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The Association of D-Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase with Phosphoribulokinase[1]

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ABSTRACT

When Ribulose-1,5-bisphosphate carboxylase/oxygenase was purified from spinach leaves (*Spinacia oleracea*) using precipitation with polyethylene glycol and $MgCl_2$ followed by DEAE cellulose chromatography, 75% of phosphoribulokinase and 7% of phosphoriboisomerase activities copurified with ribulose-1,5-bisphosphate carboxylase/oxygenase. This enzyme preparation showed ribose-5-phosphate and ribulose-5-phosphate dependent carboxylase and oxygenase activities which were nearly equivalent to its corresponding ribulose-1,5-bisphosphate dependent activity. The ribose-5-phosphate and ribulose-5-phosphate dependent reaction rates were stable and linear for much longer time periods than the ribulose-1,5-bisphosphate dependent rates. When sucrose gradients were used to purify ribulose-1,5-bisphosphate carboxylase/oxygenase from crude stromal extracts, phosphoribulokinase was found to cosediment with ribulose-1,5-bisphosphate carboxylase. Under these conditions most of the phosphoriboisomerase activity remained with the slower sedimenting proteins. Ammonium sulfate precipitation resulted in separation of the ribulose-1,5-bisphosphate carboxylase peak from phosphoribulokinase peak. Crude extracts of peas *Pisum sativum* and spinach contained 0.725 to 0.730 milligram of phosphoribulokinase per milligram of chlorophyll, respectively, based on an enzyme-linked immunosorbent assay.

Enzymological studies that have focused on the catalytic properties of RuBP carboxylase/oxygenase (Rubisco)[3] have generally been done utilizing dilute solutions of the highly purified protein (20). These circumstances tend to minimize protein-protein interactions and provide for abundant aqueous pools of substrates. However, when the protein, metabolite, and water concentrations in the stromal milieu are considered, it is unlikely that there would be abundant aqueous pools of intermediary metabolites. It was hypothesized by Ashton (2) that because of the high concentration of Rubisco in the stroma, and its capacity to bind free metabolites, the effective concentration of free metabolites in the stroma may be very low. In an environment such as this it might be necessary to have the enzymes carrying out sequential reactions organized into multienzyme complexes or clusters such that metabolic channeling would be facilitated (25). Given the high concentration of Rubisco it is highly unlikely that Rubisco molecules would be devoid of extensive protein-protein interactions or would be capable of free diffusion in the stromal environment. We have recently reported (23) that Rubisco, when purified from stromal extracts of pea chloroplasts on sucrose gradients, showed Rib-5-P and Ru-5-P dependent CO_2 fixation activities. The expression of these activities required in addition to Rubisco either phosphoribulokinase (kinase) (Ru-5-P dependent CO_2 fixation) or kinase and phosphoriboisomerase (isomerase) (Rib-5-P dependent CO_2 fixation). This Rubisco preparation showed equivalent rates of CO_2 fixation using RuBP or Ru-5-P when treated with 10 mM DTT. Also, the Ru-5-P dependent activity was linear for longer time periods than the RuBP dependent activity (25). These studies have suggested some binding specificity between kinase and carboxylase and some kinetic benefit to the carboxylase reaction. The work reported here is an extension of these studies using both spinach and peas. It is an effort to further characterize the association and the kinetics of this multiple reaction sequence.

MATERIALS AND METHODS

Chloroplasts were isolated from 6 to 8 week old spinach leaves (*Spinacia oleracea*) and 10 to 20 d old pea shoots (*Pissum sativum*) according to Cerovic *et al.* (4). Chloroplasts were lysed in 25 mM Bicine buffer (pH 8). The membranes were removed by centrifugation (25,000*g* for 15 min) and supernatants were processed on sucrose gradients (5–30%) made either in 25 mM Bicine (pH 8) containing 20 mM each of $MgCl_2$ and bicarbonate (activating), or in 25 mM Bicine (pH 8) alone (nonactivating). Gradient separation was done using a TV 865 vertical rotor for 1 h at 52,500 rpm in a Sorvall SA 60 ultracentrifuge. Rubisco was purified from 130 g of freshly harvested greenhouse-grown spinach leaves by the procedure of Hall and Tolbert (8) using PEG-$MgCL_2$ for the initial precipitation of Rubisco. The enzyme obtained from the DEAE column was made 10 mM with DTT and then passed through a G-25 column equilibrated with 100 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM EDTA, 1 mM DTT, and 1 mM ATP (pH 8). ATP was included to prevent inactivation of the kinase (22). Aliquots of activated enzyme were used for both oxygenase and carboxylase assays.

Carboxylase Assay

The RuBP dependent assay mixture contained 50 mM Bicine, 1 mM EDTA, 10 mM DTT, 10 mM $MgCl_2$, 2 mM RuBP, and 20 mM ^{14}C $NaHCO_3$ (2 μCi). For Rib-5-P and Ru-

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[3] Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; Rib-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; fr wt, fresh weight.

5-P dependent assays RuBP was replaced by 10 mM Rib-5-P or Ru-5-P and 4 mM ATP. In all cases activity of Rubisco was based on the formation of acid stable ^{14}C label which was measured by scintillation spectrometry.

Oxygenase Assay

The oxygenase assays were done in the same buffer as the carboxylase assays and used the same substrate concentrations except that bicarbonate and DTT were not added. The reaction rates were monitored by measuring O_2 consumption with an O_2 electrode (11). The reaction was initiated by activated enzyme.

Phosphoribulokinase was assayed according to Krieger and Miziorko (14) with the following modifications. Enzyme was preincubated for 30 min with 100 mM DTT before assay. The assay mixture contained 5 mM DTT, 0.4 mM Ru-5-P, or 10 mM Rib-5-P and 1 unit of isomerase, 4 mM phosphoenolpyruvate, 28 units of lactate dehydrogenase and 31 units of pyruvate kinase in 1 mL of assay mixture. Phosphoriboisomerase was measured according to Wood (28). SDS-PAGE was done by the method of Laemmli (15). Rib-5-P (sodium salt) and Ru-5-P (sodium salt) from Sigma were used for all the experiments. RuBP was also synthesized according to Horecker *et al.* (12) and was purified by the procedure of Wong *et al.* (27). This RuBP was stored in the acid form at −20°C. This RuBP or Sigma RuBP was used wherever mentioned.

ELISA

The amount of phosphoribulokinase was measured in crude extracts of peas and spinach leaves by ELISA. Phophoribulokinase from peas was purified according to the method of Krieger and Miziorko (14) up to the step of acid precipitation. At this point the preparation was further purified by chromatography over DEAE cellulose and reactive red 120-agarose type 3000-CL (Sigma) columns as described by Porter *et al.* (22). The fraction that gave a single band of approximately 44,000 mol wt and which reacted with the antibody supplied to us by Porter in Western blots, was used as a standard. Crude extracts run on SDS-PAGE were used on Western blots to determine whether the antikinase antibody reacted with any other proteins in spinach or pea. A standard curve was prepared by coating the wells with different amounts of pure kinase. The specific steps in the ELISA procedure were as follows: (a) The wells Immunolon I plates (96 Wells-Dynatech) were coated overnight at 4°C with 50 μL/well of various concentrations of the pure kinase (0.078–5 μg/mL) diluted in 0.05 M borate buffer pH 8.6. (b) The next day the plates were washed twice with 0.01 PBS containing 1% tween 20, followed by two washes with PBS. (c) The plates were blocked for 1 h at room temperature with 100 μL 0.01 M phosphate buffer containing 3 mM EDTA and 0.1% BSA (pH 8). (d) The plates were washed again with PBS as in step 2. (e) Rabbit antikinase antibody was diluted 1:4000 in PBS containing 1% BSA and 0.05% Tween; 50 μL were added to each well and the plates were incubated at room temperature for 5 h. (f) The plates were washed with PBS. (g) Goat antirabbit IgG conjugated to alkaline phosphatase (Boehringer Mannheim Biochemicals No. 605230) was diluted 1:2000 in PBS-BSA-Tween mixture; 50 μL were added to each well and plates kept overnight. (h) The plates were incubated next day with Sigma substrate 104 (*p*-nitrophenyl phosphate), 1 mg/mL in bicarbonate buffer pH 9.5. The color development was read after 10 min on a Dynatech ELISA reader. Crude extracts were diluted to give protein concentrations of 0.5 to 2 μg/mL and processed as above.

Western Blot Analysis

Crude extracts of pea and spinach were subjected to SDS-PAGE performed according to the method of Laemmli (15). Mol wt were calculated using Dalton Mark VII-L (Sigma) standards. The proteins were transferred to cellulose nitrate paper by the procedure of Towbin *et al.* (26). The kinase was treated on nitrocellulose filters with kinase antibody. Peroxidase-conjugated goat-anti-rabbit secondary antibody and reagents were purchased from Biorad (Immun-Blot kit) and were used according to manufacturer's instruction.

RESULTS AND DISCUSSION

Sucrose Gradient Sedimentation Patterns of Kinase, Isomerase and Rubisco

Figure 1 shows the sucrose gradient sedimentation pattern of kinase and isomerase activities from crude spinach chloroplast extracts. It was observed that when the gradients contained Mg^{2+} and bicarbonate (activating conditions for Rubisco), kinase activity cosedimented with the major protein peak. Previous work (23) and the SDS-PAGE results shown in Figures 3 and 4 demonstrated that the majority of the 280 nm absorbing material in this peak was Rubisco. Under nonactivating conditions kinase activity often showed a slightly slower sedimentation rate than Rubisco; however, it still moved with the rapidly sedimenting major protein peak (data not shown). Under both activating and nonactivating conditions the majority of the isomerase activity was localized at the top of the gradient with other slowly migrating proteins. We have also conducted these experiments using pea chloroplasts (data not shown). The pea kinase activity showed three peaks of activity under nonactivating conditions whereas

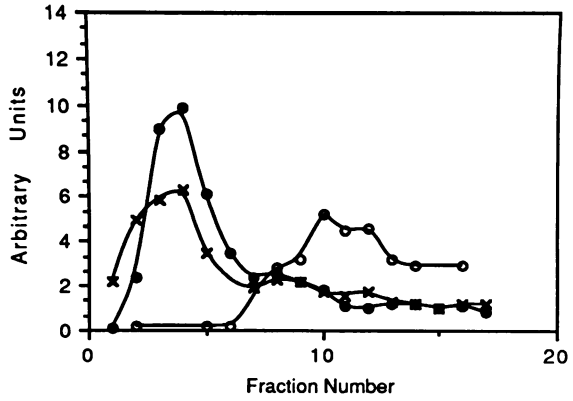

Figure 1. Analysis of the fractions obtained following sucrose density gradient separation of stroma from spinach chloroplasts. Arbitrary units: one kinase unit is 0.10 μmol per min (×), one protein unit is 0.05 OD at 280 nm (●), one isomerase unit is 0.27 μmol per min (○).

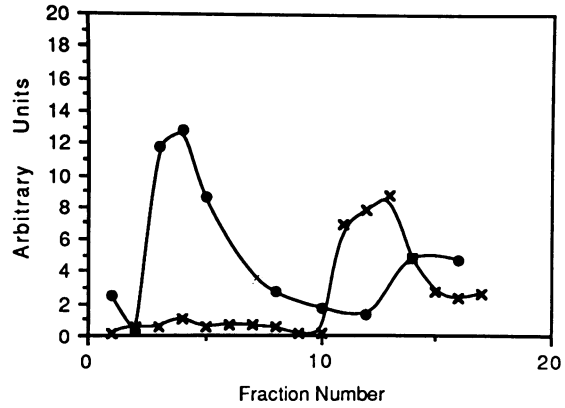

Figure 2. Sucrose density gradient separation of an ammonium sulfate precipitate of a crude extract of spinach. This extract was dissolved and dialysed against 25 mM Bicine buffer (pH 8) prior to centrifugation. Gradient separation was done under nonactivating conditions for Rubisco. Arbitrary units: one kinase unit is 0.05 μmol per min (×). One protein unit is 0.005 OD at 280 nm (●). Sedimentation pattern of the dialyzed ammonium sulfate precipitate was similar under activating conditions.

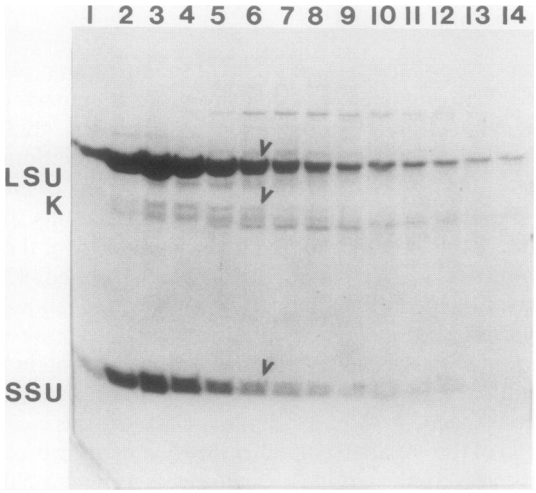

Figure 3. Ten percent SDS-PAGE of the fractions (1–14) obtained following density gradient centrifugation of spinach extracts precipitated with PEG-MgC12. Left to right, 30 to 5% sucrose. Arrows indicate positions of kinase (K) and the large (LSU) and small (SSU) subunits of Rubisco.

under activating conditions all the kinase activity cosedimented with Rubisco. Also, as was the case in spinach, the main peak of pea isomerase migrated with the slower sedimenting proteins. This isomerase pattern was also not altered by the inclusion of Mg^{2+} and bicarbonate in the sedimentation buffer. Since the main isomerase peak did not show any tendency to sediment with Rubisco, subsequent experiments were concerned principally with kinase and Rubisco.

One possible explanation for these results with kinase was that the gradient conditions resulted in the self-association of kinase into high mol wt aggregates that sedimented with Rubisco independent of any physical interaction between the proteins. In an effort to determine if this was possible, a series of experiments were conducted with partially purified spinach kinase (Sigma). We followed the sedimentation pattern of

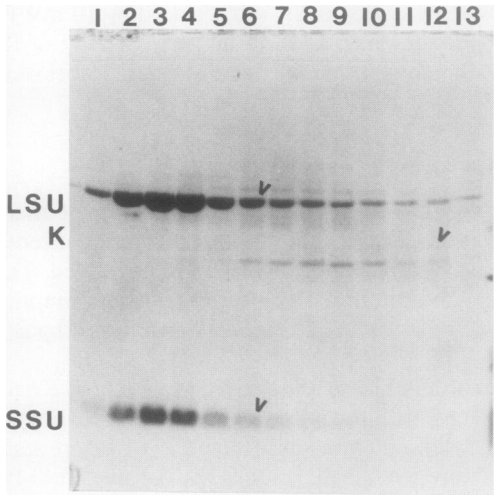

Figure 4. Ten percent SDS-PAGE. Same as Figure 3 except that the spinach extracts were pecipitated with ammonium sulfate prior to density gradient centrifugation and one less fraction was analyzed. Positions of kinase (K) and the large (LSU) and small (SSU) subunits of Rubisco.

partially purified kinase under activating and nonactivating conditions. It was observed that Sigma kinase had a low sedimentation coefficient regardless of the gradient conditions (data not shown).

If the crude spinach chloroplast extract was precipitated with 50% ammonium sulfate, dialyzed against Bicine buffer, and analyzed on sucrose gradients under activating and nonactivating conditions, the main kinase peak was observed with the lower mol wt proteins (Fig. 2). Thus, treatment with ammonium sulfate was found to result in a change in the sedimentation behavior of kinase, presumably due to a disruption of the kinase, Rubisco association. The sucrose gradient fractions of the ammonium sulfate treated and untreated samples were analyzed on 10% SDS-PAGE (Figs. 3, 4). The samples not treated with ammonium sulfate showed the presence of several bands in the Rubisco fractions (Fig. 3). Ammonium sulfate precipitation resulted in greater Rubisco homogeneity and also appeared to decrease the yield of kinase (Fig. 4). The bands identified as kinase (K) in Figs. 3 and 4 had a calculated mol wt of 43,660 which is very similar to that previously reported (44,000) for kinase (22). Also, the bands at this location in SDS gels have consistently shown cross-reactivity on Western blots with antikinase antiserum.

These studies on the sedimentation properties of kinase and carboxylase on sucrose gradients have suggested that: (a) the sedimentation of kinase with the Rubisco is due to an association between Rubisco and kinase rather than an independent sedimentation due to similar sedimentation coefficients; (b) the association of kinase with carboxylase shows some specificity, *e.g.* isomerase does not sediment with Rubisco; (c) the molecular forces stabilizing the Rubisco association are dissipated by high salt concentration.

Precipitation of Rubisco by PEG-$MgCl_2$

The specificity in the copurification of kinase and Rubisco was further studied by utilizing properties of PEG which have been reported to promote enzyme associations. PEG appar-

Table I. *Total Number of Units[a] of Phosphoriboisomerase and Phosphoribulokinase Recovered with Rubisco After Purification Using PEG-$MgCl_2$ Technique (8)*

	Isomerase	Kinase	RiBisCO	Protein
				mg
Crude	6684	2476	317	1045
PEG-$MgCl_2$ ppt	1250	1785	414	840
DEAE Cellulose	457	1874	341	250

[a] One unit is equivalent to a turnover of 1 μmol of substrate per minute.

ently acts through a steric exclusion mechanism in which long chains of PEG confine proteins to relatively small pockets. This, in turn, results in self-association and precipitation of associating proteins (9, 19). Table I shows the activities of kinase and isomerase that copurified with Rubisco prepared by the PEG-$MgCl_2$ technique. On the average, 7% of the isomerase and 75% of the kinase activities found in the crude extracts could be detected in Rubisco prepared by this technique. If ammonium sulfate, Sepharose, and DEAE chromatography were used to purify Rubisco (17), kinase and isomerase activities were not detectable in the Rubisco fraction (data not shown). It is also worth noting that Rubisco purified with PEG-$MgCl_2$ could be freed from kinase and other proteins if pooled DEAE fractions were precipitated with ammonium sulfate and separated over sucrose density gradients. These results demonstrated that the use of the nonionic polymer PEG as the precipitating agent resulted in most of the kinase and part of isomerase activities in the Rubisco fraction. This copurification of kinase and Rubisco by PEG may reflect a specific association between these proteins or it may simply be a case of copurification. In this regard we have found that purified kinase was not precipitated by the PEG-$MgCl_2$ technique; however, this does not prove that kinase was not coprecipitated with Rubisco under the conditions existing in the crude extract.

Stoichiometry of Kinase in Crude Extracts

The amount of kinase in crude extracts of peas and spinach was determined by ELISA using antikinase antibody raised against purified kinase (Fig. 5, left). The antikinase antibody was found to be specific in that it only showed one crossreacting band in Western blots of crude extracts of leaves (Fig. 5, right). There was evidence for additional bands in the stromal extracts which we suspected to be kinase degradation products. This suggestion is supported by the fact that one would not expect the less purified crude extracts to show greater antisera specificity. In this regard it is important to note that it was only the crude extracts of spinach and peas that were used for the kinase ELISA. Lane 1 in Figure 5, right, shows the high degree of antisera specificity seen in spinach crude extracts. The purified kinase, which also yielded only a single band on Western blots, was used to generate standard curves such as are shown in Figure 6. The crude extracts showed 0.730 and 0.725 mg of kinase per mg Chl in spinach and peas respectively (Table II). If we assume that the total amount of carboxylase in green leaves is about 6 to 18 mg per mg Chl (5, 6) then there appears to be sufficient kinase to approach a 1:1 to 1:3 molar ratio with carboxylase. We are aware that

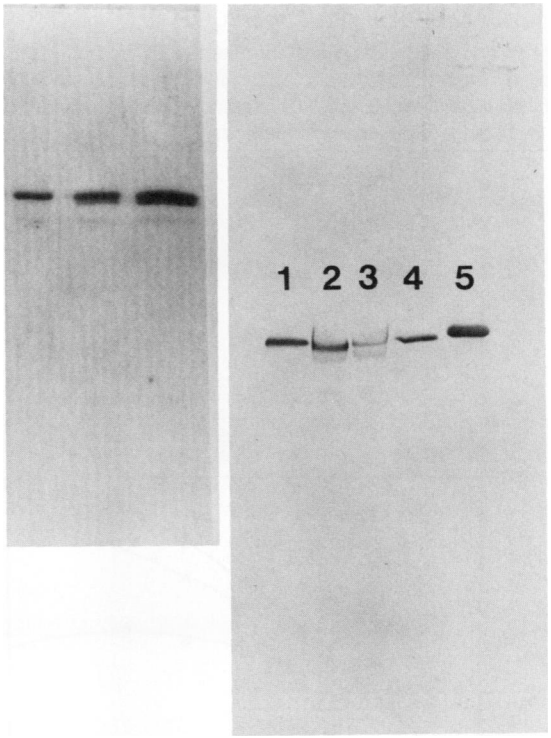

Figure 5. Left, Kinase purified from peas analyzed on 10% SDS-PAGE (5, 10, 15 μg protein). Right, Western blot using kinase antibody: 1, spinach crude extract; 2, spinach stromal extract; 3, pea stromal extract; 4, partially purified spinach kinase; 5, pure pea kinase.

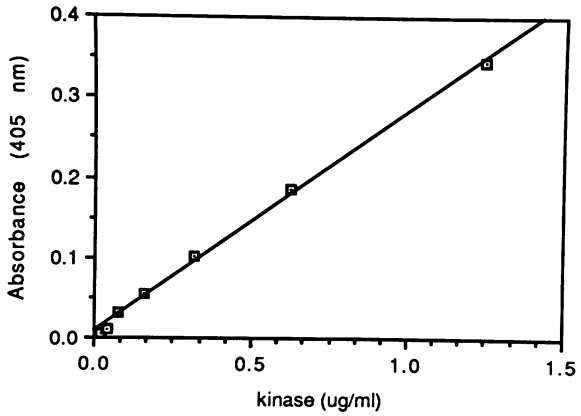

Figure 6. ELISA standard curve used for the determination of kinase concentrations in crude leaf extracts. Generated using purified pea kinase (see Fig. 5).

this molar ratio is at odds with the values calculated on an activity basis but it should be noted that determination of the amounts of enzymes based on activity can be difficult with enzymes such as kinase whose catalytic activity is subjected to regulation.

Catalytic Stabilization of Rubisco in the Linked Assay Involving Kinase and Isomerase

If an enzyme association has an *in vivo* significance it should exhibit kinetic properties that (a) are not seen in the separated

Table II. *The Amounts of Kinase on mg Chl Basis*

Spinach and pea leaves were extracted in Bicine buffer (25 mM) pH 8 containing 1 mM EDTA and 10 mM β-mercaptoethanol. Chl was determined by extraction with 80% acetone. The kinase was determined as given in "Materials and Methods."

	mg Kinase/mg Chl	*mg Chl·/g fr wt*
Peas		
1	0.66	1.15
2	0.79	1.27
Spinach		
1	0.702	1.21
2	0.702	1.14
3	0.773	1.42
4	0.742	1.05

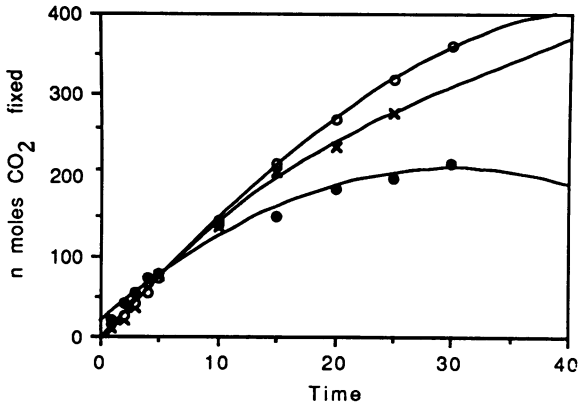

Figure 7. Time course for Rib-5-P, Ru-5-P and RuBP dependent carboxylase activity. Protein assay of 0.0125 mg used per assay mixture. Rib-5-P (○), Ru-5-P (×), RuBP (●). Concentrations: Rib-5-P (10 mM), Ru-5-P (10 mM), RuBP (2 mM). *Reaction initiated with activated enzyme.*

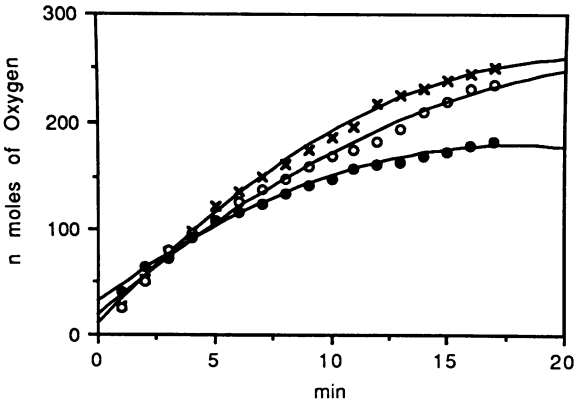

Figure 8. Time course for Rib-5P, Ru-5-P, and RuBP dependent oxygenase activity. Protein assay of 0.385 used per assay mixture. Rib-5-P (○), Ru-5-P (×), RuBP (●). Concentrations; Rib-5-P (10 mM), Ru-5-P (10 mM), reaction initiated with activated enzyme.

enzymes and (b) better reflect the properties of the living state. Previous studies on purified Rubisco have revealed certain inconsistencies in the properties of the pure enzyme when compared to its predicted *in vivo* behavior. One is the non-physiological concentrations of CO_2 and Mg^{2+} needed for full activation of the enzyme (20). The other is the rapid decay of

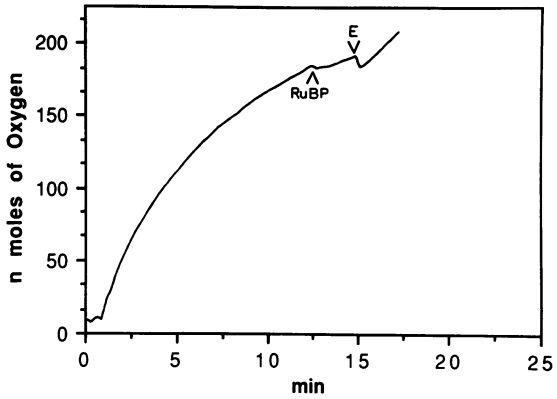

Figure 9. Time course for RuBP oxygenase. Protein assay of 0.385 mg used per assay mixture. Effect of addition of activated Rubisco (E) (0.385 mg) or RuBP (2.5 μmol) on the rate of reaction. Reaction initiated with activated enzyme. RuBP concentration (2 mM).

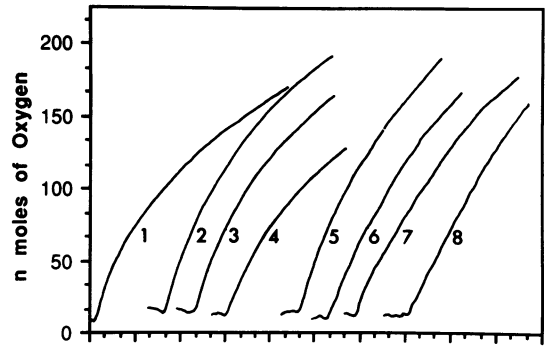

Figure 10. Comparison of RuBP oxygenase activities using several combinations of substrates. 1, RuBP; 2, RuBP+ATP; 3, RuBP+Rib-5-P; 4, RuBP+Ru-5-P; 5, Rib-5-P+ATP+RuBP; 6, Ru-5-P+ATP+RuBP; 7, Rib-5-P+ATP; 8, Ru-5-P+ATP. Concentrations: Rib-5-P (10 mM), Ru-5-P (10 mM), ATP (4 mM), RuBP (2 mM). Reaction initiated with activated enzyme.

enzyme activity during catalysis (17). This rapid decay is inconsistent with the sustained rates of carbon assimilation generally observed in photosynthesizing leaf tissues (10). Figure 7 shows the time course of Rib-5-P and Ru-5-P and RuBP dependent CO_2 fixation activities. It was observed that Rib-5-P and Ru-5-P dependent activities were less rapidly inactivated than RuBP dependent activity. It was of interest that, in spite of the fact that only 7% of the total isomerase activity was detected in the Rubisco preparation, this was sufficient to sustain a reaction rate almost equivalent to the Ru-5-P dependent activity. In all these cases, the initial velocity obtained with RuBP as substrate were more than that obtained with Rib-5-P or Ru-5-P as substrate (Fig. 7). However, these initial RuBP dependent carboxylase and oxygenase rates decreased rapidly with time whereas the Rib-5-P and Ru-5-P dependent activities were stabilized at either the same or at somewhat higher rates than that observed in the first minute of assay. The Rib-5-P and Ru-5-P dependent carboxylase activities showed 60 to 75% of their initial activity even after 20 to 25 min of active catalysis. This can be contrasted with RuBP dependent carboxylation which at the same time point

showed only 10 to 12% of its original activity (Fig. 7). During the course of this work we observed that the Rib-5-P dependent activity commenced without apparent lag. This was somewhat surprising, given that we were monitoring the consumption of substrate (CO_2 or O_2) by the third enzyme of a three-reaction sequence.

Previous work has shown that, in spite of the fact that RuBP is the substrate for Rubisco, its activity becomes nonlinear after only 90 s in the assay medium (17). We observed such typical RuBP dependent inactivation in our assays when either Sigma RuBP or laboratory made RuBP was used (data not shown).

The assay of RuBP oxygenase has certain intrinsic difficulties that stem from the fact that the continuous presence of CO_2 is required to sustain the activation state of the enzyme. This is problematical because CO_2 is a competitive inhibitor of oxygenase with respect to O_2 (20). Therefore, in the oxygenase assays conducted here, the concentration of bicarbonate was kept at a low level to minimize the competitive inhibition. These circumstances would, however, lead to enzyme inactivation by loss of activator CO_2. There was also the problem of the continual depletion of O_2 from the reaction mixture during the prolonged assay. When this O_2 depletion was combined with the high K_m of the oxygenase for O_2, significant departures from linearity were inevitable. However, even with these constraints, the differences in Rib-5-P and Ru-5-P *versus* RuBP dependent assays were quite obvious (Fig. 8). In RuBP dependent assays, the oxygenase reaction was mainly limited by active enzyme (Fig. 9) in that addition of RuBP did not affect the rate of significantly. However, the rate of O_2 consumption could be increased by the addition of active enzyme.

Using Rib-5-P and Ru-5-P dependent oxygenase assays, we tried to investigate whether Rib-5-P, ATP, or Ru-5-P individually offered any protection to RuBP dependent activity. The results, which are shown in Figure 10, demonstrated that Rib-5-P or Ru-5-P individually did not protect Rubisco from the inhibitory effect of RuBP. ATP offered some protection but typical RuBP effects were still evident. It was possible that the RuBP effects observed here were due to inhibitory contaminants in the RuBP preparations (21). We tested this by adding RuBP to Rib-5-P and Ru-5-P assay mixtures. The results in Figure 10 showed that RuBP did not inhibit Rib-5-P or Ru-5-P oxygenase activities. Since externally added RuBP failed to inhibit these reactions, it seemed unlikely that the typical inhibitory effects of RuBP were due to the presence of an inhibitory contaminant in RuBP. Paech *et al.* (21) had also observed that Rubisco inactivated during an RuBP dependent assay showed 80 to 90% of its original initial velocity during a subsequent Rib-5-P, ATP-dependent assay. However, the same enzyme showed a considerably lower percentage of its original activity when the inhibited enzyme was assayed with RuBP as substrate.

One possible interpretation of the apparent stabilization of Rubisco in Rib-5-P and Ru-5-P dependent assays is that the Rubisco may be utilizing freshly synthesized RuBP in preference to externally added RuBP. This preferential use could be explained in two ways. One is that in the coupled assays there was channeling of RuBP to Rubisco active sites. This would implicate the formation of a permanent or transient complex of these enzymes. This would help in maintaining high concentration of RuBP at the active sites even though the total intracellular concentration is low (17). The other advantage to such complex is that it would prevent Rubisco from interacting with free RuBP, which previous work has shown, can be deleterious. For example, binding of RuBP to the nonactivated enzyme was shown to cause inactivation of Rubisco (13) and, as discussed earlier, RuBP has also been shown to inactivate Rubisco during catalysis (17). The complex formation might also prevent RuBP from existing free in solution, a situation which may lead to the spontaneous formation of inhibitors (21). The other possibility is that the commercially available or laboratory made RuBP assumes a different chemical configuration after storage. Recently, Anderson (1) has shown that Ru-5-P produced from the Rib-5-P and isomerase is a better substrate for kinase than commercial Ru-5-P.

Lilley and Walker (16) while developing the spectrophotometric assay for carboxylase had observed that if reaction was initiated by Rib-5-P and ATP instead of RuBP the reaction rate was linear for a longer period of time. Bahr and Jensen (3) demonstrated stabilization of a low K_m form of RuBP carboxylase by Rib-5-P and ATP in the lysed chloroplasts of spinach. In the present and in the earlier work (24) we have demonstrated that the carboxylase as well as oxygenase activities of Rubisco do not decay as rapidly if assayed with sequential pairs of Calvin cycle enzymes. This apparent stabilization occurred even in partially purified preparations. The biochemical basis for the stabilization of Rubisco activity in a linked assay is unknown. The discrepancies in the behavior of purified Rubisco from its predicted *in vivo* properties may be, in part, due to its separation from other interacting proteins during purification.

CONCLUSIONS

This and our previous work has demonstrated that (a) kinase and carboxylase have a tendency to copurify unless treated with high salt; (b) PEG-$MgCl_2$ precipitation of Rubisco favors copurification of kinase; (c) there is sufficient kinase in the chloroplast stroma to approach 1:1/1:3 molar ratio of kinase:Rubisco; (d) the kinase linked carboxylase and oxygenase assays show enhanced stability during catalysis.

Enzyme associations like this, although difficult to detect *in vitro* because of the relatively weak forces of association, may provide the real key to understanding the regulation of metabolic processes such as photosynthetic carbon reduction cycle.

Note Added in Proof. After submission of this manuscript, Gontero *et al.* published a paper ([1988] Eur J Biochem 173: 437–443) which reported evidence for a 5-enzyme complex of Calvin cycle enzymes which included Rubisco and phosphoribulokinase.

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