식물학회지 제36권 제4호 (1993)
Korean J. Bot. 36(4) : 371~375

The Reason for the Loss of Photosynthetic Activity in Isolated Spinach Chloroplasts during Photosynthesis

Jun, Sung-Soo, Chin Bum Lee and Young-Nam Hong*
(Department of Biology, Dongeui University, Pusan and
**Department of Biology, Seoul National University, Seoul)*

분리된 시금치 엽록체에서 광합성중 광합성능이 소실되는 이유

全 星 守·李 鎭 範·洪 英 男*
(東義大學校 自然科學大學 生物學科, *서울大學校 自然科學大學 生物學科)

ABSTRACT

CO_2 fixation of isolated intact spinach chloroplasts under saturating light began to decrease after 20 min, and stopped completely after 1 h. To identify the lesion sites for the die off, reconstituted chloroplast system was used with chloroplasts collected at several phases of time course. CO_2 fixation was inhibited in the reconstituted chloroplasts made of thylakoids and stroma in the later phases, but showed a higher degree of inhibition by the participation of thylakoids than that of stroma in the later phases. Measurement of photophosphrylation and NADP reduction revealed that a severed thylakoidal damage was occurred at the later phases. This results indicate that the lesion sites for the die off are in the thylakoid.

INTRODUCTION

Studies with isolated chloroplasts have greatly facilitated to understand the biochemistry and physilogy of chloroplasts, e.g. the regulation of carbon metabolism and Calvin cycle enzymes. However, unlike intact plants, isolated chloroplasts rapidly lose their photosynthetic activity both in the light and dark (Jun, 1991). The lack of stability in isolated chloroplasts has been a drawback for the researchers who have been working with isolated chloroplasts. Hence, much efforts were made to keep isolated chloroplasts active for longer time. Effects of externally added Pi, Mg-ATP, or sugar-P on maintaining isolated chloroplasts active were reported (Bucke *et al.*, 1966; Walker and Robinson, 1978; Mourioux and Douce, 1981; Gibbs *et al.*, 1986; Fu and Gibbs, 1988; Jun, 1991).

In many cases, the photosynthetic activity of isolated spinach chloroplasts, when kept on ice in the dark, did not last more than 2 hrs (Jun, 1991). Regardless of incubation time in the dark, the time course of CO_2-supported O_2 evolution or CO_2 fixation under saturating light by isolated chloroplasts exhibited the disappearance of photosynthetic activity after 20 to 40 min depending on the preparation (Gibbs *et al.*, 1986; Seftor and Jensen, 1986; Jun, 1991). The reason for the loss of activity has been attributed to many factors. One of the causes suggested is a decrease of the RuBP level to support CO_2 fixation in the chloroplasts (Sicher and Jensen, 1979). However, even under low CO_2 concentration where the RuBP level can be maintained high, the die off was still observed (Stumpf and Jensen, 1982). Seftor and Jensen (1986) reported a decrease in the activity of Rubisco when measured as an active form, Enzyme-CO_2-Mg^{2+} complex (E-C-M) after 20 min, and attributed it as a cause for the die off. Significant leakage of Rubisco as well as other stromal proteins into the suspending medium was subsequently occurred (Seftor and Jensen, 1986). However, it is

This work is partially supported by a grant from Dongeui University '93.

generally known that thylakoid is more susceptible to damage than components in the stroma. Yet thylakoidal function with regard to this phenomenon has not been examined. Therefore, it is necessary to investigate thylakoidal function as well.

In this study, changes in the pattern of CO_2 fixation by isolated spinach chloroplasts are first examined. With reconstituted chloroplast system made of chloroplasts at different phases during CO_2 fixation, evidence is given to show that damage in the thylakoidal function occurs at the later phases of photosynthesis in the isolated chloroplasts. Measurement of thylakoidal function, photophosphorylation and NADP reduction, was also performed to support this result.

MATERIALS AND METHODS

Plant material and chloroplast isolation. Spinach (*Spinacia oleracea* L. var. "Long Standing Bloomsdale") seeds were germinated and grown on a rectangular plastic containers (60×40×10 cm) filled with a mixture of vermiculite and soil in a ratio of 2 : 1 in a controlled environment chamber for 8 to 10 weeks until maturity. Randomly selected mature leaves were used for chloroplast isolation. Intact chloroplasts were isolated according to the modified method of Mills and Joy (1980). Deribbed leaves (about 10 g fresh weight) were washed with D.W., and sliced and homogenized at 4℃ for 5 s in 50 mL of prechilled 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 4 layers of cheesecloth and 2 layers of Miracloth (Calbiochem, Inc.). After centrifugation for 50 s at 750 *g*, the supernatant was discarded and the pellet was resuspended in 25 mL of grinding medium. This resuspended material was layered onto 15 mL of a 40% Percoll mixture which has the same composition as grinding medium, and was centrifuged in a swinging bucket for 2.5 min at 2,500 *g*. The resulting pellet was resuspended in reaction buffer containing 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 concentration of Chl around 1 mg/mL. Chl concentration was determined according to Arnon (1949).

Measurement of $^{14}CO_2$ fixation. CO_2 fixation was measured by adding intact chloroplasts (around 50 μg Chl) to a 1 mL reaction mixture containing 50 mM Tricine-NaOH (pH 8.1), 0.33 M sorbitol, 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.25 mM KH_2PO_4, 10 mM $NaH^{14}CO_3$ (1 μCi/μmol), and 1,000 units of catalase. The reaction was performed at 25℃ and initiated by turning on the light at the intensity of about 600 W/m^2. An aliquot of 50 μL was taken from each reaction tube at the specified time and placed in an planchet or a vial containing 100 μL of 0.5 N HCl. After sample was air-dried in the hood, radioactivity was determined with either a Nuclear Chicago Q-gas end window counter or scintillation counter. The rate of $^{14}CO_2$ fixation was calculated from the linear portion of the time course.

The Reconstituted chloroplast system. The reconstituted chloroplast system was modified from Bassham *et al.* (1974). The intact chloroplast pellet after Percoll cushion was osmotically broken by adding 0.5~1 mL of reaction buffer containing 50 mM Tricine-NaOH (pH 8.1), 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 10 mM DTT (dithiothreitol). The resulting supernatant fraction after centrifugation at 12,000 *g* for 10 min in a swinging bucket was used as the stromal fraction and the remaining pellet was the thylkoidal fraction. CO_2 fixation was measured by combining 0.5 mL of the stromal fraction with 0.5 mM of a reaction mixture containing 50 mM Tricine-NaOH (pH 8.1), 5 mM $MgCl_2$, 0.2 mM ADP, 1 mM NADP, 2 mM PGA (3-phosphoglyceric acid), 15 μM spinach Fd (ferredoxin), 10 mM $NaH^{14}CO_3$ (1 μCi/μmol), 0.25 mM KH_2PO_4, 1,000 units of catalase, and thylakoids (30 μg Chl).

NADP reduction and coupled photophosphorylation. NADP reduction and photophosphorylation were measured with the same preparation of thylakoids but in two separate tubes. Each measurement was done in 4 duplicates. The reaction mixture contained 50 mM Tricine-NaOH (pH 8.1), 5 mM $MgCl_2$, 2 mM ADP, 5 mM KH_2PO_4, 0.5 mM NADP and 15 μM spinach Fd. For the measurement of photophosphorylation, 2 μCi of ^{32}P (NEN-054) were included in the reaction mixture. Reaction was initiated by turning on the light after adding thylakoids (about 30 μg Chl) to make a total volume 1 mL. After 1 min illumination under a saturating light intensity (600 W/m^2) at 25℃, the tubes were placed on ice and the reaction was immediately stopped. NADP reduction was terminated by adding 50 μL of 1 N NaOH and the mixtures were kept on ice in the dark for 10 min, then neutralized by adding 150 μL of 1 M Tris-HCl (pH 7.4). The neutralized mixtures were centrifuged at 5,000 *g* for 10 min and the increase in absorbance at 339 nm was read. Photophosphorylation was terminated by the addition of 1 mL "precipitation" solution containing 0.66 M HCl, 24 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 72 mM triethylamine, and 1%

saturated bromine water (Sugino and Miyoshi, 1964). The mixtures were left at room temperature for 10 min to allow complete precipitation of the carrier-free ^{32}P remnants in the pellet fraction. After centrifugation at 5,000 *g* for 10 min, each 200 μL of the supernatant was placed on an planchet and dried in the hood. Radioactivity was counted with a Chicago nuclear counter.

RESULTS

Time course of CO_2 fixation. As established in many laboratories, the typical time course of CO_2 fixation with isolated intact chloroplasts at optimal temperature (25℃) and under saturating light intensity (600 W/m^2) usually shows a biphasic pattern with a lag period prior to a linear phase. After about 20 min, the CO_2 fixation decreases and eventually ceases (the late phase). The time course of CO_2 fixation of isolated intact spinach chloroplasts revealed a similar pattern as shown in Fig. 1. CO_2 fixation of chloroplasts began to decrease after 20 min and stopped completely about 1 h after the initiation of the reaction. Addition of 5 mM Mg-ATP did not change the pattern of CO_2 fixation at the early phase, but delayed the die off (Fig. 1). The possible role of Mg-ATP here will be discussed later.

The reason for the loss of activity in the isolated chloroplasts is not yet clearly known. Loss of intactness was proved not to be a reason by FeCN (ferricyanide) assay (Fu, 1986; Seftor and Jensen, 1986). Decrease of the RuBP level in the chloroplasts or inactivation of active form of Rubisco, E-C-M, was reported to be responsible for the cessation of CO_2 fixation at the late phase (Sicher and Jensen, 1979; Seftor and Jensen, 1986). However, changes in the thylakoidal function at the late phase has not been tested in this regard. With reconstituted chloroplast system, overall function of thylakoid and stroma can be tested easily, and it is possible to distinguish which part of chloroplasts is damaged between thylakoid and stroma.

The reconstituted chloroplast system. Chloroplasts were collected from each stages as indicated in Fig. 1 (0 min, 10th mim, 30th min and 60th min), and thylakoids and stroma were obtained to make various reconstituted chloroplasts. Table 1 shows the results of CO_2 fixation by various reconstituted chloroplasts.

CO_2 fixation of the reconstituted chloroplasts was inhibited 58% and 77%, respectively, by the participation of thylakoids at 30th and 60th min in contrast to 26% and 42% with stroma at that stage. Overall, decrease in CO_2 fixation was greater in the reconstituted chloroplasts made of thylakoids from later stages (stages 2~4) than those made of stroma at the same stage. This indicates that damage in the thylakoids is more severe than that

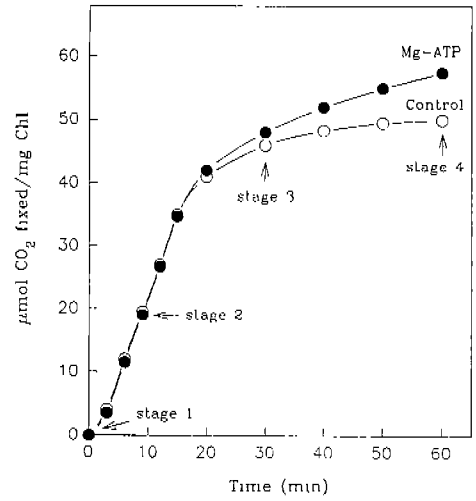

Fig. 1. The time course of CO_2 fixation by isolated intact spinach chloroplasts in the control and with 5 mM Mg-ATP at 25℃. CO_2 fixation was performed using a single preparation of chloroplasts containing 50 μg Chl under saturating light intensity (600 W/m^2) and CO_2 (10 mM).

Table 1. CO_2 fixation of the reconstituted chloroplasts made of thylakoids and stroma isolated from chloroplasts at various stages during time course of CO_2 fixation

Reconstituted chloroplast	CO_2 fixation rate	% of decrease
	μmol/mg Chl·h	
Thylakoid 1+Stroma 1[a]	79.2	–
Thylakoid 1+Stroma 2[b]	77.1	2.7
Thylakoid 1+Stroma 3[c]	58.8	25.8
Thylakoid 1+Stroma 4[d]	45.7	42.3
Thylakoid 1[a]+Stroma 1	87.7	–
Thylakoid 2[b]+Stroma 1	62.5	28.7
Thylakoid 3[c]+Stroma 1	37.1	57.7
Thylakoid 4[d]+Stroma 1	20.0	77.2

Intact chloroplasts were allowed to perform CO_2 fixation under the conditions described in Fig. 1. At each time point ([a]0 min, [b]10th min, [c]30th min, and [d]60th min), chloroplasts were collected and thylakoids and stroma were prepared as described in the Methods. For the reconstituted chloroplasts, 30 μg Chl of thylakoids and 500 μL of stroma (obtained from intact chloroplasts containing 500 μg Chl) were used.

Table 2. Photophosphorylation and NADP reduction of thylakoids isolated from chloroplasts at various stages during time course of CO_2 fixation

Thylakoid	Photophosphorylation	NADP reduction	P/2e
	μmol/mg Chl·h		Ratio
Thylakoid 1[a]	78.4	181.0	0.87
Thylakoid 2[b]	68.1	154.2	0.88
Thylakoid 3[c]	27.0	85.7	0.63
Thylakoid 4[d]	0	64.1	0

The conditions of experiment are same as described in the Table 1. Photophosphorylation and NADP reduction were measured using 30 μg Chl of thylakoids ([a]obtained from chloroplasts at 0 min, [b]10th min, [c]30th min, and [d]60th min).

in the stromal components. To confirm this result, thylakoidal function was measured.

Measurement of thylakoidal function. Using thylakoids isolated from chloroplasts at each stages, photophosphorylation and NADP reduction were measured. Both photophosphorylation and NADP reduction were gradually decreased during time course, and photophosphorylation was stopped completely at the 60th min (Table 2), coinciding with complete cessation of CO_2 fixation. Decrease in P/2e ratio implied photophosphorylation was affected more than electron transport. These results confirmed that thylakoidal damage occurred at the late phase (stages 3 and 4). Thus the cessation of CO_2 fixation of the isolated chloroplasts at the late phase seemed to be caused largely by the malfunction of thylakoids, especially leakage in photophosphorylation, rather than that of stroma.

DISCUSSION

As demonstrated here, the loss of photosynthetic activity at the late phase (stages 3 and 4) during photosynthesis occurs primarily by damage to the thylakoids although some decrease in the stromal function was observed (Table 1). The primary site of lesion seems to be photophosphorylation (Table 2). This result is in contrast to the previous reports that stromal components are the labile sites (Sicher and Jensen, 1979; Seftor and Jensen, 1986). CO_2 fixation by reconstituted chloroplasts also exhibited some loss in the stromal function. This could be due to the inactivation of stromal enzymes, especially Rubisco or leakage of stromal components into the medium (Seftor and Jensen, 1986). As a result, the actual amount of stromal components we used for this experiment at the late phase (stages 3 and 4) may have been decreased compared with those of the early phase (stages 1 and 2). However, loss in the thylakoidal function occurred to a greater extent (Table 1), suggesting damage in the thylakoid is more responsible for the die off in isolated chloroplasts than inactivation of stromal components. The measurement of P/2e ratio confirmed that damage in the thylakoid indeed occurred (Table 2). Both photophosphorylation and electron transport were harmed, but photophosphorylation was inhibited to a higher degree. The complete blockage of photophosphorylation at 60th min coincides with the complete cessation of CO_2 fixation at 60th min, supporting that inactivation of thylakoidal function is the main reason for the die off. The P/2e ratio has to stay at 1~1.5 for normal operation of photosynthesis, and decrease in P/2e ratio may be the direct cause for the die off. Since isolated chloroplasts were exposed to strong light to perform CO_2 fixation under experimental conditions, it is likely that thylakoids went through severe photoinhibition causing damage in the thylakoids, and eventually resulting in the complete cessation of CO_2 fixation.

The addition of Mg-ATP, as shown in Fig. 1, did not show any effect on the lag and lnear phase, but it prolonged the linear phase and delayed the die off at the late phase. Mg-ATP was indicated to be transported into the chloroplasts (Piazza and Gibbs, 1983). Externally added Mg-ATP may enable the chloroplasts to retain CO_2 fixation at a lower capacity at the late phase when photophosphorylation was stopped. It may not be enough to restore the photosynthetic rate at the late phase to the linear phase level. On the other hand, Mg-ATP was shown to protect thylakoids from photoinhibition (Fu, 1986), therefore delaying the onset of lesions in the thylakoids and the die off of chloroplasts.

Isolated chloroplasts lose their photosynthetic activity in the dark as well (Jun, 1991). The reason for this seems to be different from that in the light during photosynthesis. Experiments are in progress to identify the lesion sites in the dark.

적 요

시금치에서 분리한 엽록체의 탄소고정을 관찰하면 반응 시작 후 20분 정도가 지나면 고정율이 떨어지기 시작하여 1시간 정도가 지나면 고정이 완전히 멈춘다. 그 원인을 규명하기 위해 광합성 도중 시간별로 여러 단계의 엽록체에서 틸라코이드와 스트로마를 분리하여 재조합한 엽록체

의 탄소고정을 조사하였다. 후기 단계의 틸라코이드를 사용하여 재조합한 엽록체의 탄소고정능이 후기 단계의 스트로마를 사용하여 재조합한 엽록체의 탄소고정능보다 더 많이 떨어진 것으로 미루어 그 저해 원인이 스트로마보다는 틸라코이드에 의한 것으로 짐작된다. 각 단계별로 틸라코이드의 기능을 조사한 결과 후기 단계에서의 전자전달과 광인산화반응이 크게 감소하였으며 반응 후 60분 시에는 광인산화반응이 완전히 멈추어 P/2e 비율이 0이 되었다. 따라서 분리한 엽록체에서 광합성 도중 광합성능이 떨어지는 이유는 스트로마보다는 틸라코이드 기능, 특히 광인산화 반응의 저하에 있다고 사료된다.

REFERENCES

Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* **24**: 1-15.

Bassham, J.A., G. Levine and J. Forger III. 1974. Photosynthesis *in vitro*. I. Achievement of high rates. *Plant Sci. Lett.* **2**: 15-21.

Bucke, C., D.A. Walker and C.W. Naldry. 1966. Some effects of sugars and sugar phosphates on carbon dioxide fixation by isolated chloroplasts. *Biochem. J.* **101**: 636-641.

Fu, C.F. 1986. Temperature and photosynthetic carbon assimilation by spinach chloroplasts. Ph. D. thesis. Brandeis University, Waltham.

Fu, C.F. and M. Gibbs. 1988. Effects of temperature pretreatment in the dark on photosynthesis of the intact spinach chloroplast. *Plant Physiol.* **88**: 207-212.

Gibbs, M., C.F. Fu and S.-S. Jun. 1986. Effect of temperature on photosynthesis by the isolated chloroplasts. *In*, Hungarian-USA Binational Symposium on Photosynthesis, M. Gibbs (ed.). pp. 114-120.

Jun, S.-S. 1991. The influence of high temperature on photosynthesis in spinach. Ph. D. thesis. Brandeis University, Waltham.

Mills, W.R. and K.W. Joy. 1980. A rapid method for isolation of purified, physiologically active chloroplasts, used to study the intracellular distribution of amino acids in pea leaves. *Planta* **148**: 75-83.

Mourioux, G. and R. Douce. 1981. Slow passive diffusion of orthophosphate between intact isolated chloroplasts and suspending medium. *Plant Physiol.* **67**: 470-473.

Piazza, G.J. and M. Gibbs. 1983. Influence of adenosine phosphates and magnesium on photosynthesis in chloroplasts from peas, *Sedum*, and spinach. *Plant Physiol.* **71**: 680-687.

Sefter, R.E. and R.G. Jensen. 1986. Causes for the disappearance of photosynthetic CO_2 fixation with isolated spinach chloroplasts. *Plant Physiol.* **81**: 81-85.

Sicher, R.C. and R.G. Jensen. 1979. Photosynthesis and ribulose 1,5-bisphosphate levels in intact chloroplasts. *Plant Physiol.* **64**: 880-883.

Stumpf, D.K. and R.G. Jensen. 1982. Photosynthetic CO_2 fixation at air levels of CO_2 by isolated spinach intact chloroplasts. *Plant Physiol.* **69**: 1263-1267.

Sugino, Y. and Y. Miyoshi. 1964. The specific precipitation of orthophosphate and some biochemical application. *J. Biol. Chem.* **239**: 2360-2364.

Walker, D.A. and S.P. Robinson. 1978. Regulation of photosynthetic carbon assimilation. *In*, Photosynthetic Carbon Assimilation. H.W. Siegelman and G. Hind (eds.). Plenum Press, New York, pp. 43-59.

(Received September 16, 1993)