserum was shown to be approximately the same in the presence or absence of $MnCl_2$, as shown in Table III. Thus, the antiserum must attack at a site between the Mn^{2+} donation and DPC donation sites. Together, all the above experiments indicate strongly that the antiserum against an extract from repeatedly frozen and thawed chloroplasts, similar to the antiserum against PS II particles, is acting on a component inactivated by Tris-washing (or close to this component on the water side, *i.e.*, after the Mn^{2+} donation site). Whether these antisera are acting directly on the oxygen-evolving component or indirectly through membrane perturbation remains to be seen.

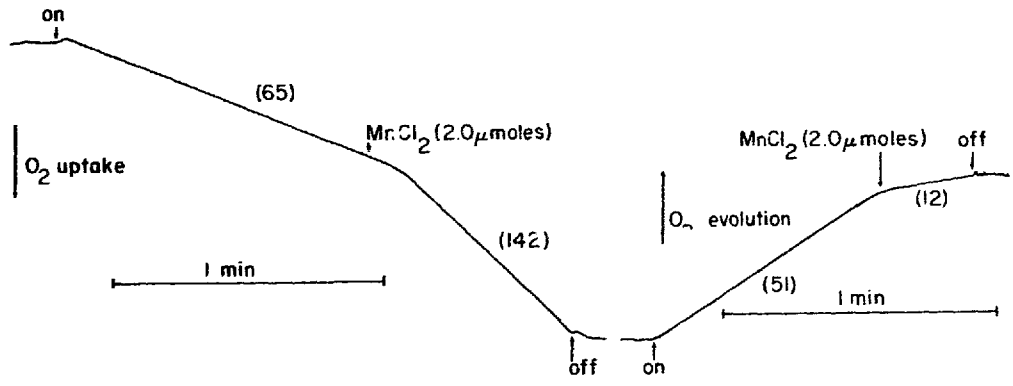

Fig.1. (Left trace), Effect of Mn^{2+} on O_2 uptake in the presence of methyl viologen. Reaction mixture contained: Tricine (pH 8.0), 45 μmoles; NaCl, 60 μmoles; methyl viologen, 0.03 μmoles; NaN_3, 3 μmoles and chloroplasts containing 60 μg chlorophyll, in a total volume of 3.0 ml. Numbers in parentheses represent rates of O_2 uptake expressed in μmoles per mg Chl per hour. (Right trace), Effect of Mn^{2+} on O_2 evolution with electron transport to $NADP^+$. Reaction mixture contained: Tricine (pH 8.0), 45 μmoles; NaCl, 60 μmoles; $NADP^+$, 0.5 μmole; saturating amounts of PPNR and chloroplasts containing 60 μg Chl in 3.0 ml volume. Numbers in parentheses are initial rates of O_2 evolution expressed in μmoles O_2 per mg Chl per hour.

TABLE III

INHIBITION OF DCPIP REDUCTION BY THE ANTISERUM IN THE PRESENCE AND ABSENCE OF $MnCl_2$ [a]

Additions	μmoles $DCPIPH_2$/mg Chl/h	% inhibition of control serum
None	278 ± 5	
0.04 ml control serum	238 ± 5	
0.04 ml antiserum	198 ± 4	16.8 ± 2
0.04 ml control serum + $MnCl_2$	224 ± 5	
0.04 ml antiserum + $MnCl_2$	185 ± 4	17.4 ± 2

[a] 1-ml of sample contained 8 μg Chl in 50 m*M* Tris—Cl buffer, pH 7.2, 0.01 *M* NaCl, and $4.5 \cdot 10^{-5}$ *M* DCPIP; where indicated, 0.3 μmoles of $MnCl_2$ are added. These results represent an average of 5 measurements.

Operating on the principle that the levels of inhibition (approx. 16%) seen were low because of the limited accessibility of the antigenic site(s) in the chloroplast membrane to the antibody(ies), we then looked at DCPIP reduction (water as donor) of PS II particles incubated in the presence of the two types of antisera (as well as the control serum). As indicated in Table IV, the percent inhibition increased almost 2-fold (approx. 30% inhibition), perhaps because the antigenic site(s) is more readily exposed in the particles than in the broken chloroplasts. On the same line of thinking, the lack of total inhibition may be explained by surmising that the site is yet

TABLE IV

EFFECT OF ANTISERA ON DCPIP REDUCTION BY PS II PARTICLES[a]

Additions	μmoles $DCPIPH_2$/ mg Chl/h	% inhibition of control serum
None	92.0	
0.02 ml control serum	80.3 ± 2.0	
0.02 ml antiserum against PS II	60.7 ± 1.5	24
0.02 ml antiserum against soluble extract	58.5 ± 1.5	27

[a]1-ml of sample contained 8 μg Chl. Reaction mixture and measurement as indicated in Table I (A). These values represent an average of 10 measurements.

only partially accessible in the particles (that may, in addition, contain some PS I), and that the antibodies are against somewhat denatured components and, thus, do not have a strong affinity for the native intermediate.

Finally, as to the nature of the soluble extract from repeatedly frozen and thawed chloroplasts, it can be stated with near certainty that the antibodies elicited against this extract were not made in response to some contaminating chlorophyll containing membrane fragment present in the extract. Careful absorption spectra measurements have shown a very slight absorbance detectable at 680 nm in most preparations. A typical extract contained 0.5 μg Chl/ml (based on an extinction coefficient of 100 mM^{-1} cm^{-1} for Chl *a*) for every mg of protein, *i.e.*, 0.05% Chl. Comparing this Chl content with that of the chloroplast membrane itself (Chl/protein = 20%), one can see that the Chl concentration of the extract is very small, *i.e.*, the Chl/protein ratio of the extract being less than 0.5% of the Chl/protein ratio of the membrane. With some reservations, one can nearly eliminate the possibility of a Chl–protein membrane fragment contaminant as being the antigenic source. It is quite possible that if anything, the amount of total contaminant present in the immunization course might elicit a low dose tolerance in the rabbit rather than an immune response [29].

Obviously, the immunochemical technique used here is only an indirect means of looking at the unknown intermediate involved in O_2 evolution. However, the commonly employed, more direct biochemical handles used in isolation and characterization of intermediates have not been successful in this case. We have shown that it is possible to obtain antisera against either the oxygen-evolving component itself or some structural protein whose conformational alteration by the antiserum in turn effects inhibition of oxygen evolution by challenging rabbits with such a crude antigen as PS II particles [7], and that it is feasible (as shown here) to obtain similar antisera by the use of simpler antigens, such as that against extracts from frozen and thawed chloroplasts.

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