Short Communication

Photoreduction Sites for 2,6-Dichlorophenolindophenol in Chloroplasts

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2,6-Dichlorophenolindophenol (DPIP) is one of the many commonly used electron acceptors for the light-induced reactions in chloroplasts (2). Until recently, it has generally been assumed that it accepts electrons from the short-wavelength pigment system 2 (PS2). Since the finding that indophenol reduction can be coupled with photophosphorylation [see review by Vernon and Avron (8)], the possibility of its reduction by the long-wavelength pigment system 1 (PS1) has also been suggested.

Recently Kok et al. (5) reported a fast photoreduction of DPIP in chloroplasts by using pulse illumination. The fast reduction was completely absent in the Scenedesmus mutant No. 8 which lacks the long-wavelength reaction center P700.

This note shows that by means of sensitive difference spectrophotometry, photoreduction of DPIP at sites belonging to the 2 pigment systems in chloroplasts can readily be demonstrated.

Experiments

Experiments were carried out with broken chloroplasts prepared from spinach as previously described (4) or with subchloroplast particles fractionated by Triton X-100 treatment (9). DPIP (grade "P"), antimycin A and 2-n-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) were obtained from Sigma Chemical Co. m-Chlorocarbonyl cyanide phenylhydrazone (CCCP) was a gift from Dr. P. G. Heytler. The dye reduction was followed directly at 590 mμ by difference spectrophotometry (3); both steady and pulse illuminations were used.

Results

Separation of Reduction Sites by Excitation Wavelengths. When the chloroplasts are illuminated by a polychromatic (e.g., 650-750 mμ) light in the presence of DPIP, the dye reduction follows a biphasic course consisting of a rapid and a slow reduction. The same reduction kinetics can be obtained when the chloro-

plants were illuminated with monochromatic (10 mμ bandwidth) 650 mμ light, as shown by the upper recording in figure 1. However, when 720 mμ light was used, only the rapid phase was observed, as shown by the lower recording in figure 1. These results indicate that under the usual conditions for observing DPIP photoreduction, namely, when both pigment systems are activated, reduction by both PSI (the rapid phase) and PS2 (the slower phase) can be observed on an instrument of adequate sensitivity and time resolution. The magnitude of the rapid phase in figure 1 amounts to $8 \times 10^4$ in optical density, which would escape detection on most conventional spectrophotometers adapted for such studies. Sonicated chloroplasts, in which the electron-transport linkage is known to be interrupted through the release of plastocyanin (4), also yielded the biphasic reduction profile, with somewhat lesser rates.

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3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) is known to inactivate PS2 exclusively. Examination of DPIP reduction by broken chloroplasts containing 5 \( \mu \text{M} \) DCMU showed a complete disappearance of the slow-phase reaction, with the rapid-phase almost intact (fig 3). Heating the chloroplasts at 45\(^\circ\) for 5 minutes yielded similar results (fig 4), as such heating also preferentially inactivates PS2. Some other phosphorylation inhibitors, such as antimycin A and HOQNO, appeared to have only a small effect on the biphasic reaction. At 1 \( \mu \text{M} \), CCCP is known to be effective in uncoupling photophosphorylation, but it has negligible effect on the electron transport reaction as manifested by the biphasic reaction kinetics. However, as seen in figure 3, 10 \( \mu \text{M} \) CCCP abolished the slow reaction.

\[ \text{Photoreduction and Photooxidation of 2,6-Dichlorophenolindophenol.} \]

Vernon and Zaugg (6) showed that ascorbate and DPIP can replace water as the electron donor for NADP reduction when PS2 is inactivated. As shown by the top curve in figure 4, chloroplasts heated at 45\(^\circ\) for 5 minutes showed only the rapid-phase reduction of DPIP. However, when both reduced and oxidized DPIP are present, the

\[ \text{Separation of Reduction Sites Through Detergent Fractionation.} \]

The separate sites for DPIP reduction are further demonstrated with subchloroplast particles prepared by fractionation by the detergent Triton X-100 (10). The light particle (designated as P-D10) has activities exclusively associated with PS1, namely, P700 photooxidation, type-I (or type-R) ESR signal, and NADP photoreduction activity with an appropriate electron donor system. The heavier particle (e.g., P-1) was reported earlier as inactive in DPIP reduction when examined on a conventional spectrophotometer.

Photoreduction of DPIP by the light particles, P-D10, and a heavy particle, P-1, induced by polychromatic red light is shown in figure 2. Recordings obtained by 2-second steady illumination as well as 20-\(\mu\text{s}\) second pulse illumination are presented. The light particle, even under conditions where light for PS2 was provided, showed only the rapid-phase reduction associated with PS1. The magnitude of the rapid reduction signal was \(2.6 \times 10^{-3}\) in optical density, several times greater than that observed with broken chloroplasts in figure 1. The heavy particle showed a slow-phase reduction which is much slower compared with unfractionated chloroplasts, but no trace of the rapid-phase reduction was detectable.

By means of flash illumination, the onset of the reaction was shown to be as rapid as the time response of the instrument allows (10\(^4\) sec). The half decay time of the dark reaction estimated from figure 2 was 0.6 \(\pm\) 0.1 second. Flash illumination of the heavy particle, however, yielded no signal. This is consistent with the low rate observed with steady illumination.

\[ \text{Preferential Inhibition of the Reduction Sites.} \]

3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) is

\[ \text{Fig. 2. Photoreduction of DPIP by subchloroplast particles fractionated by the detergent treatment.} \]

\[ \begin{align*}
\text{Exc.} & \quad \text{Flash} \\
\text{Light} & \quad \text{2x10^{-9} sec} \\
\text{HEAVY} & \quad \text{4 second} \\
\text{TIME SPAN} & \quad \text{1 second}
\end{align*} \]

\[ \Delta A \]

\[ \begin{align*}
\text{ON} & \quad \text{- DPIP} \\
\text{- DCMU} & \quad \text{5x10^{-6} M} \\
\text{OFF} & \quad \text{+ DPIP} \\
\text{+ DCMU} & \quad \text{5x10^{-6} M} \\
\text{+ HOQNO} & \quad \text{10^{-4} M} \\
\text{+ ANTIMYCIN} & \quad \text{5x10^{-5} M} \\
\text{+ CCCP} & \quad \text{10^{-6} M}
\end{align*} \]

\[ \Delta A \]

\[ \begin{align*}
0 & \quad 1 \quad 2 \quad 3 \quad 4 \quad \text{SEC}
\end{align*} \]

\[ \text{Fig. 3. Photoreduction of DPIP by spinach chloroplasts in the absence and in the presence of inhibitors.} \]

\[ \text{2-second light of 10-m\mu bandwidth at 680 m\lambda was used for illumination. Incident intensity was 6 \times 10^4 \text{ erg/cm}^2\text{sec. Concentrations of chlorophyll and DPIP and the instrument sensitivity were the same as in figure 1.} \]
initial rapid reduction became somewhat less and was followed by an oxidation. Addition of 5 μM benzyl viologen (BV) doubled the slope of the oxidation reaction. The 590 mAU absorbance change was solely due to DPIP re-oxidation and not due to BV reduction, since negligible absorbance change was observed at 385 mAU.

Discussion

Reduction of DPIP at sites belonging to the 2 pigment systems has been demonstrated by selective excitation, by physical fractionation of the 2 pigment systems, and by selective inhibition. The 2 reduction sites can apparently function in isolated systems, as evidenced with the subchloroplast particles and sonicated chloroplasts.

The PS1 reaction has a rapid rate of onset. The amount of DPIP reduced was approximately equivalent to P700 photoreduced, indicating that DPIP probably was reduced by the primary electron donor of PS1. This was further supported by experiments showing that addition of benzyl viologen accelerated the kinetics of the rapid DPIP reduction.

Reduction of DPIP is presumably effected by some photoreductant which receives electrons from PS2.

The action of DCMU, HQNO and antimycin A appear to be consistent with their known functions and support the concept of 2 sites for DPIP photoreduction. CCCP at 10 μM, however, eliminated the PS2 reduction. This agrees with the finding of DeKiewiet et al. (1) that a lower concentration of CCCP uncouples photophosphorylation, but a higher concentration also inhibits \( \text{O}_2 \) evolution.

The photooxidation shown in figure 4 in the presence of both oxidized and reduced dyes indicate that photoreduction of DPIP and photooxidation of \( \text{DPIP}^2+ \) probably form a sequential electron transfer, possibly similar to that responsible for the oxygen-catalyzed photophosphorylation found by Trebst and Eck (7). Increased rate of photooxidation of \( \text{DPIP}^2+ \) in the presence of BV (shown by the third recording in fig 4) is consistent with the suggestion of a sequential reaction, in which BV is reduced by the primary photoreductant of PS1 and reoxidized by oxygen, thus enhancing the rate of entry of reduced DPIP on the oxidizing side of PS1.

**Literature Cited**


