

Photoreduction of 2,6-Dichlorophenolindophenol by Diphenylcarbazide: A Photosystem 2 Reaction Catalyzed by Tris-Washed Chloroplasts and Subchloroplast Fragments

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Abstract. The use of diphenylcarbazide as an electron donor coupled to the photoreduction of 2,6-dichlorophenolindophenol by tris-washed chloroplasts or subchloroplast fragments provides a simple and sensitive assay for photosystem 2 of chloroplasts. By varying the concentration of tris buffer at pH 8.0 during an incubation period it is shown that the destruction of oxygen evolution activity is accompanied by a corresponding emergence of an ability to photooxidize diphenylcarbazide, as evidenced by absorbance changes due to diphenylcarbazide at 300 nm. The temperature-sensitive oxidation of diphenylcarbazide is inhibited by DCMU and by high ionic strengths. This activity appears to measure the primary photochemical reaction of photosystem 2.

Success in studying the individual photoreactions in plant chloroplasts is greatly facilitated by the availability of assay systems which are specific for 1 of the 2 photosystems which operate in normal photosynthesis. The reaction discovered by Vernon and Zaugg in 1960 (15), wherein DPIP² in the presence of ascorbate formed an efficient electron donor couple for photosystem 1 has allowed the mechanism of NADP photoreduction by this photosystem to be studied in great detail.

A number of investigations have shown that it is also possible to employ artificial electron donors for photosystem 2, which system ordinarily oxidizes water preliminary to oxygen evolution. The criterion most often used for donation of electrons into photosystem 2 is the sensitivity of the reaction to DCMU (or *o*-phenanthroline), a poison which is specific for the oxygen evolution system of plants (2, 16). From experiments which showed a DCMU-sensitive (or *o*-phenanthroline-sensitive) oxidation of ascorbate in the presence of autooxidizable quinones (3, 5, 9) it was postulated that ascorbate donated electrons prior to the DCMU block. In 1962, Trebst

and Wagner (10) studied the photooxidation of hydroquinones by isolated chloroplasts, and showed that the oxidation reactions were sensitive to DCMU. Subsequent experiments by Katoh and San Pietro (6, 7) demonstrated that ascorbate was directly oxidized in a DCMU-sensitive reaction by *Euglena* chloroplasts which had been inhibited by a heat treatment. Habermann (4) showed by experiments with oxygen isotopes that ascorbate decreased the rate of oxygen evolution while increasing the rate of oxygen uptake coupled to oxidation of the primary photoreductant, which indicated an oxidation of ascorbate as an alternate to oxygen evolution.

Another approach involves the use of washing in tris buffer to inactivate the oxygen evolution system. Nakamoto *et al.* (8) showed that ascorbate served as a donor to riboflavin phosphate in such preparations, and proposed that ascorbate was oxidized by the primary photooxidant. Yamashita and Horio (19) later showed that washing chloroplasts with 0.8 M tris buffer at pH 8.0 abolished the ability of such chloroplasts to evolve oxygen, but in the presence of DPIP or TMPD and an excess of ascorbate, photophosphorylation of the non-cyclic type was restored, which indicated that electrons were entering into the system at the level of photosystem 2. This conclusion was supported by the fact that the phosphorylations were sensitive to DCMU.

The use of tris-washing to inactivate chloroplasts was used by Yamashita and Butler (17) to study the photoreduction of NADP by artificial electron donors in the absence of oxygen evolution. Donor systems such as ascorbate-DPIP and ascorbate-TMPD could feed electrons into photosystem 2, but also had the ability to feed electrons into photosystem 1 in the presence of DCMU. The donor *p*-phenylenediamine in the presence of ascorbate,

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² The abbreviations used in this paper are: DPIP, 2,6-dichlorophenolindophenol; TMPD, N,N,N',N'-tetramethylphenylenediamine; DPC, 1,5-diphenylcarbazide; SC, semicarbazide; DPSC, 1,4-diphenylsemicarbazide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll; TSF-2, the subchloroplast fragment containing photosystem 2 and produced by the action of Triton X-100 upon chloroplasts (13). (The TSF-2 fragment was originally designated the P-1 fraction.)

however, was specific in donation to photosystem 2. A subsequent publication reports that other compounds function to feed electrons rather specifically into photosystem 2 (18), including *p*-hydroquinone, *p*-aminophenol, benzidine, semicarbazide and dihydroxydiphenyl. All these compounds supported NADP photoreduction, and ferricyanide reduction was also observed with benzidine and semicarbazide, 2 donors which do not react chemically with ferricyanide.

We have studied the effect of related donors on the photoreduction of DPIP by tris-washed chloroplasts and by photosystem 2-enriched subchloroplast particles prepared by the action of Triton X-100. The most efficient donor for this reaction is 1,5-diphenylcarbazide, and the characteristics of this reaction have been studied.

Methods

Chloroplasts were prepared from market spinach in 0.35 M NaCl and 0.02 M tris buffer pH 8 as previously described (12,13). Chlorophyll was determined by the method of Vernon (11). DPIP photoreduction was followed at 590 nm in 2.0 ml of reaction medium containing 0.25 M sucrose, 0.03 M phosphate buffer pH 6.4, 0.1 mM DPIP and 0.5 mM DPC where indicated. Assays were performed with a Beckman DB spectrophotometer which was adapted for illumination as previously described (13). Illumination was with a tungsten microscope lamp coupled with a Corning filter (No. 2403) which allowed red light of intensity 2×10^6 ergs/cm²·sec to fall on the reaction system.

Tris-washed chloroplasts were prepared by treatment with 0.2 M tris for 5 min with chloroplasts being present at a concentration sufficient to give 28 µg of Chl per ml of tris buffer. The TSF-2 subchloroplast fragment containing photosystem 2 was prepared by treatment of chloroplasts with Triton X-100 as previously described (13). (In our earlier publications this fragment was called the P-1 fragment.) Semicarbazide, 1,5-diphenylcarbazide and 1,4-diphenylsemicarbazide were obtained from Eastman Organic Chemical Company. Semicarbazide was recrystallized from propanol water and 1,5-diphenylcarbazide from methanol water. DCMU was obtained from DuPont Chemical Company, DPIP from Sigma Chemical Company and Triton X-100 from Rohm and Haas.

Results

Yamashita and Butler (18) reported that ferricyanide photoreduction by tris-washed chloroplasts was supported by the presence of either benzidine or semicarbazide as donor molecules. In extending these studies we have used the donor molecules given in table I to photoreduce DPIP, a reaction which is much easier to follow spectrophotometrically. Ex-

amination of subchloroplast fragments obtained by treatment with Triton X-100 showed that the one containing photosystem 2, TSF-2, was also capable of performing this reaction. Because of the ease of preparing and maintaining the TSF-2 fractions and the fact that such particles are not significantly contaminated with photosystem 1 activity, we have used this material to carry out most of the tests we have performed on the DPC-coupled photoreduction of DPIP, a reaction which is specific for photosystem 2.

Table I shows the effects of ionic strength and pH upon DPIP photoreduction catalyzed by TSF-2 fragments in the presence of 3 different donor molecules. With all 3 donors the reactions proceeded best at low ionic strengths and at low pH. Reactions run at pH 6.1 showed essentially the same activity as at pH 6.4. It was not possible to go lower than 6.1 in pH because of the ionization of DPIP itself. We have used pH 6.4 for our routine assays. The decreased activity observed at high phosphate concentrations is not an inhibition by phosphate, since similar results were obtained in tris-maleate or pyrophosphate buffer. The addition of NaCl to low concentrations of phosphate buffer also served to inhibit the reaction.

A marked increase in activity at lower ionic strengths was shown with all 3 donor molecules. The cause of this sensitivity to ionic strength is not immediately apparent. One possible explanation is that the ionic strength affects the degree of aggregation of the TSF-2 particles. Since a similar re-

Table I. *Photoreduction by DPIP by Photosystem 2 Particles (TSF-2) Obtained by Triton Treatment of Spinach Chloroplasts; the Effect of Ionic Strength and pH With Various Donor Molecules*

The reaction mixture contained 0.1 mM DPIP, phosphate buffer of the pH and ionic strength indicated (the molarities for ionic strengths of 0.01, 0.05 and 0.10 were 0.007, 0.0034, and 0.066, respectively), TSF-2 particles containing 37 µg Chl and where indicated 2.5 mM semicarbazide, 0.5 mM diphenylsemicarbazide or 0.5 mM diphenylcarbazide in 2.0 ml volume.

Donor	Ionic strength	DPIP photoreduction activity		
		pH 6.4	pH 7.0	pH 7.4
		<i>µmoles/hr·mg Chl</i>		
...	0.01	55	47	32
...	0.05	51	33	8
...	0.10	31	16	0
SC	0.01	83	67	53
SC	0.05	65	55	30
SC	0.10	44	28	18
DPSC	0.01	109	83	61
DPSC	0.05	85	73	34
DPSC	0.10	63	38	26
DPC	0.01	210	144	91
DPC	0.05	173	127	71
DPC	0.10	126	97	69

sponse was observed with tris-washed chloroplasts it would seem that aggregation is not the primary mode of action of the salt added as buffer. A more reasonable explanation is that salts influence the nature of the reactive surface of the TSF-2 particle or tris-washed chloroplasts. This could be done either by changing the conformation of some component or by absorption of 1 of the ions, thus decreasing the reactivity of the chlorophyll system with 1 of the reagents.

The DPC-coupled photoreduction of DPIP is a relatively simple reaction specific for photosystem 2, since it is not supported by TSF-1 particles which contain photosystem 1 (14). It would be helpful if the reaction could also be followed by means of absorbance changes of the donor molecule, DPC. For this purpose the data of Fig. 1 were obtained,

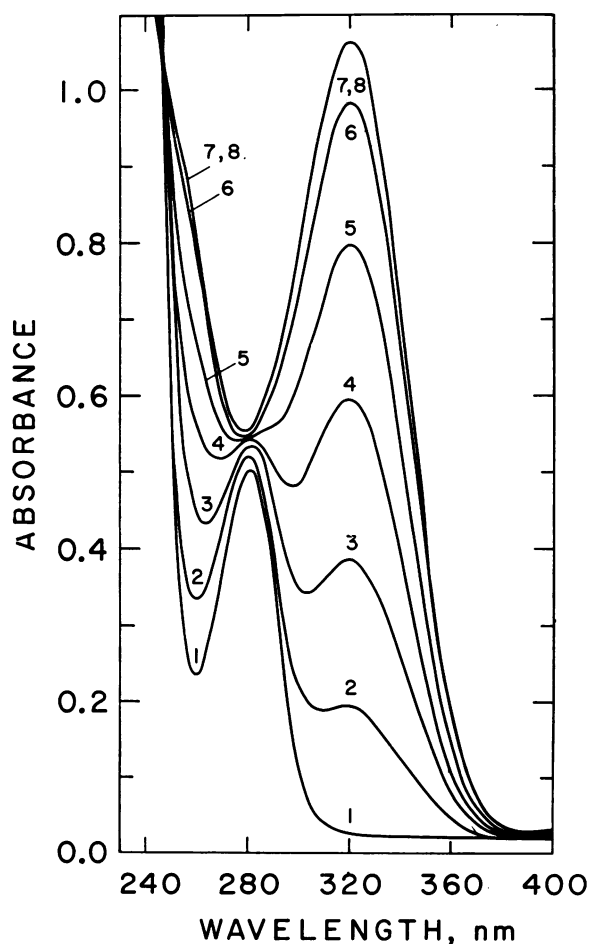


FIG. 1. Absorption spectra of DPC in dilute phosphate buffer at pH 6.4 after addition of varying amounts of hypochlorite. The phosphate buffer, of ionic strength 0.01 (0.007 M), contained 0.5 μ mole of the DPC in 3.0 ml of solution. Curve 1 shows the spectrum of DPC alone, while curves 2 through 8 show the results of addition of increments of 10 μ l of 20 \times diluted commercial chlorox containing 5.25% sodium hypochlorite.

showing the absorption spectra of DPC during various stages of oxidation with hypochlorite. Oxidation causes the appearance of a new band at 320 nm, which offers a means of measuring the oxidation reaction. In applying these data, however, a complication of an absorbance change at 320 nm due to DPIP reduction was observed. The reduction of DPIP results in a significant decrease in absorbance at this wavelength, which complicates the use of this wavelength for measuring changes in the oxidation state of DPC. A better wavelength for measurement was 300 nm, where the change due to DPC oxidation was still considerable and the absorbance change due to DPIP reduction was minimal. Accordingly we selected this wavelength to follow DPC oxidation in a coupled reaction with DPIP.

Fig. 2 shows the absorbance changes observed at 300 nm, due to DPC oxidation, which accompany the usual absorbance changes at 590 nm which reflect the reduction of DPIP. For these experiments TSF-2 fragments were used. The similar response to the addition of DCMU shows that the 2 reactions are coupled and are driven by photosystem 2. On the basis of this method for assay of DPC oxidation it was possible to follow the disappearance of oxygen

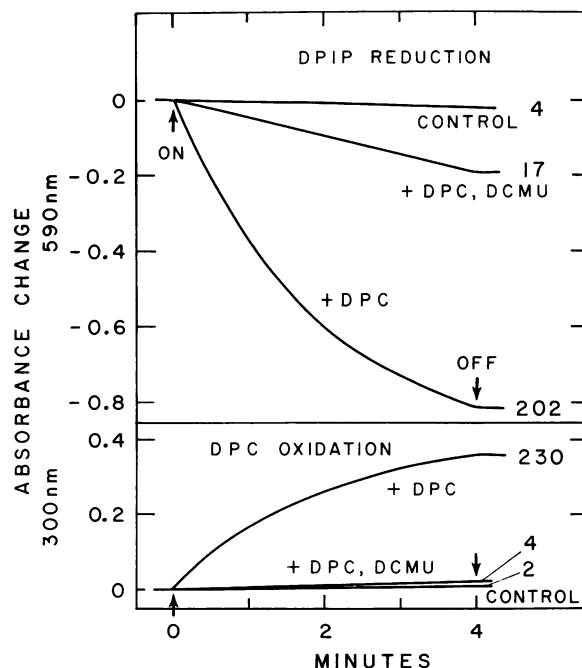


FIG. 2. Demonstration of the coupled photooxidation of DPC and photoreduction of DPIP by photosystem 2 particles. The reaction mixture employed is described in the Methods section. Where present DCMU was 10 μ M. TSF-2 fragments containing 17 μ g Chl were used in these experiments. The numbers opposite each curve are the rates of DPIP reduction or DPC oxidation, in μ moles/hr \cdot mg Chl, based on the absorbance change for the first 30 sec.

evolution activity during exposure of chloroplasts to tris buffer at high pH and simultaneously observe the related appearance of DPC photooxidation coupled to the reduction of DPIP. Table II shows the data obtained in this way. For these experiments a constant exposure time was used, but the concentration of tris was varied to give different degrees of inhibition of the oxygen evolution apparatus. As this activity decreased, as evidenced by a loss in ability of the chloroplasts to reduce DPIP using water as the donor molecule, the ability of the same chloroplasts to oxidize DPC emerged. In the presence of DPC, the rate of DPIP reduction was fairly constant for the various treatments, indicating that the total potential for DPIP reduction remained about the same during inactivation of the oxygen evolving apparatus.

The data in table II were obtained from initial rates, using a change in millimolar absorptivity coefficient of 5.4 for DPC at 300 nm and a value of 16 (1) for DPIP at 590 nm. At the concentrations of tris which more completely inhibited the oxygen evolution apparatus, an apparent higher rate of DPC oxidation than DPIP reduction was observed. This most likely derives from the use of initial rates for these calculations, since the observed absorbance changes at 300 nm are more subject to error because of the low absorbance change of DPC and the possible small absorbance change inherent in the chloroplast system itself at this wavelength. When the extents of the reactions at 590 and 300 nm were compared, they agreed with the values expected

Table II. *Appearance of DPC Photooxidation Activity as DPIP Hill Activity (Oxygen Evolution) is Inhibited by Incubation of Spinach Chloroplasts in Tris Buffer*

In both cases DPIP photoreduction was followed at 590 nm using the assay system given in the Methods section. Spinach chloroplasts containing 15 μg were used in these experiments, and where present DPC was 0.5 mM. DPC oxidation was followed at 300 nm, using a determined millimolar change in absorptivity of 5.4. The chloroplasts were incubated in pH 8.0 tris buffer of the indicated molarity for 5 min, after which the chloroplasts were removed from the medium by centrifugation and resuspended in a solution which contained 0.5 M sucrose and 0.05 M Tricine buffer pH 7.6. The activities were determined on the resuspended chloroplasts.

Tris concn. for incubation	Photoactivities		
	DPIP reduction — DPC	DPIP reduction + DPC	DPC oxidation
	$\mu\text{moles/hr}\cdot\text{mg Chl}$		
M	146	138	0
0.01	97	153	64
0.02	58	174	80
0.05	59	153	128
0.1	33	158	248
0.2	14	164	272

from the ratios of the specific absorptivities at these wavelengths for DPIP and DPC.

The temperature sensitivities of both tris-washed chloroplasts and TSF-2 fragments for the DPC-supported photoreduction of DPIP were tested. Both systems are inhibited in a parallel manner by heating (table III). These data show that this relatively simple electron transfer reaction, which is catalyzed by photosystem 2, has a requirement for an ordered structure in the tris-washed chloroplasts and the TSF-2 particles.

Discussion

The photoreduction of DPIP by DPC, which is catalyzed by either tris-washed chloroplasts or subchloroplast fragments containing photosystem 2, is a sensitive and easy means for assay of photosystem 2 activity under conditions where the enzymic apparatus responsible for oxygen evolution is destroyed. This activity is observed in subchloroplast fragments prepared by the action of either Triton X-100 or digitonin (14), and in magnitude it is observed at about the same level as in the tris-washed chloroplasts. It appears, therefore, that the method will have utility as a measure of photosystem 2 activity and will facilitate further experimentation into the nature of this photosystem.

Experiments performed with TSF-2 fragments indicate that the apparatus responsible for the initial electron transfer reactions of photosystem 2 is stable at ordinary temperatures. The data of table III show, however, that heating will destroy this activity. We have assayed TSF-2 fragments which have no

Table III. *Temperature Sensitivity of DPC-supported Photoreduction of DPIP by Tris-washed Spinach Chloroplasts and TSF-2 Fragments*

The assay method is reported in the Methods section. Tris-washed chloroplasts were prepared by incubation for 5 minutes in 0.2 M tris buffer pH 8, and the TSF-2 fragments were prepared as previously described (13). The chloroplasts or TSF-2 fragments were incubated for 5 min at the temperature indicated prior to determination of the activities. The chlorophyll contents in the assay mixtures were 18 and 35 μg Chl for chloroplasts and TSF-2, respectively.

Incubation temperature	DPC-supported DPIP photoreduction	
	TW chloroplasts	TSF-2
<i>deg</i>	$\mu\text{moles/hr}\cdot\text{mg Chl}$	
Room temp	220	254
30	220	230
35	167	198
40	146	170
47	108	79
50	96	47
55	63	15
60	17	4

residual oxygen evolution activity after having been stored frozen for periods of up to 2 years, and have found that all such preparations have shown good activity in the DPC-coupled reduction of DPIP.

The DPC-supported photoreduction of DPIP is quite specific for photosystem 2, as shown by the DCMU sensitivity of the reaction (Fig. 2) and the fact that isolated subchloroplast particles containing photosystem 1 (TSF-1) do not catalyze the reaction (14). Only as the oxygen evolution system is inactivated does the ability of the DPC to interact with photosystem 2 become apparent, indicating that in the intact chloroplast this system is not available to external agents and is "covered up" by the enzymes responsible for oxygen evolution. A reasonable assumption is that these enzymes, or factors, are removed by the tris treatment, and thus make the photosystem 2 apparatus available for the added DPC. The availability of this assay system, in which reactions at both termini of the reaction chain can be followed, should facilitate investigation into the mechanism of this photosystem.

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