Manifestation of a Prolonged Lag in the Photosynthesis of Heated Spinach Chloroplasts

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When the time course for CO_2 fixation and O_2 evolution in isolated intact spinach chloroplasts was examined, we found a prolonged lag time in the early phase of photosynthesis after heat-treatment in the dark as well as an expected time-dependent decrease in the rate during the subsequent linear phase. Because the lengthening of the lag period was generally attributed to the depletion of sugar phosphates in the chloroplasts, we tested for the possible involvement of Calvin cycle intermediates in the change of the lag phase by heat-treatment. When triose phosphate was added to the heated chloroplasts, the lag time was re-shortened without the rate in the linear phase being elevated to that measured in the control. Mg-ATP or triose phosphate plus oxaloacetate (previously known as protective chemicals) prevented the lengthening of the lag time when added prior to heat-treatment. Quantification of some metabolites in the chloroplasts confirmed that heavy losses had occurred for triose phosphate, fructose-1,6-bisphosphate, glucose-6-phosphate, and fructose-6-phosphate. However, the level of 3-phosphoglyceric acid was increased. The presence of Mg-ATP during heat-treatment alleviated the losses of those sugar phosphates. Therefore, we conclude that the decrease in sugar phosphates in the chloroplasts, as part of the negative effect from heat-treatment, is the primary cause of the lengthened lag time during the initial phase of photosynthesis.

Keywords: Heat-treatment, isolated chloroplast, lag period, photoassimilation

The adverse effect of high temperature on photosynthesis has been widely studied in various plant species. The decrease in photosynthetic activity after heat-treatment was attributed to the uncoupling of photophosphorylation and subsequent thermal inactivation of electron transport from water to NADP in isolated thylakoids (Katoh and San Pietro, 1967; Emmett and Walker, 1969, 1973; Berry and Björkman, 1980). Research on intact chloroplasts has demonstrated the occurrence of thermal uncoupling in photophosphorylation, loss of Hill activity with a damaged water splitting system in addition to the inactivation of PS II activity and noncyclic photophosphorylation (Katoh and San Pietro, 1967; Krause and Santarius, 1975; Santarius, 1975). In contrast, PS I activity remained unaffected (Katoh and San Pietro, 1967; Thomas et al., 1986).

Under the conditions used to cause heat-induced photosynthetic inhibition of isolated chloroplasts, Calvin cycle enzymes appear to remain stable (Krause and Santarius, 1975; Santarius, 1975; Chollet and Anderson, 1976; Fu and Gibbs, 1988). However, in some species, the activities of NADP-G3P dehydrogenase, Ru5P kinase, and NADP malate dehydrogenase declined in a pattern similar to that found with photosynthesis (Bauer and Senser, 1979; Berry and Björkman, 1980). Furthermore, after mild heating of spinach chloroplasts, a reversible inhibition of photosynthesis was accompanied by inactivation of RuBP carboxylase (Weis, 1981a, 1981b). Light-activation of RuBP carboxylase was also inhibited by heat-treatment above 25°C, and light activation of RuBP carboxylase was suggested as one of the most heat-sensitive sites (Weis, 1982). Recently, it was shown that inhibition of Rubisco activase was correlated with a decline in leaf photosynthesis due to heat stress (Feller et al., 1998; Law and Crafts-Brandner, 1999; Crafts-Brandner and Law, 2000). Therefore, it is most likely that heating affects both the thylakoidal and the stromal components.

Leaf photosynthesis exhibits delayed response in reaching its maximal photosynthetic rate after the onset of illumination. This so-called induction was first demonstrated in *Ulva* (Osterhout and Haas, 1918),

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Abbreviations: Chl, chlorophyll; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; OAA, oxaloacetic acid; PGA, 3-phosphoglyceric acid; Ru5P, ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; R5P, ribose-5-phosphate; sugar-P, sugar phosphate; triose-P, triose phosphate.

and had also been observed in photosynthesis by isolated chloroplasts (Turner et al., 1962; Baldry et al., 1966a). During the initial lag period, adequate levels of metabolites are accumulated and stromal enzymes become light-activated. The exogenous addition of chloroplast membrane-penetrating sugar-Ps, such as triose-P and R5P, eliminates the induction period without affecting the linear rate (Bamberger and Gibbs, 1965; Baldry et al., 1966b; Walker, 1976). Therefore, manifestation of lengthened lag is generally thought to result from the depletion of Calvin cycle intermediates.

As established in many laboratories, the typical time course for either CO_2 fixation or CO_2-supported O_2 evolution with isolated intact chloroplasts at an optimal temperature usually exhibits a triphasic pattern-i.e., an initial lag period, a linear phase, and a late phase during which the rate decreases and eventually ceases. Nonetheless, when the adverse effect of environmental factors is studied, most of the attention is usually paid to the photosynthetic rate during the linear phase while ignoring its impact on the lag phase. Considering the multifaceted negative influence of heat-treatment on photoassimilation by isolated chloroplasts, it is necessary to examine changes in the lag phase as well. In this regard, we examined the entire time course for CO_2 fixation and O_2 evolution in heated chloroplasts as an effort to elucidate the effect of heat-treatment on photosynthesis by isolated chloroplasts. Here, we demonstrate that heat-treatment leads to the lengthening of the lag period as a consequence of sugar-P depletion in heated chloroplasts.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea* L.) plants were grown for 8 to 10 weeks in pots containing a mixture of 2:1 vermiculite: soil, and were held to maturity in a controlled-environment chamber maintained at 20°C/15°C (light /dark), with a 10-h photoperiod. Light was provided by banks of fluorescent lamps interspersed with incandescent lamps at the intensity of ~200 μmol m^{-2} s^{-1}. Randomly selected, fully expanded leaves were collected after being exposed to illumination for at least 2 h, and were used for chloroplast isolation.

Chloroplast Isolation

Intact chloroplasts were isolated using a Percoll gradient as described by Jun et al. (1994). About 10 g of deribbed leaves were homogenized for 5 s in a Waring blender with 50 mL of an ice-chilled grinding medium containing 50 mM Hepes-NaOH (pH 6.8), 0.33 M sorbitol, 2 mM Na_2EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. The resulting homogenate was filtered through four layers of cheesecloth and two layers of Miracloth (Calbiochem, Inc., USA). After centrifugation at 750*g* for 50 s, the supernatant fluid was discarded and the pellet was resuspended in 25 mL of the grinding medium. This resuspended material was layered onto 15 mL of a 40% Percoll mixture that had the same composition as the grinding medium, and was centrifuged in a swinging bucket (Sorvall HB-4) at 2500*g* for 3 min. The resulting pellet was resuspended in a reaction buffer composed of 50 mM Tricine-NaOH (pH 8.1), 0.33 M sorbitol, 2 mM Na_2EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ to yield a Chl concentration of ~1 mg mL^{-1}. Chl concentration was determined according to Arnon (1949).

Temperature Pretreatment of Chloroplasts

Isolated chloroplasts suspended in the reaction buffer were subdivided and kept on ice until the experiment began. When other chemicals were to be added before the onset of heat-treatment, the additional volume was adjusted by adding the same amount of the reaction mixture. Heat-treatment in the dark was initiated by transferring the samples to a water bath maintained at 35 ± 1°C, and was terminated by returning them to ice. Subsequent measurements of CO_2 fixation and photosynthetic O_2 evolution were performed immediately.

Measurement of $^{14}CO_2$ Fixation and Photosynthetic O_2 Evolution

CO_2 fixation was measured as described earlier (Jun et al., 2001). About 50 μg Chl of intact chloroplasts were added to 1 mL of a reaction buffer containing 0.25 mM KH_2PO_4, 10 mM $NaH^{14}CO_3$ (1 μCi μmol^{-1}), and 1000 units of catalase. After the tubes were immersed in the water bath, the reaction was initiated by turning on the lights, provided by Sylvania reflector indoor flood lamps at the intensity of ~500 μmol m^{-2} s^{-1}. Aliquots of 100 μL were taken from the reaction tube at 1- min intervals, and placed in a vial containing 200 μL of 0.5 N HCl to stop the reaction. Radioactivity was measured after adding 5 mL of Optiphase 'HiSafe' III scintillation cocktail (Wallac, Inc., Finland) to the vial with Wallac 1409 scintillation counter.

Photosynthetic O_2 evolution was carried out in a 1-mL reaction buffer containing 0.25 mM KH_2PO_4 and intact chloroplasts (about 30 μg Chl), using either 10 mM $NaHCO_3$ or 5 mM PGA as the electron acceptor. The evolved O_2 was measured polarographically with a Clark type electrode (Hansatech, Ltd., UK) at 25°C. Light (600 μ mol m^{-2} s^{-1}) was provided from a Schott illuminator (Schott Glass, Stafford, UK). Rates of $^{14}CO_2$ fixation and O_2 evolution were calculated from the linear portion of the time course, and the lag time was estimated by extrapolating the linear portion to the x-axis.

Measurement of Sugar-P and PGA Contents

Samples for quantifying sugar-Ps were prepared as follows: Aliquots of isolated chloroplasts (1 mg Chl mL^{-1}) were mixed with 70% $HClO_4$ to yield a final concentration of 10% $HClO_4$. After centrifugation at 5000*g* for 10 min, the supernatant fluid was neutralized to pH 7.0 with 5 M K_2CO_3 and recentrifuged at 5000*g* for 10 min. The resulting supernatant fraction was used to estimate the amount of sugar-Ps.

FBP and triose-P contents were measured according to the method of Lowry and Passonneau (1972). Triose-P was monitored as the increase in absorbance at 339 nm by NAD reduction. To initiate the reaction, 3 units of triose-P isomerase and 2 units of G3P dehydrogenase were added to a 1-mL cuvette containing 100 μL of sample and 900 μL of a reaction mixture composed of 50 mM imidazole-HCl (pH 7.5), 1 mM sodium arsenate, 1 mM EDTA, 2 mM mercaptoethanol, and 1 mM NAD. The reaction was continued until no change in absorbance was observed. After triose-P was measured, FBP content was determined by adding 1 unit of aldolase to the cuvette.

Measurements of G6P and F6P followed the method of Michal (1984). To 600 μL of a reaction mixture containing 0.2 M TEA-NaOH (pH 7.6), 0.2 mM NADP, and 5 mM $MgCl_2$, 400 μL of sample was added. G6P content was monitored by adding 0.2 units of G6P dehydrogenase; thereafter, 1 unit of phosphoglucose isomerase was included to measure the level of F6P.

PGA content was estimated by monitoring the decrease in absorbance at 339 nm according to the method of Czok (1984). In a 1-mL cuvette, 200 μL of sample was added to 800 μL of a reaction mixture containing 0.1 M TEA-NaOH (pH 7.6), 1 mM EDTA, 0.1 mM NADH, 8 mM $MgSO_4$, 7 mM ATP, 4 units of G3P dehydrogenase, 24 units of triose-P isomerase, and 1 unit of glycerol-3-P dehydrogenase. The reaction was initiated by adding 18 units of PGA kinase.

RESULTS

Time Course of Subsequent Photoassimilation after Heat-Treatment

A typical time course for CO_2 fixation or CO_2-supported O_2 evolution by isolated intact chloroplasts at optimal temperature usually exhibits a triphasic pattern with lag, linear, and late phases (see Control in Fig. 1). After exhibiting lower rates during the induction period of 1 to 2 min, much higher maximal rates in the linear phase were achieved and maintained for

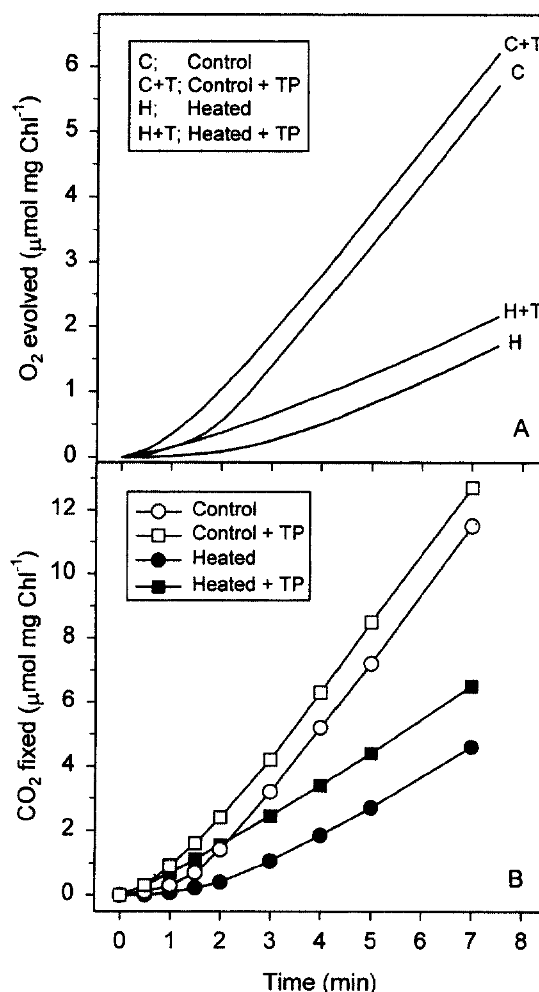

Figure 1. Time course of (A) CO_2-supported O_2 evolution and (B) CO_2 fixation by control (unheated) and heated (35°C, 10 min, pH 8.1) chloroplasts. CO_2 fixation and O_2 evolution were measured using intact chloroplasts containing 50 μg Chl and 30 μg Chl, respectively. Where indicated, 1 mM FBP (aldolase) was included in the medium. Note the lengthened lag time in the heated chloroplasts and its abolishment by the addition of FBP.

a while. The rates then gradually decreased, and photosynthesis eventually ceased in the late phase. Yet when studying the adverse effect of abiotic factors on the photosynthesis by isolated chloroplasts, most of the attention was usually paid to the photosynthetic rate in the linear phase. In this study, therefore, not only changes in the linear phase of CO_2 fixation and photosynthetic O_2 evolution but in the lag phase were assessed.

A notable change in the time course was observed when photoassimilation was measured in the heated chloroplasts. The time courses for CO_2 fixation and CO_2-supported O_2 evolution in the control and heated chloroplasts were compared in Figure 1. The triphasic patterns constituting lag, linear and late phases of the time course were similar in both chloroplasts, but the heated chloroplasts showed two distinct differences: a significantly longer lag period and a ~50% reduction in the linear rate (Fig. 1 and Table 1). The lag time for CO_2-dependent O_2 evolution in the control chloroplasts was about 1 min while that of the heated chloroplasts was nearly doubled (Table 1). PGA-dependent O_2 evolution by the control and heated chloroplasts produced similar results even though the lag time was significantly shorter in both chloroplasts (Table 1). The addition of chloroplast membrane-penetrating sugar-P (triose-P given as FBP with aldolase or R5P) to the reaction medium shortened the lag time for both chloroplasts (Fig. 1 and Table 1). However, adding these compounds after heat-treatment did not restore the photosynthetic rate of the heated chloroplasts to that of the control during the linear phase (Fig. 1 and Table 1). These findings suggest that one of the consequences of heat-treatment is the depletion of chloroplastic sugar-Ps, which results in a prolonged lag time. Inasmuch as the addition of FBP (with aldolase) or R5P after heat-treatment did not elevate the reduced rate to the control rate although the lag time was re-shortened, the depletion of sugar-P was not taken as the cause for the heat-induced inhibition of the photosynthetic rate in the linear phase, but the cause for the lengthened lag time. Notably, the low rate of CO_2 fixation during the lag phase was not further decreased by heat-treatment (Fig. 1B).

Table 1. Lag time and linear rate of CO_2-supported and PGA-supported O_2 evolution exhibited by control and heated chloroplasts. Triose-P (added as FBP with aldolase) or R5P was added to the reaction mixture to make a final concentration of 1 mM. Data are mean values with standard deviations for five measurements.

Treatment	Lag time (s)	Rate of O_2 evolution (μmol mg Chl^{-1} h^{-1})
CO_2-supported O_2 evolution		
Control (unheated)	65 ± 5	76.7 ± 4.2
Control + triose-P	45 ± 5	77.8 ± 4.7
Control + R5P	48 ± 5	82.8 ± 5.5
Heated (35°C, 10 min)	115 ± 9	37.7 ± 2.9
Heated + triose-P	54 ± 3	39.2 ± 4.1
Heated + R5P	46 ± 4	43.0 ± 3.2
PGA-supported O_2 evolution		
Control	28 ± 2	76.6 ± 4.4
Control + triose-P	26 ± 1	72.5 ± 3.6
Heated	40 ± 3	40.9 ± 2.9
Heated + triose-P	29 ± 1	37.8 ± 3.2

Change in Chloroplastic Sugar-P Content after Heat-Treatment

The re-shortening of the lag time by the addition of sugar-Ps, as shown in Figure 1 and Table 1, suggests their direct involvement in the lag phase. Because of such observations, we measured the amounts of various sugar-Ps and PGA in the control and heated chloroplasts. Triose-P and FBP contents were decreased to 60% of the control level; hexose-P in the heated chloroplasts was reduced to <50% of the control value (Fig. 2). In contrast, PGA increased >50% after heat-treatment (Fig. 2). This could have been due to enhanced chloroplastic dark respiration, but even under anaerobic

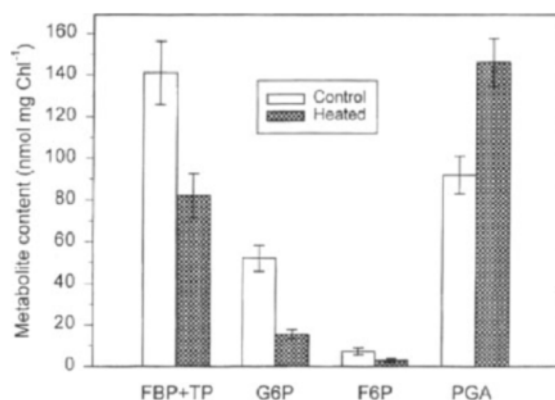

Figure 2. Change in the content of triose-P, FBP, hexose 6-P, and PGA during heat-treatment (35°C, 10 min). Obtaining chloroplast extracts and metabolite determinations were done as described in the Methods using 200 μg Chl for FBP and triose-P, and 400 μg Chl for hexose 6-P. The experiment was repeated three times with four duplicates, but because of variations in the control values, mean values for all were not taken. The results shown here are mean values with standard deviations for a representative one. Other experiments showed similar results.

conditions similar results were obtained (data not shown). Therefore, the decline in sugar-P was not taken as a result of enhanced respiration in heated chloroplasts. It may have been caused by the impaired reductive phase of the Calvin cycle. However, the increase in PGA did not account for the total sum of missing sugar-Ps, suggesting that a considerable amount have been degraded or drained into some unknown sinks. Whatever the reason for the sugar-P loss in the heated chloroplasts may be, the direct cause for the lengthening of the lag time clearly involves the depletion of sugar-Ps.

Effect of Protective Agents on Lag Time and Sugar-P Content

Some combinations of certain chemicals, such as Mg-ATP or triose-P (FBP with aldolase) and OAA, both of which are physiologically relevant, can prevent a decrease in photosynthetic rates if added prior to heat-treatment (Fu and Gibbs, 1988). Therefore, we tested the effect of these protective chemicals on lag time. Surprisingly, a heat-induced prolonged lag was indeed prevented by adding the protective agents during heat-treatment. Chloroplasts protected by Mg-ATP or FBP and OAA had lag periods comparable in length to those for the control chloroplasts (Table 2). Because lag time was correlated with the level of carbon intermediates along with the heat-depleted sugar-Ps in the chloroplasts, the presence of protective agents might possibly have prevented a heat-induced decline in Calvin cycle intermediates. To examine this possibility, we determined the changes in sugar-P levels in the presence of Mg-ATP, which would not directly affect sugar-P content. The presence of Mg-ATP during heat-treatment stabilized sugar-P levels in the chloroplasts (Fig. 3). Amounts of triose-P and FBP in the presence of Mg-ATP decreased only 20% after heat-treatment, compared with a 50% reduction when Mg-ATP was absent. Furthermore, the decrease in hexose-Ps (G6P and F6P) was completely prevented (Fig. 3). Mg-ATP addition increased the amount of hexose 6-Ps in the control chloroplasts by >50% (data not shown), possibly because Mg-ATP may have stimulated phosphorylation of the stromal free sugars derived from starch. Nevertheless, in the presence of Mg-ATP, the amount of chloroplastic hexose 6-Ps did not decrease at all during heat-treatment (Fig. 3). Therefore, it is highly likely that no manifestation of a lengthened lag time in the protected chloroplasts was due to the prevention of sugar-P losses by Mg-ATP during heat-treatment.

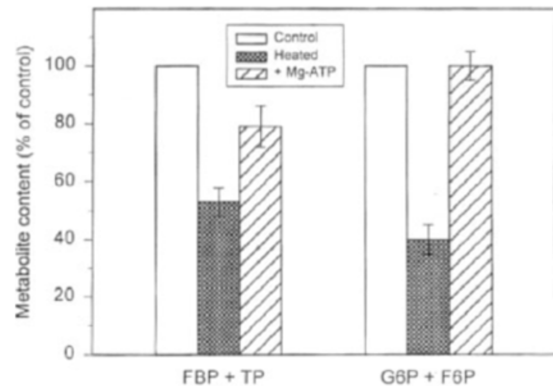

Figure 3. Maintenance of sugar-P content in heated chloroplasts by Mg-ATP. All experimental conditions are as described in Figure 2. Data were shown in relative values because the amount of hexose 6-P in the control was less than that with Mg-ATP in all three measurements with different preparations of chloroplasts.

Table 2. Lag time and linear rate of CO_2-supported O_2 evolution exhibited by control, heated, and protected chloroplasts. Intact chloroplasts were heated at 35°C for 10 min at pH 8.1 prior to measurement of O_2 evolution. Mg-ATP or triose-P (added as FBP with aldolase) plus OAA were added prior to heat-treatment to make a final concentration of 5 mM, but they were removed prior to onset of O_2 evolution. Data are mean values with standard deviations for five measurements.

Treatment	Lag time (s)	Rate of O_2 evolution (μmol mg Chl^{-1} h^{-1})
Control (unheated)	71 ± 4	69.7 ± 5.3
Heated (35°C, 10 min)	115 ± 8	31.7 ± 2.8
Heated + Mg-ATP	78 ± 6	70.2 ± 6.1
Heated + triose-P + OAA	60 ± 7	61.9 ± 4.3

DISCUSSION

When plants are illuminated after prolonged exposure to darkness, they usually undergo a period of induction before reaching their maximal photosynthetic rate. This initial lag has been observed not only in intact leaves but also in isolated chloroplasts (Turner et al., 1962; Bamberger and Gibbs, 1965; Baldry et al., 1966b; Walker, 1976). In the current study, heat-treatment of spinach plants led to a lengthening of the induction period for leaf CO_2 photoassimilation as well as a lower rate (data not shown). Similarly, heating the intact chloroplasts caused a decline in the maximal rate and

a significantly longer lag period in the time course for CO_2 fixation and O_2 evolution (see Fig. 1). Decreased photoassimilation of chloroplasts after heating has been attributed to either a change in adenine nucleotides (Fu and Gibbs, 1988) or inactivation of the Calvin cycle (Wise, 1981a, 1981b). Recently, inhibition of Rubisco activase was correlated with a decline in leaf photosynthesis by heat stress (Feller et al., 1998; Law and Crafts-Brandner, 1999; Crafts-Brandner and Law, 2000). However, these affected sites do not change the length of the lag period. Osterhout and Hass (1918) originally proposed that the time required for light-activation of Calvin cycle enzymes and the build-up of intermediates was the main cause for the induction period. Observation of shortened lag period by pre-illumination was supportive of the catalyst activation (Walker, 1976), and abolishment of the lag phase but with no effect on the linear rate by chloroplast penetrating sugar-Ps (triose-P, R5P) was seen as crucial evidence for the limitation in Calvin cycle intermediates during the lag period (Bamberger and Gibbs, 1965; Baldry et al., 1966b; Bucke et al., 1966). Since sugar-P content was directly involved in the manifestation of the lag period, we tested the effect of exogenous sugar-Ps added after heat-treatment on photosynthesis. A mere addition of triose-P or R5P, both of which are chloroplast permeable, to the heated chloroplasts re-shortened the lag time, but without restoring the rate (see Fig. 1 and Table 1). Therefore, it was very likely that the loss of sugar-Ps was another heat-induced event, but not the direct cause for the decline in photosynthesis.

To test whether sugar-Ps were involved in the lengthened lag time, we directly measured several metabolites in the chloroplasts prior to and after heat-treatment. Levels of triose-P, FBP, G6P, and F6P were all greatly decreased whereas PGA content increased (see Fig. 2). The decreased amount in those sugar-Ps, however, was not quantitatively correlated with the increased amount of PGA. Furthermore, increased PGA was observed even under anaerobic condition (data not shown). Therefore, the decrease in sugar-Ps did not appear to result from their conversion to PGA via enhanced chloroplastic respiration during heat-treatment. Impairment of the reductive phase in the Calvin cycle was likely responsible for the increased PGA and decreased sugar-P contents. In addition, some portion of the sugar-Ps was drained into an unknown sink, possibly by phosphatase action.

The adverse effect of heat-treatment is not reversed but prevented by the addition of Mg-ATP or FBP plus OAA prior to heat-treatment (Fu and Gibbs, 1988). In view of those results, we examined the effect of these protective chemicals on the lag period and sugar-P content. Neither a longer lag period nor lower photosynthetic rates were observed in the protected chloroplasts (see Table 2). Furthermore, sugar-P contents were not reduced as much in the presence of Mg-ATP. Levels of triose-P and FBP were slightly decreased while those of G6P and F6P were maintained at the control level (see Fig. 3). Therefore, we conclude that heating induces a decline in sugar-Ps in the chloroplasts, which is manifested as a prolonged lag in the time course. Elucidation of the cause for this sugar-P loss is a subject of further investigation. In addition, decreased sugar-P contents apparently are not the reason for the lower photosynthetic rate incurred by heating. Imbalanced adenine nucleotides, inactivation of the Calvin cycle, and impairment in Rubisco activase may all contribute to the reduction in photoassimilation.

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