Site-specific Uncoupling of Photosynthetic Phosphorylation in Spinach Chloroplasts*

L. J. Laber and Clanton C. Black

From the Department of Biochemistry, University of Georgia, Athens, Georgia 30601

SUMMARY

In spinach chloroplasts which have been shaken with n-heptane, photophosphorylation coupled to the reduction of nicotinamide adenine dinucleotide phosphate is inhibited about 95% while the photoreduction of NADP+ (and ferricyanide) is stimulated as much as 50%. Photophosphorylation catalyzed by phenazine methosulfate, however, is inhibited only 50%. These data are interpreted to indicate that there are two sites of photophosphorylation in spinach chloroplasts. Both sites are utilized in cyclic photophosphorylation, whereas one of the sites is common to both cyclic and noncyclic photophosphorylation. In heptane-treated chloroplasts the only site functioning is that site associated exclusively with cyclic electron flow. Heptane treatment also has a differential effect upon the adenosine triphosphatase activities of chloroplasts. Trypsin-activated Ca++-dependent ATPase is not affected by heptane treatment, whereas Mg++-dependent ATPase activated by light in the presence of dithiothreitol is inhibited 80 to 90%

After heptane treatment, the ability of chloroplasts to accumulate protons upon illumination is inhibited 40 to 60% and their ability to synthesize ATP when transferred from acidic to alkaline environment is inhibited 50%.

This paper presents data which indicate that treatment of spinach chloroplasts with n-heptane has a preferential uncoupling effect upon photophosphorylation coupled to the reduction of NADP+ and ferricyanide. Since heptane treatment appears to distinguish between two sites of photophosphorylation, experiments were performed to test the influence of heptane treatment upon other activities related to photophosphorylation. In particular the two adenosine triphosphatases, the proton uptake capacity, and the acid-base transition phosphorylation of chloroplasts were investigated relative to the effect of heptane treatment. Preliminary reports of these effects have been made (1-3).

EXPERIMENTAL PROCEDURE

Preparation of Chloroplasts—Chloroplasts were prepared daily from market spinach as described previously (4). The chloroplasts were suspended finally in 0.085 M NaCl and 0.002 M Tris hydrochloride, pH 7.6 to 7.7. This suspension contained 0.7 to 1.1 mg of chlorophyll per ml. Chlorophyll was determined by the method of Arnon (5).

Heptane Treatment of Chloroplasts—The standard treatment consisted of layering 0.1 ml of n-heptane over 1 ml of chloroplasts (about 0.9 mg of chlorophyll per ml) in a test tube, 15 x 150 mm. The tube was put on a Rurrall wrist action shaker (about 300 strokes per min) for 10 sec. The tube was then stored in an ice bucket, and aliquots of chloroplasts were pipetted from beneath the heptane layer immediately before use in the various reactions. The chlorophyll concentration was not affected by this treatment. Time after treatment was measured from the start of the shaking to the time that the chloroplasts were removed from the tube containing the heptane. Fisher Scientific “certified spectranalyzed” n-heptane was used.

NADPH Assay—The reduction of NADP+ was assayed in the presence of the following reagents, in a 1-ml volume: 50 μmoles of Tricine-KOH, pH 7.9; 2 μmoles of MgCl₂; 0.3 μmole of NADP+; 0.1 mg of spinach ferredoxin, 65% pure; and about 20 μg of chlorophyll, as chloroplast fragments. The optical density change at 340 με of the suspension after 1 min of illumination at 2500 foot-candles was taken as a measure of NADP+ reduction.

Ferricyanide Reduction—The reduction of ferricyanide was measured in solutions containing, in 3 ml: 100 μmoles of Tricine-KOH, pH 7.9; 5 μmoles of MgCl₂; 1.2 μmoles of K₃Fe(CN)₆; and chloroplasts (about 30 μg of chlorophyll). The change in optical density at 420 με during 1 to 3 min of illumination at 2500 foot-candles was taken as proportional to ferricyanide reduced.

ATP Assay—ATP formation was measured during noncyclic electron transport. The reaction mixture contained, in 3 ml: 100 μmoles of Tricine-KOH, pH 7.0; 5 μmoles of MgCl₂; 3 μmoles of 32P; 3 μmoles of ADP; and chloroplasts containing about 30 μg of chlorophyll. Where applicable, either 1.2 μmoles of K₃Fe(CN)₆ or 0.5 μmole of NADP+ and 0.3 mg of spinach ferredoxin were added. The reactions were stopped by the addition of 0.3 ml of 20% trichloracetic acid after 1 min of illumination at 2500 foot-candles. ATP was determined by the method of Nielsen and Lehninger (6) as modified by Avron (7).

Assay of ATPase Activity—Two ATPases were investigated. The Mg++-dependent, dithiothreitol- and light-activated ATPase was activated and assayed by the method of McCarty and Racker (8) with a light activation stage of 5 min at 2500 foot-candles. Aliquots of the activation mixture were assayed for ATPase by
incubation with ATP in the dark for 20 min at 37°C. Components of the Mg++-dependent ATPase assay reaction were, in 0.9 ml: Tris hydrochloride, pH 8.0, 50 μmoles; MgCl₂, 5 μmoles; ATP, 5 μmoles; and ATPase, a 0.1-ml aliquot of the activation mixture (about 25 μg of chlorophyll). The ATPase reaction was stopped by the addition of 0.1 ml of 20% trichloracetic acid.

Ca++-dependent ATPase was activated by partial digestion of chloroplasts with trypsin (9). Components of the Ca++-dependent ATPase activity were measured by the procedures of Vambutas and Racker (9). Components of the Ca++-dependent ATPase reaction were, in 0.9 ml: Tris hydrochloride, pH 8.0, 50 μmoles; CaCl₂, 5 μmoles; ATP, 5 μmoles; and ATPase, a 0.2-ml aliquot of the activation mixture (about 25 μg of chlorophyll). The ATPase reaction was stopped by the addition of 0.1 ml of 20% trichloracetic acid.

The extent of hydrolysis of ATP by each ATPase was determined by assaying for P<sub>i</sub> by the method of Taussky and Shorr (10).

**Assay of Light-induced pH Rise**—The pH changes in suspensions of chloroplasts brought about by illumination (11) were observed with the use of an Orion 801 digital pH meter, equipped with a Beckman combination glass electrode, at 20°C in a water-jacketed chamber at an intensity of about 3000 foot-candles.

**Acid-Base Transition Phosphorylation Assay**—Phosphorylation brought about by transition from acidic to basic conditions was investigated by the procedure of Jagendorf and Uribe (12). Chloroplasts (200 μg of chlorophyll) were added to a mixture containing 5 μmoles of glutamic acid, 10 μmoles of succinic acid, 5 μmoles of MgCl₂, and 0.03 μ mole of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (final volume, 0.9 ml). The pH of the mixture of glutamic acid and succinic acid was adjusted with KOH prior to the addition of MgCl₂, dichlorophenylidimethylurea, and chloroplasts such that the final pH was about 4.0. An alkaline mixture was injected into the above preparation 20 sec after the addition of the chloroplasts. This alkaline mixture contained 10 μmoles of Tris hydrochloride, 0.2 μmole of ADP, 2 μmoles of NADP<sup>+</sup>, and sufficient NaOH to bring the total reaction mixture to pH 8.0. After 20 sec at pH 8.0 the reactions were stopped by the addition of 0.2 ml of 20% trichloracetic acid. The <sup>32</sup>P incorporated into ATP was determined as described under “ATP Assay.”

**RESULTS**

**Photophosphorylation and Electron Transport**—Treatment of chloroplasts with heptane as described in “Experimental Procedure” inhibits cyclic photophosphorylation only 50%, even after several hours (1). This level of inhibition is reached within 10 min of the start of the treatment. The addition of 6 μM DCMU and 3 mM ascorbate to the reaction mixtures during assay for cyclic phosphorylation did not affect the extent of the inhibition. In contrast, phosphorylation associated with NADP<sup>+</sup> reduction was inhibited as much as 95%.

Chloroplasts treated with heptane continued to function in electron transport, as measured by the reduction of NADP<sup>+</sup> and ferricyanide. The data presented in Fig. 1 show that NADP<sup>+</sup> reduction in the absence of ADP and P<sub>i</sub> was stimulated for the first hour after heptane treatment. The stimulation in some experiments was as great as 50% above the control rates. Ferricyanide reduction followed a similar trend. Thus, the initial effect of heptane treatment is the inhibition (uncoupling) of ATP synthesis with a resultant stimulation of electron transport.

**TABLE 1**

**Stoichiometry of ferricyanide- and NADP<sup>+</sup>-dependent photophosphorylation in heptane-treated chloroplasts**

Reduction and photophosphorylation with the indicated electron acceptors were carried out on heptane-treated chloroplasts as described under “Experimental Procedure.” After measurement of the absorbance change at the appropriate wave length, the reactions were stopped by the addition of trichloroacetic acid and the amount of NADP incorporated was determined.

<table>
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<th>Electron acceptor</th>
<th>Time after treatment</th>
<th>Treatment</th>
<th>Reduction</th>
<th>ATP formation</th>
<th>NADP&lt;sup&gt;+&lt;/sup&gt;:P&lt;sub&gt;i&lt;/sub&gt;</th>
<th>δ&lt;sup&gt;32&lt;/sup&gt;P incorporation</th>
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heptane treatment upon ferricyanide photophosphorylation is similar to its effect upon phosphorylation catalyzed by NADP+.

In other experiments the duration of the shaking treatment was investigated. Shaking for 30 sec or more eliminated the stimulation of electron transport. We also found that layering the heptane over the suspensions (without shaking) or suspending the chloroplasts initially in buffer saturated with heptane (5.2 X 10^{-4} M heptane) had no effect at all.

In order to be certain that heptane was solely responsible for the uncoupling action, we purified some heptane by the method of Herold and Wolf (13). Treatment of chloroplasts with such heptane produced the same results as treatment with the Fisher spectranaalyzed solvent. Likewise, "critical reactivity reagent" heptane, 99.98 mol %, purchased from Matheson, Coleman and Bell, had the same effects upon photophosphorylation and electron transport in spinach chloroplasts.

Other nonpolar organic solvents were tested with the standard heptane treatment described in "Experimental Procedure." Hexane stimulated NADP+ reduction by about 35% while inhibiting the associated photophosphorylation by about 80% in 15 min. Hexane, however, also inhibited phenazine methosulfate-catalyzed phosphorylation to the same extent during the same time period. Hexane would, therefore, be classified as an uncoupler, but it does not display the selectivity of heptane.

Some uncoupling activity by cyclohexane was also noted. Other organic solvents tested were carbon tetrachloride, benzene, and toluene, all of which inhibited NADP+ reduction. The inhibition by benzene was the greatest: 45 min after treatment, benzene treatments were 30 min after the start of heptane treatment, the Mg++-dependent ATPase activity was inhibited 80 to 90%.

ATPases—The Ca++-dependent ATPase of chloroplasts was found (Fig. 2) to be completely insensitive to the heptane treatment, while 30 min after the start of heptane treatment the Mg++-dependent ATPase activity was inhibited 80 to 90%. The decline of Mg++-dependent ATPase activity during heptane treatment paralleled the decline in ATP synthesis activity more closely than any other activity we investigated.

**Fig. 2.** Effect of heptane treatment upon ATPase activities of chloroplasts. The Ca++-dependent ATPase (activated by tryptophan) and Mg++-dependent ATPase (activated by light in the presence of dithiothreitol) activities were assayed in chloroplasts which had been treated with heptane at zero time. Details of the heptane treatment and assay procedures are described in "Experimental Procedure." The control rates were 300 and 275 μmoles of P_i released per mg of chlorophyll per hour for Ca++-dependent ATPase and Mg++-dependent ATPase, respectively. The Ca++-dependent ATPase activity decreased in the controls by less than 10%, during 3 hours, whereas the Mg++-dependent ATPase activity was constant for the duration of the experiment.

**Fig. 3.** Effect of heptane treatment upon light-induced pH rise and acid-base transition phosphorylation. The measurements of these activities are described in "Experimental Procedure." The extent of the pH change for control chloroplasts was 0.27 pH unit per reaction mixture (200 μg of chlorophyll). The pH rise approached 50% of control within 10 min, and sometimes the yields decreased so slowly that 1 hour after heptane treatment the rates appeared to return to a rate close to that of the control within an hour after heptane treatment. These observations are consistent with the behavior of other uncouplers (11). That is, the pH rise associated with electron transport is inhibited by uncouplers and closer inspection of the process reveals that the inhibition is due to a stimulation of the return of protons to the external medium.

Acid-Base Phosphorylation—The synthesis of ATP induced by rapidly changing the pH of a suspension of chloroplasts from acidic to alkaline (12) also is affected by prior heptane treatment. The data of Fig. 3 show that after heptane treatment the yields of ATP decreased until the yield approached 50% of the control. The data for rate of decay of yield of ATP in these acid-base transitions were variable. Sometimes the yields approached 50% of control within 10 min, and sometimes the yields decreased so slowly that 1 hour after heptane treatment the rates remained slightly above 50% of control. However, the extent of decay always approached 50%.

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**Discussion**

Evidence for two sites of photophosphorylation in chloroplasts of higher plants has been controversial at best. The two types of arguments used have been based upon measurements of stoichiometry or upon differential effects of uncouplers of photophosphorylation. Foremost among the first class have been Izawa, Winget, and Good (14), who observed ATP:2e- ratios as high as 1.3 with ferricyanide as the electron acceptor; correcting for a "basal" electron flow which is not coupled to phosphorylation, they obtained ATP:2e- ratios of 2. However, Del Campo, Ramirez, and Arnon (15) and Black (16) have failed to observe ratios above 1.0. Furthermore, ratios greater than 1 are not seen during NADP+-dependent phosphorylation with the type of chloroplasts used in the present experiments (1).
Several compounds differentially inhibit noncyclic and cyclic photophosphorylation. For example, Bamberger, Black, Fewson, and Gibbs (17) reported that carbonyl cyanide m-chlorophenylhydrazone, 2-heptyl-4-hydroxyquinoline-N-oxide, and 4-nonyl-4-hydroxyquinoline-N-oxide each inhibited NADP+-catalyzed photophosphorylation more strongly than they did that catalyzed by phenazine methosulfate. Similar observations were made by Avron and Shavit (18) for the uncouplers carbonyl cyanide p-trifluoromethoxyphenylhydrazone, octylguanidine, and gramicidin S. Desaspidin was reported by Baltscheffsky and deKiewiet (19) and by Gromet-Elhanan and Arnon (20) to inhibit strongly cyclic phosphorylation, at certain low concentrations, while not affecting noncyclic phosphorylation at all. The dramatic selectivity of desaspidin, however, has been placed in question by the later reports of Gromet-Elhanan and Avron (21) and by Avron and Shavit (22), and others. Thus it appears doubtful that the action of desaspidin indicates the existence of 2 sites of phosphorylation.

In another experimental approach Schroeder and Racker (23) subjected chloroplasts to physical disruption in a Nossal shaker. Photophosphorylation with phenazine methosulfate was inhibited from 30 to 70%, while photophosphorylation coupled to NADP+ reduction was inhibited 70 to 85%. Since these values were made by Avron and Shavit (18) for the uncouplers carbonyl cyanide p-trifluoromethoxyphenylhydrazone, octylguanidine, and gramicidin S. Desaspidin was reported by Baltscheffsky and deKiewiet (19) and by Gromet-Elhanan and Arnon (20) to inhibit strongly cyclic phosphorylation, at certain low concentrations, while not affecting noncyclic phosphorylation at all. The dramatic selectivity of desaspidin, however, has been placed in question by the later reports of Gromet-Elhanan and Avron (21), Urbach and Simonis (22), and others. Thus it appears doubtful that the action of desaspidin indicates the existence of 2 sites of phosphorylation.

Until the effect of heptane was found, no inhibitor or uncoupler of photophosphorylation had had the dramatic differential effect in higher plant chloroplasts that valinomycin has upon two different types of phosphorylation in Rhodospirillum rubrum chromatophores (observed by Baltscheffsky and Arwidsson (24)).

The scheme in Fig. 4 summarizes our interpretation of the data presented. There are two sites of phosphorylation in chloroplasts. One site is associated with an electron transport pathway between the two photosystems. This site is also functional in cyclic photophosphorylation. The second site is on a pathway unique to cyclic electron transport. This scheme predicts that it should be impossible to find an uncoupler which would inhibit phenazine methosulfate-catalyzed phosphorylation by greater than 50% while leaving noncyclic phosphorylation intact. Likewise, any general uncoupler would be expected to inhibit noncyclic phosphorylation by a greater degree than it would cyclic phosphorylation.

**Fig. 4.** Scheme of electron transport, showing two sites of ATP synthesis in spinach chloroplasts. PMS, phenazine methosulfate.
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