Effects of Temperature Pretreatment in the Dark on Photosynthesis of the Intact Spinach Chloroplast

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ABSTRACT
Isolated, intact spinach (Spinacia oleracea L. var. "Long Standing Bloomsdale") chloroplasts were heated in the dark and the effect of this treatment on photosynthetic activities was determined at 25°C. Dark incubation of the chloroplasts for 10 minutes at 35°C and pH 8.1 resulted in a 50% decline in CO2 photoassimilation. This decline in photosynthetic performance was dependent upon time, temperature, and medium pH with the optimum effect at acidic pH values. Photosynthetic decline was not observed if MgATP, MgADP, or a mixture of fructose 1,6-bisphosphate, aldolase, and oxaloacetate or ribose 5-phosphate and oxaloacetate was added prior to but not after the temperature pretreatment. A chloroplast preparation reconstituted with thylakoids and stroma from pretreated (35°C, 10 minutes, pH 8.1) intact chloroplasts and supplemented with ferredoxin, ADP, and NADP was photosynthetically competent, indicating that ATP-coupled electron flow and the enzymes comprising the Benson-Calvin cycle remained stable during the dark treatment. In contrast, exposure of isolated thylakoids to 35°C for 10 minutes uncoupled photosphorylation from NADP and ferricyanide reduction. We propose that the decline of intact chloroplast photosynthesis is the result of a decrease in the content of or a change in the ratios of the adenine nucleotides. Maintenance of an adequate supply of adenine nucleotide is the effect of the externally added MgATP or of chloroplastic respiration of a sugar phosphate.

Many studies have been reported on the effect of temperature pretreatment on photosynthetic electron transport and phosphorylation in isolated thylakoidal preparations (13, 16, 25, 27), but less attention has been paid to relate these findings to CO2 photoassimilation in the parent chloroplast (7, 28–30). Of the thylakoidal reactions measured, there appears to be a collective agreement that phosphorylation is the most susceptible to thermal inactivation, followed by PSI and with PSI the most stable. The isolated stromal enzymes that comprise the Benson-Calvin cycle when assayed individually were found to be comparatively stable to high temperature (18, 28, 29). This approach makes evaluation of the temperature-sensitive steps of photosynthesis within the intact chloroplast difficult inasmuch as there is evidence that soluble stromal compounds (sugar, proteins) are able to protect cell structures against heat inactivation (18, 26, 27).

We show here a quantitative study on the effects of preheating up to 40°C in the dark on the photosynthesis of the intact spinach chloroplast at 25°C. The pH of the medium during the pretreatment was varied since the stability of the thylakoidal reactions is reported to be pH sensitive (6). We then examined thylakoidal and stromal performance both individually and combined in a reconstituted system fortified with adenine and pyridine nucleotides, Fd, and Pi in order to determine pretreatment-sensitive reactions.

Lastly, results are given on two physiological systems that reverse the decline in photosynthesis of the intact chloroplast when present during but not after the pretreatment phase. Preliminary accountings of some aspects of this study have been published (10, 11).

MATERIALS AND METHODS
Plant Material and General Assay Conditions. Eight-week-old expanded spinach (Spinacia oleracea L. var. "Long Standing Bloomsdale") leaves were used for chloroplast isolation by the method of Mill and Joy (20). Intact chloroplast CO2 photoassimilation rates were measured by adding chloroplasts to a 1-mL standard reaction mixture containing 50 mM Tricine-NaOH (pH 8.1), 0.33 m sorbitol, 10 mM NaH14CO3 (1 μCi/μmol), 0.25 mM K2HPO4, 1 mM MgCl2, 1 mM MnCl2, 2 mM Na3EDTA, and 1000 units of catalase. Thirty seconds after addition of chloroplasts to the standard reaction mixture in a water bath maintained at 25°C, the reaction was initiated in saturating light of 600 W/m2 from two sides by banks of Sylvania 75-W reflector indoor flood lamps. Fixation rates were calculated by spotting 0.1 mL of chloroplast-containing mixture at appropriate time intervals on planchet containing 0.1 mL of 0.5 n HCl. Radioactivity of the air-dried planchet was measured with a Nuclear Chicago end window counter. The photosynthetic rate was calculated from the linear part of the time-course curve.

For temperature treatment in the dark prior to photosynthesis, chloroplasts suspended in 1 mL of a reaction medium were placed in darkened tubes in a temperature-controlled water bath. After designated time intervals, photosynthetic measurements were determined on an aliquot (usually 50 μL) at 25°C as indicated.

The reconstituted chloroplast preparation first described by Bassham et al. (2) was modified. The intact chloroplast pellet containing about 0.5 mg of Chl was osmotically fragmented by adding 0.5 mL of buffer containing Tricine-NaOH (pH 8.1), 10 mM DTE, 1 mM MgCl2, 1 mM MnCl2, and 2 mM Na3EDTA. The resuspension was centrifuged in an HB-4 swing-out bucket for 10 min at 12,000g. The supernatant fluid was the stroma, and the pellet contained the thylakoids. CO2 photoassimilation was reconstructed by combining 0.5 mL of stroma with 0.5 mL of a mixture containing Tricine-NaOH (pH 8.1), 0.25 mM K2HPO4, 0.2 mM ADP, 5 mM MgCl2, 1 mM NADP, 1000 units of catalase, 10 mM NaH14CO3 (1 μCi/μmol), 15 μM spinach Fd, 1 mM PGA3 and thylakoids of 70 to 100 μg of Chl. The rate of

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3 Abbreviations: G3P, glyceraldehyde 3-phosphate; R5P, ribose 5-phosphate; PGA, glyc erate 3-phosphate; OAA, oxaloacetate.
photosynthesis was monitored as described for the intact chloroplast.

Electron Transport in Intact Chloroplasts. The H$_2$O → NADP sequence coupled to ATP synthesis was followed by measuring PGA-supported O$_2$ evolution. The H$_2$O → NADP sequence without coupled phosphorylation was determined by OAA-supported O$_2$ evolution in the presence of 5 mM NH$_4$Cl.

The amount of O$_2$ evolved was measured polarographically with a Clark-type electrode at 25°C. The light intensity was 400 W/m$^2$ provided by a slide projector with a 50-W bulb focused through a heat filter of water.

P/2e Measurement. The rates of NADP reduction and $^{32}$P esterification were measured in a single thylakoid preparation. The reaction was carried out in a 1-mL solution containing 50 mM Tricine-NaOH (pH 8.1), 5 mM MgCl$_2$, 5 mM KH$_2$PO$_4$ with carrier-free $^{32}$P$_i$, 0.5 mM NADP, 2 mM ADP, 15 μM spinach Fd, and chloroplast fragments containing about 30 μg of Chl. Following illumination at 25°C for 1 min, the reaction was terminated by the addition of 50 μl of 1 N NaOH. The reaction mixture was brought to pH 8.1 by the addition of 1 M Tris-HCl (pH 7.4). The neutralized mixture was centrifuged and the increase in $A_{320}$ was taken as the amount of NADPH formed. Esterification of $^{32}$P was by a method described in an earlier report (12).

In some experiments, the rate of electron flow and coupled photophosphorylation was determined using 2 mM K-ferricyanide in place of Fd and NADP. After 1 min of illumination, the reaction was terminated by addition of cold TCA. The increase in $A_{320}$ was taken as a measurement of electron flow.

RESULTS AND DISCUSSION

Photosynthesis by Preheated Chloroplasts. Figure 1 illustrates the rate of CO$_2$ fixation at 25°C following incubation of the intact chloroplasts for 10 min in the dark at temperatures between 0°C and 40°C in the standard reaction mixture. The 40°C and 35°C treatments result in a 95% and 50% reduction, respectively, in the subsequent photoassimilatory rate. Although the inhibitory value is sharply defined for any single chloroplast preparation, it does vary a few percent with different preparations. As illustrated in this figure, chloroplasts kept at room temperature (20–25°C) in the dark for 10 min are essentially unaffected. Photosynthesis is affected when the dark incubation time at room temperature is extended (Fig. 2). The decline in the photosynthetic pattern is linear with respect to time.

Properties of Chloroplasts after Pretreatment at 35°C. One explanation for our results presented in Figures 1 and 2 is the loss of chloroplast integrity during the preheating period. On the basis of the ferricyanide assay, chloroplast intactness remained unchanged (at least 85%) up to 20 min at 35°C and pH 8.1. See reference 25 for a similar observation.

A selective depletion of photosynthetic carbon metabolites due to a leaky chloroplastic envelope is discounted since addition of 5 mM G3P, RSP, or PGA during and after preheating did not restore the photosynthetic rate to that of the untreated chloroplasts. NAD- and NADP-G3P dehydrogenases, ribulose 5-P kinase, adenylate kinase, fructose 1,6-bisphosphatase, NAD-malate dehydrogenase, ribulose 1,5-bisP carboxylase, and PGA kinase in the crude stromal extract are unaffected after 20 min of preheating the intact chloroplasts at 35°C. PGA (2–30 mM) is able to suppress CO$_2$ fixation in both the control (unheated) and preheated (10 min, 35°C) chloroplasts to the same level indicating a functional Pi translocator. Furthermore, a low rate of photosynthesis caused by a high concentration of Pi can be overcome by exogenous PGA.

The measurement of photosynthetic O$_2$ evolution in intact chloroplasts with various electron acceptors is a means of monitoring partial thylakoidal performance. Thus, with nitrite and OAA, which accept electrons via nitrite reductase and malate dehydrogenase from FdH$_2$ and NAD(P)H, respectively, the rate of photosynthetic O$_2$ evolution in chloroplasts preheated at 35°C for 20 min is unchanged (Fig. 3). These results indicate that electron flow from water to reductant is not damaged by the pretreatment. On the other hand, in chloroplasts exposed to 35°C.

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Fig. 1. CO$_2$ photoassimilation at 25°C by intact chloroplasts pretreated at different temperatures in the dark at 35°C. The standard reaction mixture contained 600 μg of Chl/mL during the pretreatment and 30 μg of Chl/mL during photosynthesis. The photosynthetic rates were linear between 4 and 18 min.

Fig. 2. Kinetics of temperature pretreatment in the dark on subsequent CO$_2$ photoassimilation by intact chloroplasts at 25°C. The standard reaction mixture contained 700 μg of Chl/mL during pretreatment and 35 μg of Chl/mL during photosynthetic assay.

Fig. 3. Photosynthetic O$_2$ evolution at 25°C by intact chloroplasts after a pretreatment at 35°C in the dark for 10 min. The standard reaction mixture contained 500 μg of Chl/mL during the pretreatment and 25 μg of Chl/mL during the assay. The concentration of PGA, KNO$_3$, and OAA was 5 mM. The KNO$_3$ and OAA reaction mixtures contained 5 mM NH$_4$Cl to uncouple phosphorylation from electron flow.
for 20 min, their subsequent ability to support O₂ evolution declines similarly in the presence of PGA and CO₂.

Of the four substrates tested for their capability of supporting O₂ evolution (Fig. 3), only PGA and CO₂ require ATP in addition to reductant. Inasmuch as there is ample evidence of thermal uncoupling in fragmented chloroplasts (13, 16), it was important to determine the status of light-driven ATP formation in thylakoids isolated from preheated intact chloroplasts. Consistent with the literature reports (13, 16), complete uncoupling is observed with ferricyanide (Fig. 4) or NADP (not shown) as Hill oxidants when the fragments are subjected to 35°C for 20 min. The P/2e ratio remains unchanged, however, in the thylakoids isolated from the intact chloroplasts exposed to the same condition. We interpret our results as evidence of a remarkable differential thermostability of the photophosphorylative process in the two thylakoidal preparations.

The Reconstituted Chloroplast System. To support the observation recorded in Figure 4 that chloroplast integrity protects thylakoidal function during the preheating period, CO₂ fixation was measured in a preparation in which thylakoids from whole chloroplasts or from ruptured chloroplasts heated 10 min at 35°C were reconstituted with “fresh” (unheated) stromal protein. The results in Table I are totally consistent with those in Figure 4. Extending the heating period of the intact chloroplast to 30 min did not affect the subsequent photosynthetic rate (data not shown). It is noteworthy that BSA partially protects the heat-treated thylakoids against thermal destruction. Exposure of the stromal fluid up to 40°C for 10 min did not affect the reconstituted system (not shown), confirming the stability of the carbon assimilatory enzymes.

Stromal Acidification. Factors such as externally added Mg²⁺ (19) and a high osmotic concentration (3) have been reported to induce a decrease in stromal pH, resulting in an increased chloroplast sensitivity to Pi because the Pi concentration for photosynthesis is shifted downward (15). Because of the increased photosynthetic response to Pi by dark-treated chloroplasts, we interpret the data in Figure 5 as evidence of an apparent decrease in stromal pH during pretreatment. Therefore, the pH dependence of the reaction mixture on subsequent photosynthesis at pH 8.1 was examined. As shown in the pH profile in Figure 6,

Table I. Photosynthesis by Thylakoids from Pretreated Intact Chloroplasts or Pretreated Isolated Thylakoids Reconstituted with "Fresh" Stroma

<table>
<thead>
<tr>
<th>Condition of Stroma and Thylakoids</th>
<th>CO₂ Fixation at 25°C, pH 8.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, 0°C, 10 min</td>
<td></td>
</tr>
<tr>
<td>Thylakoids from preheated chloroplasts with “fresh” stroma</td>
<td>74.9 µmol/mg of Chl.h</td>
</tr>
<tr>
<td>Preheated isolated thylakoids with “fresh” stroma</td>
<td>81.8 µmol/mg of Chl.h</td>
</tr>
<tr>
<td>Isolated thylakoids preheated in BSA (100 mg/mL) with &quot;fresh&quot; stroma</td>
<td>27.3 µmol/mg of Chl.h</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of Pi concentration on CO₂ photoassimilation by intact chloroplasts at 25°C after a pretreatment at 35°C for 10 min in the dark. The standard reaction mixture contained 520 µg of Chl/mL during pretreatment and 26 µg of Chl/mL during photosynthetic assay.

the optimum of the photosynthetic decline resulting from the pretreatment was 5.5 to 6.0.

Reversal of Pretreatment Effect. Photosynthesis in the intact chloroplast declined about 50% following pretreatment in the dark at 35°C for 10 min (Fig. 1). In contrast, we found that preparations of stroma and thylakoids reconstituted from “fresh” or treated (35°C, 10 min) chloroplasts and fortified with ADP, NADP, Fd, and a primer (PGA, fructose 1,6-bisP) have identical photosynthetic rates (not shown). The integrity of O₂ evolution coupled to nitrate and OAA reduction confirms retention of adequate Fd and NADP for these reactions (Fig. 3). However, the decline in O₂ evolution when PGA or CO₂ was the reductant is taken as evidence that the level of ATP may be damaged by the pretreatment. Inasmuch as the P/2e ratio was unaffected in thylakoids isolated from treated (35°C, 10 min) chloroplasts (Fig. 4), it is possible that the adenosine-P and pyridine nucleotide contents rather than phosphorylative performance is affected.
Conceivably, acidification of the stroma during pretreatment accounts for the metabolic changes of the photosynthetic cofactors. A loss in photosynthesis due to a lowering of stomatal pH by the presence of Mg$^{2+}$ was reversed by the addition of K$^+$ (10–50 mM) to the medium (19). In our preparations, K$^+$ up to 100 mM in the presence or absence of Mg$^{2+}$ added during or after a pretreatment of 35°C for 10 min did not prevent the loss of photosynthetic performance. Chloroplast envelope ATPase has been postulated to be a mediator of K$^+$ uptake (19). We found ATPase activity to be totally operative in isolated chloroplast envelopes (23) subjected to 35°C for 30 min (not shown).

The alkalization of the stroma in darkened chloroplasts by exogenous ATP has been reported (5). The addition of ATP or ADP during the pretreatment phase had no protective effect on subsequent photosynthetic decline (Table II). While an adenine nucleotide translocator has been identified in the chloroplast envelope (14), until recently scant attention has been paid to the effect of Mg$^{2+}$ in the presence of ATP even though MgATP is required for the kinases of the Benson-Calvin cycle and MgADP is used by the coupling factor. Piazza and Gibbs (22) have shown that MgATP and MgADP enhanced photosynthesis in intact spinach chloroplasts, while ATP and ADP had no effect. Furthermore, protein synthesis in the darkened pea chloroplast was accelerated by exogenous ATP but only after the addition of Mg$^{2+}$ to the reaction medium (8). MgATP and MgADP at 5 mM did reverse the pretreatment inhibition (Table II). High concentrations (10 mM) are similarly effective; at concentrations of 3 mM, MgATP and MgADP are less effective by about 25%. Mgadenosine-P can be replaced by MnATP and MnADP but not by MgGTP, MgGDP, MgGDP, Mg5'-AMP, or Mg3'-AMP. As illustrated in Figure 7, addition of MgATP during pretreatment halted further decline but restoration to the control rate was not observed. The protective system functions only when added prior to but not after pretreatment (not shown).

In view of the effectiveness of externally added MgATP and MgADP in blocking the pretreatment effect, we replaced them with triose-P (fructose 1,6-bisP and aldolase) or R5P and OAA since their respiratory conversion to PGA represents a potential means of affecting the status of ATP and ADP within the darkened chloroplast. Furthermore, Robinson (25) has reported Mg$^{2+}$ and MgADP by a factor of 2 to 3, while MgATP and 30 mM MgADP by a factor of 2 to 4.
that triose-P and OAA can increase stromal pH. Complete protection against pretreatment is observed but only in the presence of both substrate and OAA (Table III). We have measured rates of 3 to 4 μmol of PGA formed/mg Chl·h from triose-P or RSP under pretreatment conditions with a halved rate in the absence of OAA and none in the absence of sugar phosphates (K. Ahluwalia, unpublished results). The requirement of OAA and externally added sugar phosphate rules out molecular O₂ and endogenous starch as an adequate protective mechanism and may reflect the low rate of starch degradation in our intact chloroplast preparations (21).

Results are given in Figure 6 illustrating that medium pH during pretreatment determines the extent of photosynthetic inhibition. Both protective reagents (Tables II and III) abolished completely the pretreatment effect at all medium pH values tested (Table IV).

Lastly, the decline in photosynthesis following pretreatment for 10 min at 40°C is not reversed by either protective system. We interpret this result as evidence for irreversible thermal damage at 40°C.

**CONCLUSION**

We demonstrate here that a 10-min exposure of the intact darkened spinach chloroplast to 35°C results in a diminution of subsequent photosynthetic CO₂ and PGA reduction. The decline in photosynthesis is not the result of damage to (a) chloroplast integrity; (b) thylakoidal performance monitored as electron flow and phosphorylation; (c) the phosphate translocator; (d) enzymes and intermediates of the Benson-Calvin cycle; and (e) possibly the levels of Fd and NADP. Inasmuch as the extent of pretreatment damage is time-, temperature-, and pH-dependent and can be reversed by two potentially physiological systems, the immediate causal agent would appear not to be thermal but rather enzymic.

Our results do not allow for a rigorous identification of the factor that links the pretreatment effect with photosynthetic deficiency. A simple explanation for protection by Mg-adenosine-P or by chloroplastic respiration of a sugar phosphate is maintenance of the absolute concentration of the nucleotides during the pretreatment. If the level of the adenine nucleotides was the only factor, it is difficult to rationalize lack of reversibility of photosynthetic decline by MgATP added after pretreatment. A more important pace-setter may be the ATP/ADP ratio (28).

While we offer no definitive proof of stromal acidification during pretreatment, nonetheless, we remain impressed by the positive effect of low medium pH on photosynthetic decline. We propose an increase in the activity of a stromal acid phosphatase in response to stromal pH and temperature accounting for degradation of the photosynthetic cofactors. We are aware of the report of Robinson (24) claiming a small (5 μmol/mg Chl·h) activity of a stromal acid phosphatase. We have confirmed his observation but have evidence that a stromal activity exists (unpublished data).

Forte et al. (9) have purified a non-metal-requiring phosphatase from pea leaves that has an optimum activity at pH 6 on the phosphomonoesters of nucleotides including ATP, ADP, NADP, and NADPH, with lesser activity on P-enolpyruvate, glucose 6-P, and AMP. Most pertinent here is the evidence that each substrate is a competitive inhibitor of the enzymic action on the other substrates. In a preliminary study, we have detected a similar enzymic activity in crude stromal preparations of freshly ruptured spinach chloroplasts. It is possible that the protective systems of Mg-adenosine-P or a respirable substrate coupled to an OAA dismutation reaction shields the degradation of the nucleotides by alkalization of the stroma or by maintenance of nucleotide content or ATP/ADP ratio by competitive inhibition. Phosphatase activity on ATP and ADP would generate AMP. In a reconstituted system, AMP was shown to inhibit the Benson-Calvin cycle after fructose 1,6-bisP formation (17). However, isolated chloroplastic fructose 1,6-bisphosphatase of spinach (4) as well as ribulose 5-P kinase of peas (1) have been reported to be insensitive to AMP. The pertinence of these studies is questionable inasmuch as they were performed with the activated, reduced enzyme. Here, AMP would be formed internally in the dark when the enzymes were in the inactive state, and this may influence sensitivity to inhibitors.