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An Improved Spectrophotometric Determination of the Activity of Ribulose 1,5-bishosphate Carboxylase

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Abstract : Spectrophotometric assay of ribulose 1,5-bisphosphate carboxlase, Rubisco, is a very useful method for the routine determination. However, when the freshly synthesized ribulose 1,5-bisphosphate, RuBP, is used as substrate there is a lag time between the carboxylation and NADH oxidation in the assay. In this study, we found firstly that the lag time was closely related to the concentrations of phosphoglycerate kinase, PGK, glyceraldehyde 3-phosphate dehydrogenase, GAP-DH, and phosphocreatine kinase, PCK, in the assay mixture. After increasing the concentrations of the coupling enzymes of PGK and GAP-DH, the lag time was shortened, but could not be eliminated completely. By increasing the concentration of PCK, lag time decreased significantly until there was no lag time. So, to overcome the lag time, the key is to increase the use of PCK but not the coupling enzymes. In addition, the accumulation of ADP in the assay mixture proved to be the most important factor in the assay mixture that produced the lag time in the spectrophotometric assay, and an optimum condition for activation and catalysis of Rubisco were also established. With the assay conditions established, high initial and total Rubisco activities were obtained.

Key words : ADP, Lag time, Rubisco, Spectrophotometric assay.

リブロース-1,5-ビスリン酸カルボキシラーゼ活性の分光光度法を用いた測定法の改良 : 杜 玉 春*,**・野瀬昭博**・川満芳信・村山盛一・和佐野喜久夫***・内田 泰*** (**鹿児島大学連合大学院，琉球大学農学部，***佐賀大学農学部)

要 旨 : リブロース-1,5-ビスリン酸カルボキシラーゼ (Rubisco) 活性の分光光度測定法はRubisco活性測定法において重要な方法である．しかし，リボース-5-リン酸から合成したリブロース-1,5-ビスリン酸を基質として用い，分光光度測定法でRubiscoの活性を測定する時，NADHの酸化が始まるまでに数分間の遅延時間 (Lag time) が生じる．本研究では，先ず遅延時間の程度が反応液中の3-ホスホグリセリン酸キナーゼ (PGK)．グリセルアルデヒド-3-リン酸脱水素酵素 (GAP-DH)，ホスホクレチアチンキナーゼ (PCK) の濃度に密接に関係することを示した．PGKとGAP-DHの濃度を増やすと，遅延時間は短くなったが，完全になくすことができなかった．しかし，PCKの濃度を増すと，遅延時間は完全になくなった．つまり遅延時間をなくすためには反応液中のPGKとGAP-DHの濃度ではなく，PCKの濃度を増加することが最も重要であることが分かった．分光光度測定法で遅延時間が生じる原因については，反応液中のADPの蓄積が関係していることが明らかになった．以上のようなことを参考にした抽出・反応系を用いて，サトウキビの葉においてRubiscoの高いinitialとtotal活性を得ることができた．

キーワード : アデノシン5-ニリン酸，遅延時間，分光光度測定法，リブロース-1,5-ビスリン酸カルボキシラーゼ．

In many plant physiological studies, the measurement of Rubisco actibity is very important. Rubisco activity generally is measured by two methods, one is the $^{14}CO_2$ method, another is spectrophotometric assay. Compared with the $^{14}CO_2$ method, the spectrophotometric method has the advantages of being convenient and producing no

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Abbreviations : ADP, adenosine 5′-diphosphate ; ATP, adenosine 5′-triphosphate ; BSA, bovine serum albumin ; DTT, dithiothreitol ; EDTA, ethylenediamine tetraacetic acid ; GAP, glyceraldehyde 3-phosphate ; GAP-DH, glyceraldehyde 3-phosphate dehydrogenase ; HEPES, 2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid ; NAD^+, β-nicotinamide-adenine dinucleotide oxidized form ; NADH, β-nicotinamide-adenine dinucleotide reduced form ; PCK, phosphocreatine kinase ; PGA, phosphoglycerate ; PGK, phosphoglycerate kinase ; Pi, orthophosphoric acid ; R5-P, ribose 5-phosphate ; RuBP, ribulose 1,5-bisphosphate ; Rubisco, ribulose 1,5-bisphosphate carboxylase.

radioactive waste, especially Rubisco activity can be measured continuously. Although the spectrophotometric assay for Rubisco has been developed for a long time[1,9], because there was a long lag time between the carboxylation and NADH oxidation, this method had not been widely used. In shortening the lag time, Lilley and Walker[3] had made significant progress by adding an ATP-regenerating system to the assay mixture, but this procedure was often judged not acceptable because the lag time still existed[2,11]. Then, mainly based on Lilley and Walker's method, various attempts have been made to shorten the lag time. These attempts were generally made by using a large amount of PGK and GAP-DH to accelerate the reactions to the end of GAP[2,11], some results showed that the lag time was partially shortened but could not be eliminated completely by this kind of method[2]. It has been known that ADP is a very sensitive inhibitor for PGK[6,7]. We think that, instead of using a large amount of PGK and GAP-DH, increasing the concentration of PCK to reduce the accumulation of ADP in the assay mixture may be more effective for the elimination of lag time.

Another problem for this method comes from the reaction substrate, RuBP. Commercial RuBP is very expensive and unstable. Edmondson et al.[2] reported that RuBP from Sigma Chemical Co. (St. Lousi, Mo. USA) contained too much xylulose 1,5-bisphosphate and supported low carboxylase activity. Lilley and Walker[3] had suggested using R5-P, which was much cheaper, more stable and produced higher activity of Rubisco to replace RuBP as a substrate in the assay system. But because there existed a longer lag time compared with using RuBP as a substrate, R5-P has up to now seldom been directly used as a substrate.

In this experiment, we freshly synthesized RuBP from R5-P, overcame the lag time problem and obtained high Rubisco activity. Because this method does not require sophisticated equipment and special tools, we think that it can be used for routine determination of Rubisco in many laboratories.

As for the principle, spectrophotometric assay of Rubisco is based on the measurement at 340 nm of PGA-dependent NADH oxidation by a coupled enzyme system. Reactions are explained as the following equations, in which PCK is added for the regeneration of ATP in the assay mixture:

$$\text{(1)}\quad \text{RuBP}+CO_2 \xrightarrow{\text{Rubisco}} 2(\text{3-PGA})$$

$$\text{(2)}\quad 2(\text{3-PGA})+2\ \text{ATP} \xrightarrow{\text{PGK}} 2(\text{1,3-PGA})+2\ \text{ADP}$$

$$\text{(3)}\quad 2(\text{1,3-PGA})+2\ \text{NADH} \xrightarrow{\text{GAP-DH}} 2\ \text{GAP}+2\ \text{NAD}^+ +2\ \text{Pi}$$

$$*\ \text{phosphocreatine}+\text{ADP} \xrightarrow{\text{PCK}} \text{creatine}+\text{ATP}$$

Materials and Methods

1. Materials

Sugar cane (*Saccharum* sp. cv. NiF4) was raised in 14.5 L pots filled with a mixture of soil-sand-peat (1 : 1 : 1) in a glass house (College of Agriculture, University of the Ryukyus, Okinawa, Japan) under natural sun light. Water was given daily and Hoagland solution was given twice a week. Two months after planting, the upper most fully expanded leaves were used as materials.

RuBP, phosphoribuloisomerase, phosphoribulokinase, BSA and ADP were from Sigma Chemical Co. (USA). PGK, GAP-DH, PCK, R5-P, NADH and ATP were from Boehringer Mannheim Biochemical (FRG). DTT and phosphocreatine were from Wako Pure Chemical (Japan).

2. Extraction and Assay

After being illuminated under photon flux density of 1,500 μmol m^{-2} s^{-1} for one hour, leaf pieces, around 4 cm^2, were frozen and held in liquid nitrogen until analyzed. The frozen leaf pieces were rapidly homogenized in a chilled mortar with 30 mg insoluble polyvinyl polypyrrolidone, 0.2 g sea sand and 4 mL extraction buffer which contained 100 mM Tris-HCl pH 7.8, 1 mM EDTA-NaOH pH 7.0, 5 mM $MgCl_2$, 5 mM DTT, and 0.2% (w/v) BSA. The homogenate was filtered through one layer of Mira-cloth (Calbiochem-Novabiochem Co. USA) and centrifuged for 30 seconds at 12,000 rpm in a Diskboy

Kurabo Eb4000. The supernatant was used immediately for the determination of initial Rubisco activity. All processes in extraction were conducted as close to 0°C as possible.

Rubisco was assayed at 30°C in a medium containing 50 mM HEPES-KOH pH 8.0, 10 mM $NaHCO_3$, 0.2 mM NADH, 2.5 mM ATP, 10 mM KCl, 1 mM EDTA-NaOH pH 7.0, 20 mM $MgCl_2$, 5 mM DTT, 5 mM phosphocreatine, 6 units per mL of PGK and GAP-DH, 20 units per mL of PCK. Initial Rubisco activity was determined by adding 20 μL of leaf extract to the assay solution, whose final volume was 1000 μL, and the reaction was completed within 1 minute. The absorption at 340 nm (Shimadzu, UV-160) without RuBP was taken as base line and the reaction was started by adding 0.6 mM RuBP at final concentration. To measure the total activity, aliquot of leaf extract was transferred to a test tube, and Mg^{2+} and $NaHCO_3$ concentrations were brought to 30 mM and 10 mM, respectively. The Rubisco was activated on ice for 10 minutes[10] and then determined as above.

3. Synthesis of RuBP

RuBP was made from R5-P. The method of synthesis basically was as according to Sharkey et al.[11], but several aspects had been changed. Briefly, 100 μmol R5-P, 100 μmol $MgCl_2$ and 110 μmol ATP were dissolved in 4 mL distilled water. The pH of the solution was adjusted to 6.8 by 2 M KOH. When 2 M KOH was added, the solution was stirred vigorously. Then 2 units per mL of phosphoribuloisomerase and phosphoribulokinase plus 4 mM DTT at final concentrations were added. The solution was stirred continuously and pH was kept at around 6.8 by adding 0.1 mM KOH until there was no pH decline. Then the pH of the solution was adjusted to about 2 by 3.5% $HClO_4$. The solution was kept on ice for 5 minutes and centrifuged at 14,000 g for 10 minutes to remove the denatured enzymes. HEPES and KCl were added to the supernatant, and the concentrations of HEPES and KCl were brought to 150 mM and 10 mM, respectively. After the pH was raised back to 5 by 2 M KOH, the resulting solution was centrifuged again to remove potassium perchlorate. The product was stored as solution at −80°C. It was reported that making RuBP in this way could get a conversion efficiency of exceeding 80%[11].

4. Preparations of PGK, GAP-DH and PCK

PGK and GAP-DH were purchased as ammonium sulfate precipitates. Because sulfate is an inhibitor of Rubisco in competition with RuBP, it has to be removed. Precipitates were centrifuged, the pellets dissolved in 20% glycerol and stored at −80°C. PCK was purchased as powder and directly dissolved in 20% glycerol and stored at −80°C. Prepared in this way. PGK, GAP-DH and PCK can be stored for several months without loss of activity.

Results

1. Lag time

Lag time is the time difference between the carboxylation of RuBP and the oxidation of NADH. The upper parts of Fig. 1 show the typical reaction trace in spectrophotometric assay of Rubisco with lag time. The lag time generally lasts several minutes, depending on assay conditions. The long lag time in Rubisco assay not only prolongs the measurement of Rubisco, but most importantly, it makes it difficult to measure the true initial activity of Rubisco because a long time is elapsed between extraction of Rubisco and assay of its activity, in which activation of Rubisco may occur. The lower parts of Fig. 1 show the standard reaction trace in spectrophotometric assay by using the present assay method. It shows that lag time has been completely overcome.

2. Substrate

Under the conditions of the presence of a small amount of enzymes, 3 units per mL of PGK and GAP-DH, and 1.3 units per mL of PCK, freshly synthesized RuBP by the method described in Material and Method and commercial RuBP supplied by Sigma Chemical Co. (USA) were used as a substrate in the assay. Using RuBP from Sigma as the substrate gave lower activity of Rubisco but with no lag time, while using freshly synthesized RuBP caused more than twice the amount of activity in Rubisco than using commercial RuBP from Sigma, but with a lag time (Table 1). Similar results were also reported in other studies[2].

3. The concentrations of the enzymes in assay mixture

The concentrations of the enzymes, PGK,

GAP-DH and PCK, in the assay mixture were closely related to lag time (Table 2). Increasing the concentrations of the coupling enzymes, PGK and GAP-DH, in the assay mixture, the lag time was reduced, but could not be eliminated completely, even at very high concentrations of coupling enzymes. When the concentration of PCK was increased, the lag time was reduced significantly until there was no lag time. However, with the increases of the concentrations of PGK and GAP-DH, and PCK, although the lag time was reduced, at the same time the Rubisco activity was also decreased. Considering both activity and lag time, the combinations of 6 units per mL of PGK and GAP-DH, and 20 units per mL of PCK were appropriate conditions for obtaining maximum activity without lag time. Although the activity obtained is much higher than reported previously[8,12,13,14], this activity is significantly lower than with lag time (Table 2). The lower activity without lag time may result from two things. One is that the ammonium sulfate was not removed completely in the preparations of PGK and GAP-DH, the residue sulfate inhibited Rubisco activity. Recently, we found that when ammonium sulfate was removed more thoroughly by centrifuging the ammonium sulfate suspension at higher speed for a longer time (e. g. at 45,000 g for 30 minutes), Rubisco activity did not drop significantly, even large amounts of PGK and GAP-DH were used (deta not shown here). Another possibility is that higher activity with a lag time was not initial Rubisco activity, but the activity in some levels of activated state.

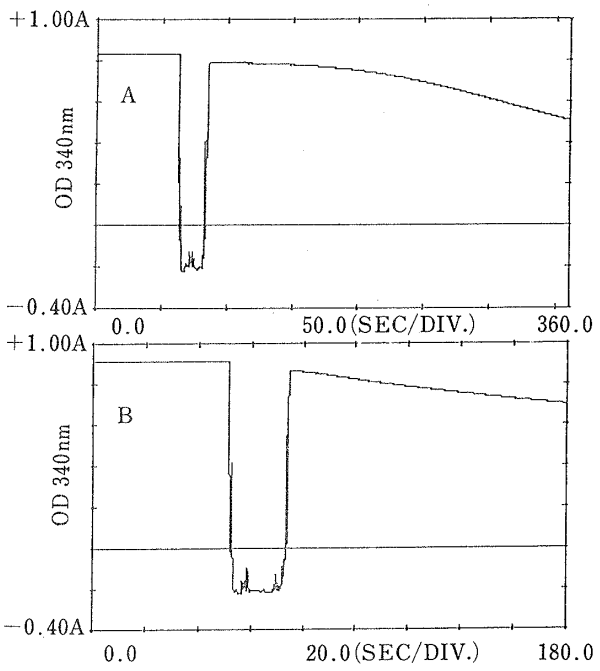

Fig. 1. Typical recorder traces of Rubisco activity. (A), Reaction with a lag time, assay mixture contained 3 units per mL of PGK and GAP-DH, and 1.3 units per mL of PCK; (B), reaction without lag time, assay mixture contained 6 units per mL of PGK and GAP-DH, and 20 units per mL of PCK. In both cases, freshly synthesized RuBP were used as substrate.

4. Optimal pHs for extraction and assay

Optimal extraction pH was 7.8 (Fig. 2). This result agreed with the results obtained from maize, spinach, soybean and wheat[8]. Optimal assay pH was 8.0 (Fig. 3). This result is consistent with the results of Sharkey et al.[11] and Usuda[13].

5. Optimal Mg^{2+} concentrations for activation and catalysis

Mg^{2+} was needed in both assay and activation processes. If the assay medium contained no Mg^{2+}, the activity of Rubisco was almost zero. Mg^{2+} did not show apparent inhibition for assay and activation up to 30 mM (Fig. 4) (data on activation were not shown here). These results agree with the results of Lorimer et al.[4,5] and Usuda[13], which showed that 20 mM was optimal for activation and assay, and 30 mM was not inhibitory for catalysis, respectively. When Mg^{2+} concentration was too high, especially over 40 mM, inhibition was

Table 1. Effects of different RuBP on the lag time and activity of Rubisco.

Substrate	Activity (μmol m^{-2} s^{-1})		Lag time (s)
	Initial	Total	
Commecial RuBP from Sigma	36.8	36.3	0
Freshly synthesized RuBP	74.5	75.5	150

The concentrations of enzymes in the assay mixture were : 3 units per mL of PGK and GAP-DH, 1.3 units per mL of PCK.

Table 2. Effects of concentrations of the coupling enzymes and PCK on lag time and initial activity of Rubisco*.

Con. of enzymes (units per mL)			Activity	Lag time
PGK	GAP-DH	PCK	(μmol $m^{-2}s^{-1}$)	(s)
1 U	1 U	1.3 U	65.7	190
2 U	2 U	1.3 U	74.7	160
3 U	3 U	1.3 U	72.6	150
6 U	6 U	1.3 U	71.3	110
9 U	9 U	1.3 U	63.0	110
15 U	15 U	1.3 U	47.1	100
3 U	3 U	1.3 U	72.6	150
3 U	3 U	6 U	67.5	40
3 U	3 U	15 U	52.8	15
6 U	6 U	15 U	56.4	10
6 U	6 U	20 U	52.8	0

*The responses of lag time and total activity of Rubisco to the concentrations of coupling enzymes and PCK were similar to those indicated above (data not shown here).

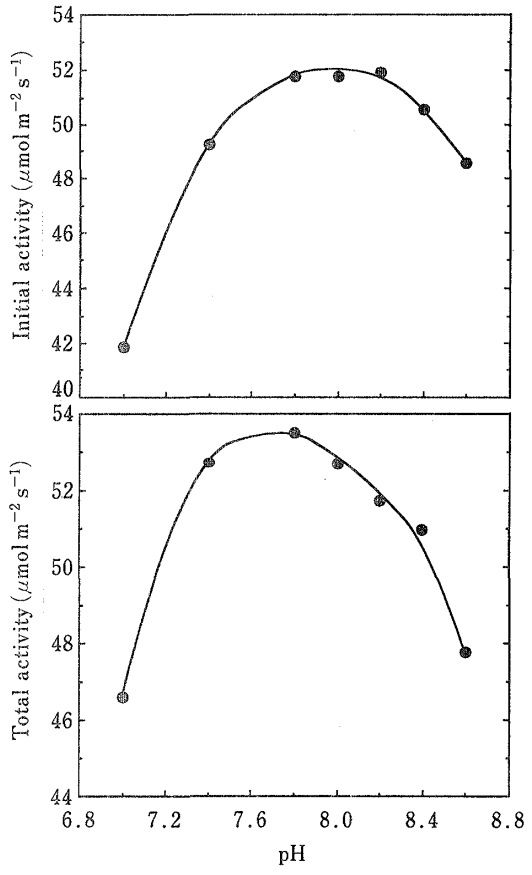

Fig. 2. Effects of extraction pH on initial and total activities of Rubisco.

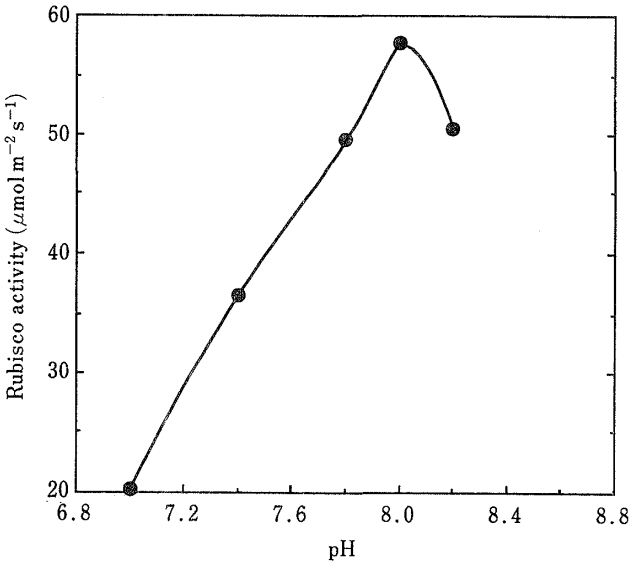

Fig. 3. Effects of assay pH on initial activity of Rubisco. The response of total activity of Rubisco to assay pH was very similar to that shown here.

shown both for the activation and catalysis.

6. Relationship between extract volume and optical density

By using the above assay method, the relationship between extract volume used in assay and the difference of optical density within one minute, $\triangle$OD, was established (Fig. 5). In Fig. 5, about 10 cm^2 of leaf pieces were extracted in 4 mL of extraction buffer. Under

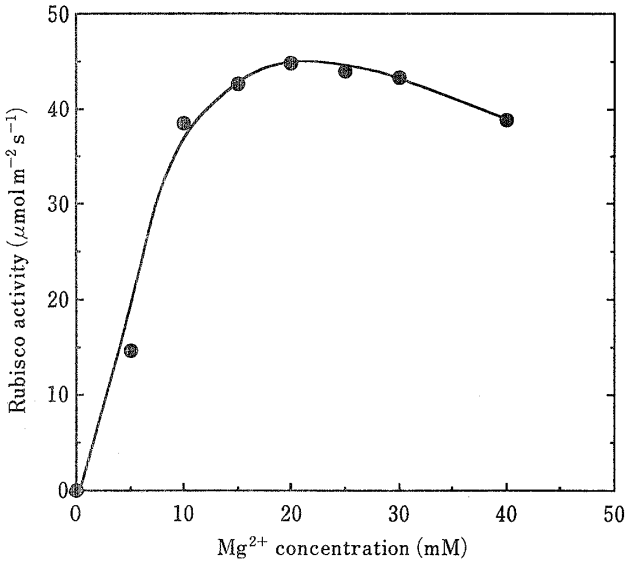

Fig. 4. Effects of Mg^{2+} concentration on total activity of Rubisco. The response of initial activity of Rubisco to Mg^{2+} concentration demonstrated a very similar way shown here.

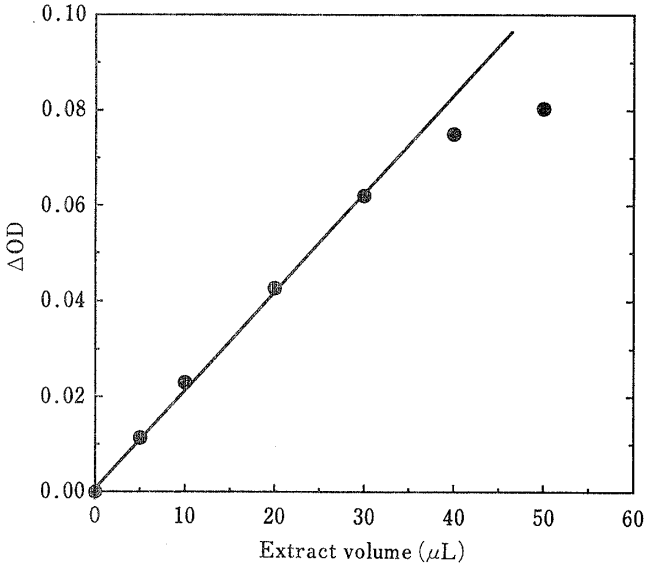

Fig. 5. The relationship between extract volume and optical density, ΔOD. Refer to "Relationship between extract volume and optical density" in Results in detail.

this conditions, the △OD was linearly related to the volume within 30 μL. Based on this relationship, for the formal experiments, the area of leaf pieces was reduced from about 10 cm^2 to about 4 cm^2, and 20 μL of extract was the selected amount to be used in assay.

Discussion

It has been established that ADP is a sensitive inhibitor for PGK[6,7]. The data in Table 2 show that the concentration of PCK was closely related to the lag time. These facts indicate that ADP played an important role in producing lag time. Table 3 shows the effect of ADP on the lag time and the activity of Rubisco. The results demonstrate clearly that the reactions in the assay mixture were very sensitive to the concentration of ADP, and ADP was an important factor in the assay mixture that produced the lag time in spectrophotometric assay. In case of using freshly synthesized RuBP as substrate, the ADP in the assay mixture could come from two sources. Firstly, when we synthesized the RuBP, a large amount of ATP had been used. With the conversion of R5-P into RuBP, ATP was converted into ADP. When this freshly synthesized RuBP was used as the substrate, the ADP would go into the assay mixture with RuBP. Secondly, when the reaction chains from RuBP to GAP were occurring, ADP could be produced with the conversion of 3-PGA to 1,3-PGA. However, when the commercial RuBP from Sigma was used as substrate, there was no lag time (Table 1 and Table 3). Therefore, it was clear that when the freshly synthesized RuBP was used as substrate, the lag time was produced by the first ADP, not by the second one under the assay condition of using a small amount of enzymes such as 3 units per mL of PGK and GAP-DH, and 1.3 units per mL of PCK. After synthesis of RuBP, because the concentrations of ADP and ATP were not determined in the final RuBP stock solution in this experiment, how

Table 3. Effects of ADP on lag time and initial activity of Rubisco.

ADP (mM)	Activity* (μmol m^{-2} s^{-1})	(s)
0.0	31.4±0.4	0
0.3	37.9±1.1	90
0.6	38.0±0.4	180
0.9	33.0±0.7	220

1) Concentrations of the enzymes in the assay mixture were: 3 units per mL of PGK and GAP-DH, 1.3 units per mL of PCK.
2) ADP concentrations were acquired by adding certain amounts of ADP with substrate into the assay mixture.
* Mean of three replicates with standard error.

much ADP and ATP were brought into the assay mixture with substrate is not clear. We think these points need to be clarified in future experiments because these kind of data are very helpful for the quantitative understanding of the effects of ADP and ATP on lag time and Rubisco activity. An interesting point in Table 3 is that by adding certain amounts of ADP in the assay mixture, for example, 0.6 mM, the measured activity of Rubisco increased significantly. This result coincided with the result that by using freshly synthesized RuBP, which contained a certain amount of ADP, higher activity of Rubisco was obtained than by using commercial RuBP from Sigma, which contained less ADP (Table 1). Why assay mixture containing a certain amount of ADP could increase the activity of Rubisco is not clear and also worthy of further research.

To overcome the lag time, previous researchers generally used a large amount of coupling enzymes to drag the reactions to the end of GAP. For example, Sharkey et al.[11] used 47 units per mL of PGK and GAP-DH, Edmondson et al.[2] used 60 units per mL of PGK and 25 units per mL of GAP-DH. By comparison, they only used 1.3 and 15 units per mL of PCK in the assay mixture, respectively. The previous study[2] and the data in Table 2 have shown that increasing the use of the coupling enzymes could shorten the lag time but could not eliminate the lag time completely. On the other hand, Table 2 has shown that using lower concentrations of coupling enzymes, higher activity of Rubisco could be obtained. This meant that even using a small amount of coupling enzymes, for example, 3 units per mL of PGK and GAP-DH, the conpling enzymes were enough to convert 3-PGA, which was synthesized from RuBP and CO_2, into GAP. The data in Table 3 have indicated that ADP was an important factor in the assay mixture which produced the lag time, and increasing the concentration of PCK in the assay mixture was more effective for the shortening of lag time (Table 2). So the conclusion is clear, to obtain higher activity of Rubisco without the lag time in the spectrphotometric assay by using freshly synthesized RuBP as substrate, a large amount of PCK, a small amount of PGK and GAP-DH should be used. Previous methods using a large amount of PGK and GAP-DH, but relatively a small amount of PCK to overcome the lag time[2,11], seems not to be appropriate.

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