A Lipid Requirement for Photosystem I Activity in Heptane-extracted Spinach Chloroplasts

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ABSTRACT

A lipid requirement for photosystem I activity in Spinacia oleracea chloroplasts has been characterized. The transfer of electrons from tetramethyl-p-phenylenediamine through the chloroplast photosystem to viologen dye was used as an assay of photosystem I activity. Activity is diminished by prolonged heptane extraction and is partially restored by readdition of the extracted lipid. Extracted chloroplasts require plastocyanin for maximal restoration of activity. The effect of lipid extract in restoration is partially replaced by triglycerides containing unsaturated, C18 fatty acids. Various potential reduct carriers which occur naturally in chloroplasts do not substitute for extracted lipid. Galactolipids, sulfolipids, and phospholipids are not involved in the restoration of activity.

In 1957, Lynch and French (12) demonstrated that lyophilized chloroplasts could be extracted with an organic solvent to remove a lipid essential for photosynthetic activity. Plastoquinone was the first compound characterized which would restore photosynthetic activity lost on solvent extraction (3, 5). Subsequent work has tended to localize the function of plastoquinone A in photosystem II. We have employed similar extraction techniques in the investigation of photosystem I.

In 1967, Henninger and Crane (8) reported a decrease in photosystem I activity after heptane extraction with ascorbic acid and TMPD as electron donor system. The effect of extraction could only be fully reversed on addition of an unspecified component of the lipid extract plus a water soluble protein fraction. Elstner et al. (6) in 1967 reported that plastocyanin restores photosystem I activity which is lost after heptane extraction.

The work to be reported in this paper amplifies and extends these observations on the role of lipids in photosystem I.

MATERIALS AND METHODS

Preparation of Chloroplasts. Fresh spinach (Spinacia oleracea) was obtained from the local market. Batches of 300 g of leaves were used for the preparation of chloroplasts by the method of Jagendorf and Avron (10). The pelleted chloroplasts were re-suspended in 150 ml of distilled water and centrifuged at 20,000 g for 20 min. The resulting broken chloroplasts were re-suspended in about 15 ml of distilled water and quickly frozen onto the walls of a 500-ml round bottom flask in a Dry Ice-acetone bath. These chloroplasts were then lyophilized. The chloroplasts were kept dark during lyophilization and all periods of storage.

Chlorophyll concentration was measured by the method of Arnon (1).

Extraction of Chloroplasts. Lyophilized chloroplasts from 500 g of spinach leaves (approximately 300 mg chlorophyll) were homogenized briefly with about 50 ml of dry, freshly distilled n-heptane in a glass homogenizer fitted with a ground glass pestle. The homogenate was placed in an Erlenmeyer flask and made up to 500 ml with n-heptane. The heptane suspension of chloroplasts was slowly stirred at 23°C on a magnetic stirrer for approximately 12 hr. Then the chloroplasts were separated from the extract by vacuum filtration on a sintered glass filter. The chloroplasts were scraped from the filter and traces of heptane removed by lyophilization for 1 hr. The extracted chloroplasts were stored below 0°C in the dark for several weeks without perceptible decrease in activity to be measured. The heptane extract was always yellow as it came through the filter in good, dry preparations. Occasionally we obtained a green heptane extract; in these instances the chloroplasts did not respond well to readdition of extracted lipid. The heptane extract from the chloroplasts was evaporated to dryness on a flash evaporator and redissolved in 10 ml of 2,2,4-trimethylpentane (isooctane). The concentrated lipid extract was always dark green in color.

Reconstitution of Extracted Chloroplasts. To test restoration of activity, an appropriate amount of the extracted lipid or of some other compound was dissolved in 0.2 ml of isooctane. This lipid solution was then added to a 15-mlg sample of extracted chloroplasts, and the isooctane was removed under vacuum. The sample was placed on a lyophilizer for 1 hr to remove the last traces of solvent. The reconstituted chloroplasts were taken up in 3 ml of 0.01 M tricine buffer, pH 7.5, which gave a chlorophyll concentration of about 0.3 mg per ml and were promptly used for activity measurements.

Assay Measurements. Photosystem I activity was measured with sodium ascorbate and TMPD as the electron donor system and methyl viologen as the electron acceptor (9). Assay components are as shown in Table I. Oxygen uptake accompanying autoxidation of methyl viologen was monitored continuously with a YSI oxygen electrode equipped with a recorder. All values reported for rate of oxygen consumption were corrected for the dark oxidation rate which never exceeded 20 μmoles oxygen per mg Chl per hr.

The reaction cell was illuminated with a 750 w projection lamp equipped with a Baird Atomic narrow band pass filter (wavelength maximum 685 μm) between the lamp and the reaction cell. Light intensity within the reaction vessel was 8 x 106 ergs per cm² per sec. The reactions were run at 25°C.

Thin Layer Chromatography. Thin layer plates 20 cm x 20 cm

1 Supported under grants from the Institute for General Medical Science, GM10741, GM01195, and K6-21,839.
2 Abbreviations: TMPD: N,N',N'',N'''-tetramethyl-p-phenylenediamine; DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl: chlorophyll.
were spread 0.5-mm thick with a 50% G-HR and 50% G-HR/UV (Brinkmann) slurry of silica gel and activated by heating at 110 C for 45 min. These were streaked with approximately 15 mg of lipid extract and developed in a 1/1 ratio of chloroform and heptane (v/v). Separated lipids were then recovered in distinct bands on the plate as indicated below.

**Preparation of Plastocyanin.** A partially purified plastocyanin preparation was made by the method described by Böger et al. (4). Plastocyanin was recovered from the pH 8 column and purified on a second diethylaminoethyl cellulose column run at pH 7.0.

**Isolation and Measurement of Complex Lipid.** The galactolipids in a total lipid extract of spinach leaves were separated from neutral lipids and phospholipids on a Florisil column (14).

Galactolipids and sulfolipids were detected on the thin layer chromatograms, eluted, and their concentration measured spectrophotometrically (15).

Phosphorus analysis was carried out by the method of Bartlett (2).

**RESULTS**

Table I shows the reaction requirements of the reconstituted system. The reaction requires all components with the exception of DCMU. DCMU does not inhibit, which shows that the reaction is exclusively based on photosystem I.

Table II shows the decrease in photosystem I activity on extraction of chloroplasts and the restoration of activity on readdition of extracted lipid. Plastocyanin also stimulates the activity of heptane-extracted chloroplasts. The control (lyophilized) rate of

<table>
<thead>
<tr>
<th>Assay System</th>
<th>μMoles O₂ Consumed per mg Chl per hr</th>
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<tbody>
<tr>
<td>Complete system</td>
<td>295</td>
</tr>
<tr>
<td>Minus DCMU</td>
<td>280</td>
</tr>
<tr>
<td>Minus methyl viologen</td>
<td>60</td>
</tr>
<tr>
<td>Minus ascorbate</td>
<td>0</td>
</tr>
<tr>
<td>Minus TMPD</td>
<td>30</td>
</tr>
<tr>
<td>Minus chloroplasts</td>
<td>0</td>
</tr>
<tr>
<td>Boiled chloroplasts</td>
<td>0</td>
</tr>
<tr>
<td>Minus light</td>
<td>0</td>
</tr>
</tbody>
</table>

Table I. Reaction Requirements for the Measurement of Photosystem I Activity in Reconstituted Chloroplasts

The complete system contains in a 3-ml assay chamber the following amounts of components in micromoles: Tricine (pH 7.5): 75; DCMU: 0.03; TMPD: 0.5; sodium ascorbate: 10.0; methyl viologen: 0.4; and chloroplasts containing 30 μg of chlorophyll.

500 reported in Table II is low since this rate can be as high as 1000 μmoles per mg Chl per hr in the best chloroplast preparations. Although we have tried a variety of extraction methods, we have been unable to get good restoration if the extracted chloroplasts show a rate much below 100. Either lipid extract or plastocyanin alone give good stimulation but maximal rates require the presence of both.

Figure 1 shows the relation between activity and amount of extract added to extracted chloroplasts in the presence and absence of plastocyanin. The graph in Figure 1 indicates that a 3-fold excess of extracted lipid over the amount extracted saturates the system either in the presence or absence of plastocyanin. In this experiment, plastocyanin gave a uniform stimulation at all concentrations of lipid extract tested. Plastocyanin always has a significant stimulatory effect but occasionally the addition of plastocyanin nearly masks the lipid effect.

Figure 2 shows saturation curves for plastocyanin in the absence and presence of added lipid extract. In these curves the activity at high plastocyanin concentrations is increased only slightly in the presence of added lipid extract.

The extracted lipid was fractionated by thin layer chromatography to yield two active fractions. One active fraction (band 1) occurs from about 2 cm above the origin to just above where plastoquinone A runs. A second active fraction (band 3) occurs near the top of the plate, within the β-carotene band. Lipids recovered from the origin and from the area between bands 1 and 3 had no stimulatory effect. As is indicated in Table III, bands 1 or 3 alone gave stimulation of activity, but the best rate occurred in the presence of lipid from both bands 1 and 3.

Several compounds known to be present in chloroplasts and implicated in electron transport were tested for activity. Each compound was tested in the concentration range in which it has been reported to occur naturally. Plastoquinone A, a mixture of

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### Table II. Effect of Heptane Extraction and Reconstitution on Photosystem I Activity

Lipid refers to heptane extract with three times the amount added back to extracted chloroplasts as that removed (3-fold excess). Plastocyanin (50 μmol) was added back where indicated. Assay conditions are as in Table I.

<table>
<thead>
<tr>
<th>Chloroplast Preparation</th>
<th>μMoles O₂ Consumed per mg Chl per hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyophilized</td>
<td>500</td>
</tr>
<tr>
<td>Heptane extracted</td>
<td>80</td>
</tr>
<tr>
<td>Extracted + lipid</td>
<td>250</td>
</tr>
<tr>
<td>Extracted + plastocyanin</td>
<td>280</td>
</tr>
<tr>
<td>Extracted + plastocyanin + lipid</td>
<td>475</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Saturation curves for heptane extract in the reconstitution of extracted chloroplasts in the presence and absence of plastocyanin. The curve with plastocyanin represents 50 μmol of plastocyanin in each assay. Assay conditions are as in Table I.
plastoquinones C and D, β-carotene, vitamin K₁, and chlorophyll gave no significant stimulation in activity. Lecitin and sitosterol had no stimulatory effect.

A survey of various simple lipid esters demonstrated that monolen, methyl oleate, tristearin, and trilaurin gave no stimulation. However, as is shown in Table IV, diolein, triolein, trilinolenin, trielaidin, and trilinolenin each gave an increase in activity. Trilinolenin, the most active, gave about 60% of the rate achieved with the unfraccionated heptane extract. All of these compounds gave maximal stimulation at about the same concentration. Figure 3 shows a saturation curve for restoration of photosystem I activity with triolein.

Chloroplasts contain large amounts of galactosyl diglycerides containing unsaturated C₁₈ fatty acids. We therefore tested these compounds for activity. A Florsil column separates neutral lipids from the more polar galactolipids, sulfolipids, and phospholipids (14). Only the neutral lipid fraction restored activity to extracted chloroplasts. This fraction gave stimulation comparable to that achieved with the unfraccionated heptane extract.

Galactolipids and sulfolipids can be detected in low concentration on thin layer chromatograms and quantitatively measured spectrophotometrically (15). Analysis of heptane-extracted lipids by these methods indicated that some galactose-containing lipid is extracted but it remains near the origin on our thin layer chromatograms.

Phosphorus analysis of the heptane extract showed that very little phospholipid was extracted.

**DISCUSSION**

Chloroplast photosystem I activity is stable and is not now known to be susceptible to specific inhibitors. The ascorbate/TMPD to methyl viologen reaction is particularly sensitive, convenient, and requires no added enzymes for measurement of photosystem I activity. The activity of this reaction in freshly isolated chloroplasts exceeds 1000 μmoles oxygen consumed per mg Chl per hr.

Lyophilization and extraction with n-heptane drastically reduce photosystem I activity, and this activity is significantly restored on readdition of the extracted lipid. Plastocyanin, known to be released by heptane extraction (6), stimulates the heptane-extracted chloroplasts and improves the restoration of activity in chloroplasts reconstituted with heptane extract.

**Table IV. Compounds which Stimulate Heptane-extracted Chloroplasts**

Rate values are expressed as percentage of the rate achieved on addition of unfraccionated heptane extract to extracted chloroplasts.

<table>
<thead>
<tr>
<th>Addition to Extracted Chloroplasts</th>
<th>Stimulation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diolein</td>
<td>25</td>
</tr>
<tr>
<td>Triolein</td>
<td>50</td>
</tr>
<tr>
<td>Trilinolenin</td>
<td>50</td>
</tr>
<tr>
<td>Trielaidin</td>
<td>50</td>
</tr>
<tr>
<td>Trilinolenin</td>
<td>60</td>
</tr>
</tbody>
</table>

**Table III. Distribution of Activity in Heptane Extract Fractionated by Thin Layer Chromatography**

Conditions of chromatographic separation are described under "Materials and Methods." Location of bands 1 and 3 are explained in text. Assay conditions are as in Table I.

<table>
<thead>
<tr>
<th>Chloroplast Preparation</th>
<th>μMoles O₂ Consumed per mg Chl per hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyophilized</td>
<td>800</td>
</tr>
<tr>
<td>Extracted</td>
<td>150</td>
</tr>
<tr>
<td>Band 1 lipids</td>
<td>320</td>
</tr>
<tr>
<td>Band 3 lipids</td>
<td>250</td>
</tr>
<tr>
<td>Bands 1 + 3 lipids</td>
<td>390</td>
</tr>
</tbody>
</table>

**Fig. 3.** Saturation curve for restoration of Photosystem I activity with triolein. Assay conditions are as in Table I.
Attempts to purify the active lipid from the heptane extract have led to the conclusion that several stimulatory compounds are present. Purification was complicated by the fact that the fractionated lipid extract lost activity after a day or two although un fractionated extract is stable for several weeks at -15 C.

On fractionation by thin layer chromatography, activity occurred in regions of the chromatating containing plastoquinones, vitamin K, α-tocopherolquinone, and β-carotene but none of these compounds in a purified state would stimulate activity.

The discovery that triglycerides would stimulate activity prompted us to test several glyc er esters for restoration of activity. Of the compounds tested, it appears that triglycerides containing unsaturated C18 fatty acids give the most dramatic stimulation. Although most of the nonstimulatory lipids tested had no measurable effect, oleic acid inhibited the residual activity of extracted chloroplasts (11).

Authentic triglycerides have chromatographic mobility which corresponds to one of the active fractions (band 1) from the chloroplast heptane extract. An assay for esters (7) in the band 1 area of a thin layer chromatogram demonstrated the presence of esters in this fraction.

One micromole of trinolenin per 15 mg of extracted chloroplasts represents about 1 μmole of triglyceride per micromole of Chl. This amount of triglyceride is nearly saturating in restoring activity lost on heptane extraction. A minimum of about 2 mg of band 1 lipid is required for restoration of activity in extracted chloroplasts. This is similar to the amount of purified triglyceride needed for similar restoration.

Triglycerides are reported to be absent (13) in chloroplasts. In contrast, galactolipids, sulfolipids, and phospholipids are all present in easily detectable concentrations (16). It was important, therefore, to test these compounds for possible ability to restore activity to extracted chloroplasts. Phospholipid does not appear to be extractable by n-heptane. Although galactose-containing lipids are extracted, they remain near the origin on our thin layer chromatograms and are not detected in active regions of the plate.

We have concluded that a fairly nonpolar lipid (or lipids) is responsible for restoration of activity and perhaps chloroplast triglycerides contribute to this response. It seems reasonable to assign a structural rather than a redox role to this lipid.

LITERATURE CITED