Photochemical Activities of Spinach Chloroplasts Following Treatment with the Detergent Triton X-100

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A number of detergents have been used to convert chloroplasts into smaller complexes for more intensive study of the properties of the photosynthetic apparatus (16). In an early series of experiments, Smith treated chloroplasts with digitonin, bile salts, sodium deoxycholate and sodium dodecyl sulfate (23-25). The first 3 detergents removed and solubilized the chlorophyll while the latter split the chloroplasts into protein units containing the pigment. Other investigators have employed Zephran chloride, Tween 20 and saponin (10), Duponol C and span 80 (4), and several anionic and cationic detergents (15). Digitonin treatment of chloroplasts has been used to release a photooxidative activity (5, 9, 13, 20) and to produce separable chloroplast fractions which contain altered chlorophyll ratios and appear to represent a separation of pigment system I (1, 3, 31).

Triton X-100 effectively ruptures intact chloroplasts, and at low concentrations disrupts the sequential electron flow required for the Hill reaction (6, 10, 22). Sauer and Park reported that in contrast to other detergents tested, Triton X-100 causes a change in the fluorescence properties of the chloroplast at concentrations below those required to inhibit the Hill reaction (22), indicating a facile interaction of this detergent with the chlorophyll of the chloroplast. The experiments of Vernon et al. showed that chloroplasts solubilized by Triton X-100 catalyze photochemical redox reactions similar to those catalyzed by purified chlorophyll a solubilized by the same detergent (28, 30). Furthermore, Kahn isolated a chlorophyll-protein complex from chloroplasts by treatments with Triton X-100 (7).

In contrast to the solubilizing effect of Triton X-100 at high concentrations, lower amounts produce rather specific effects upon the intermediate electron transport complex of chloroplasts, inhibiting photosynthesis and the related pH shift while stimulating electron flow (6, 18, 19). Because of its many effects upon the photosynthetic apparatus, an investigation into the action of this detergent upon a variety of photochemical reactions of spinach chloroplasts has been conducted and serves as the basis for this paper.

Methods

For chloroplast preparation approximately 40 g of market spinach were deveined and then macerated in a Servall Omnimixer with ice cold 0.02 M Tris, pH 7.8, which also contained 0.35 M NaCl. The macerate was strained through 8 layers of cheesecloth and centrifuged at 1000 x g for 10 minutes. The sediment was washed by centrifugation in the same buffer solution and resuspended in 2 ml of 0.5 M sucrose which was also 0.1 M in potassium phosphate buffer pH 7.5. The final suspension, which contained 2.5 to 3 mg chlorophyll per ml retained its activity in the Hill reaction for several hours.

The detergent Triton X-100, a nonionic alkylphenoxy polyethoxyl ethanol, is reported to have 9 to 10 oxyethylene groups per molecule, making an average molecular weight of 625. The molar absorptivity in water is reported to be 1.33 x 10^4 at 275,5 mJ (Rohm and Haas Company, Philadelphia, Pennsylvania, Publication SAN 200-1, SP-168) which agrees with measured values of freshly prepared dilute solutions whose percentage composition was based on volume measurements. After a few days standing a slight decrease in peak height is observed.

The photochemically induced electron transfer reactions were determined spectroscopically in 3.0 ml of reaction mixture containing either phosphate or Tris and 30 to 80 mg chlorophyll. Most experiments were performed under aerobic conditions, but some were performed anaerobically with the modified Thunberg tube previously described (30). Anaerobic conditions were obtained by alternate evacuation and flushing with argon gas. The reaction mixtures were illuminated through a red filter (Corning No. 2403) by means of a tungsten filament lamp. The light intensity reaching the reaction mixture was approximately 2 x 10^8 ergs/cm^2 per sec. The absorbancy changes caused by illumination were determined with a modified Beckman DB recording spectrophotometer as previously described (33).

Partially purified spinach ferredoxin was prepared according to the directions of San Pietro and Lang (21). This preparation also contained NADP reductase and some plastocyanin. In some experiments ferredoxin which had been purified by chromatography on DEAE columns was employed (17). Plastocyanin was prepared according to the directions of Katoh et al. (8), and cytochrome f according to the procedure reported by Keister (12). The purified spinach ferredoxin and the cytochrome f were furnished by Dr. D. Keister, and the plastocyanin

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3 The abbreviations used are as follows: DPPI, 2,6-dichlorophenolindophenol; TMPD, N,N,N',N'-tetramethyl-phenylenediamine; TMO (TMOH), the oxidized (reduced) form of trimethylphenazine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.
Results

The presence of Triton X-100 at low concentrations uniformly causes a stimulation of Hill reactions (photoreduction of an added electron acceptor coupled to O₂ evolution) by spinach chloroplasts. Figure 1 shows the data obtained for 2 typical oxidants, ferricyanide and DPIP. Although not investigated in as much detail, similar results were obtained using indigo carmine, FMN and phenazine methosulfate as Hill oxidants. The sharp stimulation of ferricyanide activity shown in figure 1, which occurs over a narrow concentration range, could only be observed by using dilute solutions of the detergent in conjunction with careful mixing techniques to prevent contact of the chloroplast with concentrated detergent. Throughout this investigation the experiments were performed without an incubation period prior to illumination (except for the anaerobic experiments in which the detergent would be in contact with the chloroplasts during the experimental manipulation). Some experiments were performed to determine the effect of a prior incubation period with the detergent. Whereas maximal stimulation for the usual procedure was normally obtained at 0.007% final concentration of Triton X-100, a 5-minute incubation period caused a loss in activity and resulted in a shift of the maximum to 0.0055% detergent with ferricyanide. Less stimulation was shown with DPIP, which is probably due to the partially uncoupled state of the chloroplasts in the presence of DPIP.

Neumann and Jagendorf (18,19) and Izawa and Good (6) have reported that low concentrations of Triton X-100 uncouple photophosphorylation and stimulate ferricyanide photoreduction by spinach chloroplasts. We have examined the effect of this detergent upon noncyclic photophosphorylation (table 1). At concentrations of Triton X-100 which stimulate ferricyanide photoreduction, the ability to form ATP is almost entirely lost, which agrees with the data previously reported (6,19). Furthermore, as shown in table II, the stimulation of ferricyanide photoreduction is elicited by either ammonium ion or detergent, but once the system is uncoupled addition of either agent causes no further increase in the rate of electron flow. These data are consistent with a close relationship between the uncoupling effect of these agents and the increase in rate of electron flow.

Table I. Inhibition of Photophosphorylation by Triton X-100

<table>
<thead>
<tr>
<th>Phosphorylation type</th>
<th>Triton X-100 %</th>
<th>ATP formed/hr per mg chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncyclic with ferricyanide</td>
<td>0</td>
<td>83.1</td>
</tr>
<tr>
<td></td>
<td>7 × 10⁻⁴</td>
<td>77.2</td>
</tr>
<tr>
<td></td>
<td>1.7 × 10⁻³</td>
<td>43.8</td>
</tr>
<tr>
<td></td>
<td>3.3 × 10⁻³</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>5.0 × 10⁻³</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>6.7 × 10⁻³</td>
<td>0</td>
</tr>
</tbody>
</table>

Table II. Effect of Ammonium Ion and Triton X-100 on Ferricyanide Photoreduction by Spinach Chloroplasts

<table>
<thead>
<tr>
<th>Addition</th>
<th>Triton X-100 %</th>
<th>Photoreduction amoles/hr per mg chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄ mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>122</td>
</tr>
<tr>
<td>0</td>
<td>6.7</td>
<td>470</td>
</tr>
<tr>
<td>0</td>
<td>13.3</td>
<td>0</td>
</tr>
<tr>
<td>0.67</td>
<td>0</td>
<td>315</td>
</tr>
<tr>
<td>1.34</td>
<td>0</td>
<td>540</td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>600</td>
</tr>
<tr>
<td>1.34</td>
<td>6.7</td>
<td>625</td>
</tr>
<tr>
<td>2.0</td>
<td>6.7</td>
<td>590</td>
</tr>
</tbody>
</table>
plast is broken down into simpler units and the chlorophyll is solubilized to such an extent that it is available for photoreactions of this type (30). For comparison purposes, chloroplast fragments obtained by sonication were also examined in this reaction, showing that such particles (which have very little ability to form ATP) are not stimulated at low detergent concentrations. They do show the usual response at high concentrations of Triton X-100, however. Further evidence of the more simple nature of the photochemical reaction at high detergent concentrations is shown by the fact that the reaction is not light saturated. At the light intensities used, all of the reactions carried out at low detergent concentrations were saturated.

Chloroplasts gain the ability to photooxidize cytochrome c after treatment with detergents. Nieman and Vennesland first described this activity for digitonin-treated chloroplasts (20), and the reaction has been studied in more detail by Katoh and Takamiya (9) who showed the dependency of the reaction on added plastocyanin. Kok et al. (13, 14) employed the detergent Tween 20 to alter spinach chloroplasts and they have studied the photooxidation of cytochrome c, cytochrome f and plastocyanin.

We have followed cytochrome c photooxidation by chloroplasts treated with Triton X-100, as shown in figure 3. In our investigation the photooxidation was coupled to either O₂ or to NADP in the absence of O₂. In both cases the activity did not appear until the concentration of detergent was greater than 0.01 %, a concentration at which activity in the Hill reactions is mostly inhibited. The inhibitor DCMU was added to the reactions at Triton concentrations below 0.02 % to inhibit the cytochrome photoreduction activity normally shown by chloroplasts.
the reaction, cytochrome f was effective only when added with plastocyanin, indicating that plastocyanin may be more closely involved with the photochemical step and cytochrome f acts via plastocyanin. Our data are in opposition to those obtained by Kok et al. with the detergent Tween 20, in which case both plastocyanin and cytochrome f individually stimulated the rate of cytochrome c photooxidation (13). We have not ruled out, however, the possibility that sufficient cytochrome f remains attached to the particle in our experiments to allow maximal rates in the absence of added cytochrome f. As expected, plastocyanin also stimulates the rate of cytochrome c photooxidation coupled to NADP photoreduction under anaerobic conditions, as shown in figure 5. To show this requirement for plastocyanin, purified spinach ferredoxin was used to couple with NADP. In the experiments reported by Whatley a partially purified preparation of ferredoxin was used, which would contain some plastocyanin (32). Consequently, the need for added plastocyanin would not be apparent in those experiments.

Fig. 4. Plastocyanin requirement for aerobic cytochrome c photooxidation by spinach chloroplasts in the presence of Triton X-100. The experimental procedures were those given for figure 3. Chloroplasts equivalent to 0.08 mg chlorophyll were present. Plastocyanin (0.003 μmole) or cytochrome f (0.77 mg of a 50% purified preparation) were added as indicated. The concentration of Triton employed was 0.1%. The photooxidation rates for the 3 illumination periods of A are, in sequence, 24, 22, and 73 μmoles/hr per mg Chlorophyll. The rates of B arc 29, 61 and 80.

Whereas the cytochrome photooxidase activity (with O₂) was maintained at higher detergent concentrations, cytochrome oxidation coupled to NADP reduction was observed only within a rather narrow range of detergent concentration. This does not represent a failure to couple to NADP (see the next section), but probably is due to a cyclic oxidation and reduction of cytochrome c under these conditions. Spinach ferredoxin must necessarily be added to allow NADP photoreduction to proceed. It will also serve as a cofactor for cytochrome c photoreduction, and thus could facilitate the cyclic flow of electrons through cytochrome c. The rates reported in figure 3 are lower than those reported previously (9, 13), but the reactions reported here are for basal reactions which have not been stimulated by the addition of plastocyanin.

Figure 4 shows the effect of adding purified plastocyanin and cytochrome f to chloroplasts treated with Triton. Whereas plastocyanin alone stimulated

Fig. 5. Plastocyanin requirement for cytochrome c photooxidation coupled to NADP under anaerobic conditions. Spinach chloroplasts (0.08 mg chlorophyll) were used. The experiments were performed as described in figure 3, except that 0.3 mg of purified spinach ferredoxin (specific activity 31) was used. The final concentration of Triton X-100 was 0.023%. Where indicated 0.003 μmole of plastocyanin was used.

Photoreduction of NAD[P]. Chloroplasts are capable of photoreducing NADP with either water or a substitute reductant as the source of electrons (29). In the former case both pigment systems of the chloroplast are required, while in the latter case only pigment system I is involved. Consequently, this reaction is much more stable. The effect of Triton upon both of these reactions has been investigated, and figure 6 presents some of these data. Both reactions show the usual stimulation at low Triton concentrations, followed by an inhibitory range. In this case the maximal stimulation occurs at 0.008 to 0.009% Triton. At high Triton levels, the photoreduction supported by DPIP and ascorbate increases in magnitude, while no NADP reduction is observed in the absence of this electron feeding system. Chloroplast fragments show only slight stimulation

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at 0.008 % Triton, which again reflects the fact that they have very weak phosphorylating ability. At high Triton, however, they also support the photoreduction of NADP in the presence of ascorbate and DPIP. All these data agree with those presented above for other Hill reagents, showing the destruction of the intermediate electron transfer complex required for photosynthesis, and the emergence of photoreactions catalyzed by pigment system I as the concentration of detergent increases. The rates shown in figure 6 are initial rates, and consequently are higher than those reported if a 1- or 2-minute period is used for calculation of rates. Typical tracings observed for NADP photoreduction are shown below in figure 9.

The photoreduction of NADP at high Triton levels has been investigated in more detail. As shown in figure 7, ascorbate alone is unable to effectively supply electrons for the reaction, but when coupled with DPIP a photoreduction of NADP occurs. This response is similar to that observed with intact chloroplasts which have been poisoned with DCMU or have been aged to destroy the O₂ evolving system (29).

Plastocyanin is also required for the photoreduction of NADP by chloroplasts at high Triton concentrations, using ascorbate-DPIP as the electron feeding system. Figure 8 shows that no reaction occurs with chloroplasts alone, and the reaction is not elicited by the addition of either purified spinach ferredoxin or plastocyanin when they are added separately. When added together, however, the usual rate of NADP photoreduction is observed. These experiments also indicate that the flavoprotein, NADP reductase, is not required. It is probably supplied by the chloroplasts themselves, a condition that again parallels the situation observed with intact chloroplasts.

**Fig. 6.** Photoreduction of NADP by spinach chloroplasts in varying concentrations of Triton X-100. The reactions were followed spectrophotometrically at 340 nm. Chloroplast fragments obtained by sonication were prepared as per figure 2. The reaction mixtures (3 ml) contained phosphate buffer and sucrose as given in figure 1 plus 0.5 mM NADP, 2 mg partially purified spinach ferredoxin and spinach chloroplasts equivalent to 0.07 mg chlorophyll. Where indicated 0.13 mM DPIP and 0.67 mM ascorbate were also added, and in these reactions 0.01 mM DCMU was added to inhibit O₂ evolution. The rates plotted are initial rates.

**Fig. 7.** Requirements for DPIP for NADP photoreduction in the presence of chloroplasts and ascorbate at high Triton X-100 concentration. The reaction system contained the components listed in figure 6 for the ascorbate-DPIP supported reactions, except that DPIP was added where indicated. Partially purified spinach ferredoxin was used, which contains the plastocyanin required for the reaction. The concentration of Triton was 0.1%.

**Fig. 8.** Plastocyanin requirement for NADP photoreduction by spinach chloroplasts with ascorbate-DPIP at high Triton concentration. The experimental procedures outlined for figure 6 were employed except that purified spinach ferredoxin was used and 0.08 mg chlorophyll was present. For experiment A, 0.003 μmole of plastocyanin was present initially and the photoreaction proceeded only when 0.3 mg purified ferredoxin was added. For experiment B, purified ferredoxin was present initially and plastocyanin added to allow the reaction to proceed. The concentration of Triton was 0.1%.
It is possible to show a stimulation by plastocyanin of NADP photoreduction by chloroplasts utilizing water as the electron donor (NADP Hill reaction). Figure 9 presents tracings obtained for NADP photoreduction, and shows both the marked stimulation of NADP reduction obtained at 0.007% Triton X-100 and the inhibition observed at 0.01% detergent. When plastocyanin was added to chloroplasts treated with 0.01% detergent a marked stimulation in rate was obtained, indicating that at this concentration some of the plastocyanin had been removed (or inactivated) and added plastocyanin was then able to stimulate the reaction. Purified ferredoxin was used in these experiments, since impure preparations contain some plastocyanin.

Figure 10 shows that a modest stimulation was observed in the rate of NADP photoreduction supported by ascorbate and TMPD. In this reaction TMPD substitutes for DPIP in coupling ascorbate to the photochemical system. This slight stimulation has significance since it is generally held that the photoreduction of NADP by ascorbate-TMPD does not involve ATP formation (26). This will be discussed below.

**Discussion**

As the concentration of Triton X-100 is increased the photochemical activities of treated spinach chloroplasts go through 3 phases: an initial stimulation of electron transfer related to an uncoupling of photo-phosphorylation, a subsequent inhibition of electron transfer reactions, followed by a reappearance of some of the simpler reactions at high Triton concentrations. These 3 responses will be discussed separately.

*Stimulation of Electron Transfer Reactions at Low Concentrations of Triton X-100.* A marked stimulation of several electron transfer reactions of chloroplasts occurs within a small range of Triton concentrations. The maximum usually occurs at about 0.007% Triton but occurs at a slightly higher value for NADP photoreduction. This difference could be due to the added spinach ferredoxin required for NADP reduction, but this possibility has not been explored. The marked increase in electron transport is accompanied by a loss in ATP forming ability, indicating that Triton is acting as an uncoupling agent, as has been proposed previously (19). What the exact relationship is between this uncoupling and the structural changes brought about by the detergent remains to be determined. It would appear, however, that the uncoupling of phosphorylation from photosynthetic electron transport may not be the only effect operative, since figure 10 shows that stimulation is also observed for ascorbate-TMPD photoreduction of NADP, a photo-reaction which is reported to proceed without an accompanying phosphorylation (26). It is possible that the same structural modification which prevents phosphorylation results in more efficient electron flow in other portions of the electron transport chain also.

The ratio of chlorophyll to Triton at the stimulating concentration is 5 detergent molecules per chlorophyll, which is higher than reported by Sauer and Park (22). This is probably a function of total chlorophyll concentration and incubation time.

*Inhibition at Intermediate Triton Concentrations.* Following the initial stimulation of electron transfer...
chloroplast architecture. All available requirements are transferal.

which does stimutilate that f-produced shows intimate indications that broken chloroplasts evolve.

The reappearance of plastocyanin in the presence of oxygen causes a profound effect. The experimental procedure was that described for figure 2. Where indicated, 0.003 \textmu mol of plastocyanin was added. The rates observed under anaerobic conditions were much faster, and consequently these experiments were performed with only 3.2 \mu g of chlorophyll in the reaction mixture. No TMQ was present and the TMQH₂ was present initially in the side arm. The rates observed were 3980 and 3600 \textmu moles cytochrome reduced per mg chlorophyll in the absence of plastocyanin. The aerobic experiments 0.077 mg of chlorophyll was present. Under these conditions faster rates were observed if both TMQ and TMQH₂ were present, and the reaction mixture contained 33 \mu M TMQ and 33 \mu M TMQH₂. The aerobic rates observed were 71 and 69 \textmu moles/hour per mg chlorophyll in the absence and presence of plastocyanin respectively. The Triton X-100 concentration used was 0.1 %. The aerobic experiments also contained 3.3 \texttimes 10^{-3} M azide to prevent a dark oxidation of the reduced cytochrome.

is a simpler reaction than either NADP photoreduction or the cytochrome c photooxidation. The cytochrome c-TMQH₂ reaction is not stimulated by either spinach ferredoxin or plastocyanin (fig 11), and is catalyzed by purified chlorophyll a solubilized by Triton (30). This reaction proceeds via a preliminary photoreduction of some TMQ contained in the TMQH₂, with the semiquinone form of TMQ then reducing cytochrome c. In this case the excited chlorophyll (P₇₆₅) reacts first with the acceptor molecule TMQI to produce oxidized chlorophyll which is restored to its original state by reaction with the donor TMQH₂.

The photoreactions involving plastocyanin may well proceed by the alternate mechanism, whereby the excited chlorophyll (P₇₆₅) first reacts with the donor molecule to produce reduced chlorophyll. This would agree with the report of Kok et al. (14) that reduced plastocyanin must form a complex with the P₇₆₅ for the photoreaction to proceed. Furthermore, the photoreduction of NADP by plastocyanin...
fragments described above, which requires plastocyanin and is not catalyzed by solubilized chlorophyll a, is clearly different from the simpler reaction catalyzed by the water soluble chlorophyllin or hematoporphyrin (27). The latter reaction does not require ferredoxin, but needs only the flavoprotein NADP reductase to couple the excited porphyrin to NADP. Also, this reaction is supported by ascorbate alone and is inhibited if DTPA is also present.

The chloroplast fragments produced by high Triton levels are probably similar to those prepared by digitonin treatment (1, 3), from which chlorophyll b has been partially removed and only reactions associated with pigment system I can be performed. The availability of such fragments will allow a close comparison to be made between photo-reactions catalyzed by isolated chlorophyll itself and the same pigment in the organization found in the fragments, and will allow more definitive experiments to be done on the mechanism of the initial photo-reduction of excited chlorophyll.

Summary

Spinach chloroplasts treated with various levels of Triton X-100 have been examined for their ability to perform a variety of photosynthetic electron transfer reactions. In general, 3 responses are elicited by increasing concentrations of the detergent.

Low levels markedly stimulate all electron transfer reactions investigated, including ferricyanide, 2,6-dichlorophenolphindolphene, NADP and cytochrome c I Hill reactions. The photoreduction of NADP by ascorbate plus 2,6-dichlorophenolphindolphene is also stimulated. These reactions show maxima at about 0.007% Triton (0.008-0.010 for NADP photoreduction). At these concentrations the photophosphorylation is almost completely uncoupled. This is the major cause of the increased rates of electron flow. However, the photoreduction of NADP supported by ascorbate plus tetramethyl-p-phenyleneediamine is also slightly stimulated in this region. Since this reaction is supposed not to support ATP formation, some additional structural modification of the chloroplast may be involved.

At Triton levels of 0.01 to 0.02% the electron transfer reactions became inhibited. There is some residual activity in the ascorbate plus 2,6-dichlorophenolphindolphene coupled reduction of NADP, however. This inhibition is most likely due to disruption of the oxygen evolving system along with interference of the electron transfer complex. Plastocyanin is removed under these conditions.

Increasing the Triton concentration above 0.02% allows the following reactions to reappear: cytochrome c reduction supported by reduced trimethylbenzoquinone, cytochrome c photooxidation by oxygen, NADP photoreduction supported by ascorbate plus 2,6-dichlorophenolphindolphene and NADP photoreduction coupled to cytochrome c oxidation. Except for the cytochrome photoreduction reaction with reduced trimethylbenzoquinone, plastocyanin is either required or markedly stimulates the reactions. Cytochrome f does not stimulate cytochrome c photo-oxidase activity when added separately, but does increase the rate observed with plastocyanin. Some observations concerning the initial photochemical reaction of chlorophyll in these systems are made.

Literature Cited


