OXIDATION OF 1,5-DIPHENYLCARBAZIDE AS A MEASURE OF PHOTOSYSTEM 2 ACTIVITY IN SUBCHLOROPLAST FRAGMENTS[1]

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Summary. The use of 1,5-diphenylcarbazide as an electron donor coupled with DPIP[2] reduction allows the detection of photosystem 2 in subchloroplast fragments which have lost the ability to evolve oxygen. Various subchloroplast fragment obtained by detergent treatment of spinach chloroplasts have been assayed in this manner. Photosystem 1 activity was measured as NADP reduction in the presence of ascorbate and DPIP. The best separation of the two photosystems was obtained with Triton X-100 at a concentration of 40 mg Chl/gram detergent.

During normal photosynthesis in plant chloroplasts, two photosystems in series transfer electrons from water to NADP. Vernon and Zaugg (1) showed that the photosystem responsible for NADP photoreduction (photosystem 1) could be studied separately by supplying the chloroplast with electrons from the donor system ascorbate-plus-DPIP. This system has subsequently been widely used to detect photosystem 1 in fragments obtained from chloroplasts through the action of detergents (2-5). Recently Yamashita and Butler (6) demonstrated that

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[2]The abbreviations used in this paper are: DPIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; SC, semicarbazide; DPSC, 1,4-diphenylsemicarbazide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TX, Triton X-100; Chl, chlorophyll; TSF-1 and TSF-2, the subchloroplast fragments (originally designated PD-10 and P-1, respectively) corresponding to photosystem 1 and photosystem 2, obtained by the action of Triton X-100 on chloroplasts (3,4).

following treatment of spinach chloroplasts with Tris buffer of high pH, a number of electron donors could couple with photosystem 2, the most effective being p-phenylenediamine. In a subsequent investigation (7) two donors, benzidine and semicarbazide were used with chloroplasts to photoreduce ferricyanide in a reaction which involved only photosystem 2. These donors could be used since they did not reduce ferricyanide in the dark under the conditions employed.

We report here the use of 1,5-diphenylcarbazide as an electron donor to photosystem 2 of chloroplasts. The oxidation of this donor is coupled to the photoreduction of DPIP in a simple and specific test for photosystem 2 activity. Using this assay system it has been possible to determine the levels of photosystem 2 activities in fragments obtained from spinach chloroplasts through the use of various detergents.

METHODS

Chloroplasts were prepared from market spinach in 0.35 M NaCl and 0.02 M Tris buffer pH 8 as previously described (3). Chlorophyll was determined by the method of Vernon (8). DPIP photoreduction was followed at 590 nm in 2.0 ml of aqueous medium containing 0.25 M sucrose, 0.03 M phosphate buffer pH 6.7, 0.1 mM DPIP and 0.5 mM DPC. NADP photoreduction was followed at 340 nm in 2.0 ml of medium containing 0.25 M sucrose, 0.03 M phosphate buffer pH 6.7, 0.4 mM NADP, 7.5 mM ascorbate, 0.05 mM DPIP, 1 µM plastocyanin and saturating amounts of crude ferredoxin (9) which also contained ferredoxin-NADP reductase. Where indicated Triton X-100 was added to the reaction mixture for NADP photoreduction. The spectrophotometric determinations of these activities was accomplished with a Beckman DB spectrophotometer adapted for

illumination as previously described (3). Illumination of the reaction mixtures was with red light obtained with Corning filter (No. 2403) and a tungsten microscope light. Light intensity at the reaction system was 2 x 10^5 ergs/cm^2.sec.

For the experiments involving treatment of chloroplasts with Triton X-100, the general procedure previously described (3) was used. The amount of detergent employed was varied, as indicated in Table 1. All incubations with detergents were performed at zero degrees, followed by centrifugation as indicated in Table 1. Digitonin fractionation was accomplished according to the direction of Anderson and Boardman (2). Ammonox Lo, lauryl dimethyl amine oxide, was used at zero degrees for 60 minutes to disrupt the chloroplasts. Semicarbazide, 1,4-diphenylsemicarbazide and 1,5-diphenylcarbazide were obtained from Eastman Organic Chemicals Company. Semicarbazide was recrystallized from propanol-water and 1,5-diphenylcarbazide from methanol-water. Digitonin and 2,6-dichlorophenol indophenol were obtained from Sigma Chemical Company and DCMU from DuPont Chemical Company.

RESULTS

Yamashita and Butler (7) employed semicarbazide as a donor for ferricyanide photoreduction by photosystem 2 with chloroplasts washed in 0.8 M Tris buffer pH 8.0, which treatment served to inactivate the oxygen evolution system. A search for other donors led us to use 1,5-diphenylcarbazide, which is the most active compound we have tested to date with Tris-washed chloroplasts. Figure 1 shows the activity of three such electron donors for DPIP photoreduction, using TSF-2 particles (containing photosystem 2) obtained by the use of Triton X-100

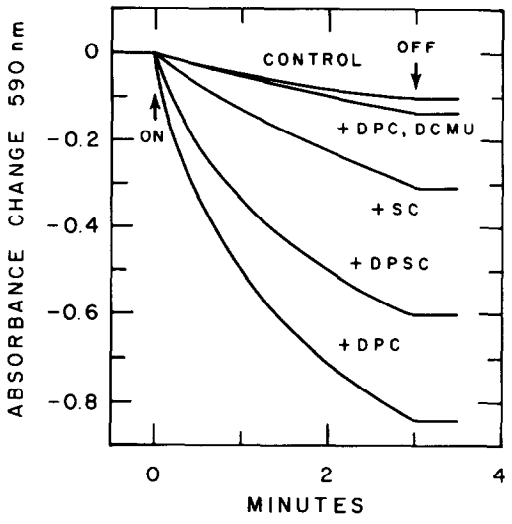

Figure 1. DPIP photoreduction by electron donors in the presence of photosystem 2 fragments (TSF-2) produced by the action of Triton X-100 on spinach chloroplasts (3). The reaction mixture employed 6 mM phosphate buffer pH 6.4, 0.08 mM DPIP, TSF-2 subchloroplast fragments containing 0.035 mg Chl and where indicated 2.5 mM semicarbazide (SC), 0.5 mM diphenylsemicarbazide (DPSC), 0.5 mM diphenylcarbazide (DPC) and 10 μM DCMU in a volume of two ml.

(3,10). The DCMU sensitivity shows the reaction is indeed mediated by photosystem 2. Of the compounds tested, DPC was the most active with these subchloroplast fragments. Whereas some residual oxygen evolution was observed for these particles, the rate of DPIP reduction was greatly stimulated by the addition of DPC.

The DPC-DPIP couple is a sensitive assay for photosystem 2, and when used in conjunction with the ascorbate-DPIP assay for NADP photoreduction the degree of separation of the two photosystems by treatment with detergents can be determined (Table I). The lower concentration of Triton was investigated since it more nearly represents the concentration employed by Briantais (11) in fragmenting corn chloroplasts. The digitonin treatment followed that described by Boardman and Anderson (12), who were the first

Table 1

Distribution of photosystem 2 and photosystem 1 activities in fractions obtained by treatment of spinach chloroplasts with detergents. The assay systems and preparation methods are described in the Methods Section. The numbers in parentheses are the μg Chl in the assay systems used to determine photosystem 1 and photosystem 2 activities.

Method of Preparation	Photoactivities, μmoles/hr·mg Chl: DPIP Reduction Photosystem 2		NADP Reduction Photosystem 1		
	- DPC	+ DPC	No TX	0.035% TX	0.1% TX
Triton X-100					
125 mg Chl/gram detergent					
10,000 g for 10 min	74	104 (38)	-	-	-
10,000 g for 30 min	72	94 (46)	0	17	81 (46)
80,000 g for 30 min	34	36 29	0	33	180 (30)
105,000 g for 5 hrs	0	3 (26)	67	128	260 (26)
40 mg Chl/gram detergent					
144,000 g for 1 hr (TSF-2)	81	262 (30)	0	0	58 (30)
144,000 g for 6 hrs	0	35 (22)	0	132	79 (22)
144,000 g for 10 hrs after dilution of 1:1 (TSF-1)	0	0 (30)	435	735	470 6
144,000 g for 12 hrs after dilution of sup from above 2:3	0	0 (43)	504	935	480 (8)
Digitonin					
60 mg Chl/gram detergent					
1,000 g for 10 min	13	80 (51)	0	38	140 (30)
10,000 g for 30 min	75	142 (30)	13	39	104 (30)
50,000 g for 30 min	0	15 (35)	-	194	194 (18)
144,000 g for 60 min	0	8 (39)	75	435	169 (16)
Ammonox Lo					
435 mg Chl/gram detergent					
10,000 g for 30 min	0	7 (26)	0	32	42 (26)
80,000 g for 30 min	0	10 (25)	12	22	51 (50)
105,000 g for 5 hrs	0	4 (73)	19	46	92 (30)

to fragment chloroplasts by means of detergent treatment.

The data of Table 1 show that the cleanest separation of the two photosystems was obtained with Triton X-100 at the level of 40 mg Chl/gram detergent, which is the amount used previously for the production of a very active photosystem 1 particle (3). The other treatments allow some photosystem 2 activity to remain in all fractions, as detected by this sensitive method. Since the TSF-1 fragment shows high photosystem 1 activity (NADP photoreduction) and no DPC-coupled photoreduction of DPIP, the later activity cannot be ascribed to photosystem 1. This shows that the DPC-coupled reduction of DPIP is specific for photosystem 2.

The data of Table 1 show that NADP photoreduction is stimulated if Triton X-100 is added to the reaction mixture. This response has been routinely observed, and most likely represents a disaggregation of these particles in the presence of the detergent. Some residual photosystem 1 activity which is apparently inaccessible to the test reagents can be assayed by use of a solubilizing amount (13) of Triton X-100 in the reaction mixture. The use of high Triton in the assay system allows some photosystem 1 activity to be observed in all fragments. However, the lowest activity for NADP-photoreduction was found in the photosystem 2 particles obtained by the standard treatment with Triton X-100. These data show that the cleanest separation of the two photosystems was obtained by the use of Triton X-100 at the concentrations previously employed in this laboratory (3). This conclusion is also in agreement with the structures observed for these fractions by electron microscopy (10).

REFERENCES

1. Vernon, L. P. and Zaugg, W. S., J. Biol. Chem., 235, 2728 (1960).
2. Anderson, J. M. and Boardman, N. K., Biochim. Biophys. Acta, 112, 403 (1966).
3. Vernon, L. P., Shaw, E. R. and Ke, B., J. Biol. Chem., 241, 4101 (1966).
4. Vernon, L. P., Ke, B. and Shaw, E. R., Biochemistry, 6, 2210 (1967).
5. Wessels, J. S., in J. B. Thomas and J. C. Goedheer (Editors), "Currents in Photosynthesis," Donker, N. V., Rotterdam, 1966.
6. Yamashita, T. and Butler, W. L., Plant Physiol., 43, 1978 (1968).
7. Yamashita, T. and Butler, W. L., Plant Physiol., 44, 435 (1969).
8. Vernon, L. P., Anal. Chem., 32, 1144 (1960).
9. San Pietro, A. and Lang, H. M., J. Biol. Chem., 231, 211 (1958).
10. Vernon, L. P., Mollenhauer, H. H. and Shaw, E. R., in Regulatory Functions of Biological Membranes, Ed. J. Järnefelt (Amsterdam:Elsevier Publishing Company, 1968) p. 57.
11. Briantais, J. M., Photochem. Photobiol., 5, 135 (1966).
12. Boardman, N. K. and Anderson, J. M., Nature, 203, 166 (1964).
13. Vernon, L. P. and Shaw, E. R., Plant Physiol., 40, 1269 (1965).