Ribulose Diphosphate Carboxylase/Oxygenase

IV. REGULATION BY PHOSPHATE ESTERS*

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SUMMARY

The stimulation or inhibition of ribulose diphosphate oxygenase by a variety of compounds is compared with the reported effects on these compounds on the ribulose diphosphate carboxylase activity. A possible transition state analog of ribulose diphosphate, 2-carboxyribitol 1,5-diphosphate, at a molar ratio of inhibitor to enzyme of 10 to 1, irreversibly inactivates the oxygenase and carboxylase activities. This is consistent with the hypothesis that there may be a single active site for both the carboxylase and oxygenase activities. Several compounds of the reductive pentose photosynthetic carbon cycle act as effectors of the ribulose diphosphate oxygenase in a manner complementary to their reported effect upon the carboxylase. Ribose 5-phosphate inhibits the oxygenase with an apparent $K_i$ of 1.8 mM, but it is reported to activate the carboxylase; fructose 6-phosphate and glucose 6-phosphate act similarly but are less effective than ribose 5-phosphate. Fructose 1,6-diphosphate stimulates the oxygenase at low magnesium ion concentrations. The stimulatory effect of 6-phosphogluconate on the oxygenase is associated with a 3-fold reduction of the $K_m$ ($CO_2$). ATP inhibits the oxygenase but has been reported to stimulate the carboxylase; pyrophosphate acts in an opposite manner. From these results it appears that the ratio of carboxylase to oxygenase activity may be a variable factor with predictable subsequent alteration in the ratio between photosynthetic CO$_2$ fixation and photorespiration.

Protein Fraction I of leaves (1) has at least two catalytic activities. It is a RuP$_2$ carboxylase (3-P-glycerate carboxylase (dimerizing), EC 4.1.1.39), as well as RuP$_2$ oxygenase (2-4). In the oxygenase reaction, molecular O$_2$ instead of CO$_2$ is apparently added at carbon number 2 of RuP$_2$ followed by cleavage between carbon atoms 2 and 3 to yield 1 molecule of phosphoglycolate from carbon atoms 1 and 2 of RuP$_2$ and one 3-P-glycerate from carbon atoms 3, 4, and 5. The phosphoglycolate so produced in this way is excreted by the chloroplast and is subsequently oxidized in the peroxisomes (6). The dual activity of isolated protein Fraction I will be referred to as RuP$_2$ carboxylase/oxygenase. Both activities have been found in enzyme preparations from spinach (2-4), soybean (2, 4), Chlamydomonas (7), Chromatium (8), and Rhodospirillum rubrum (9). The RuP$_2$ oxygenase dependent production of glycolate may accompany photosynthesis in all algae and higher plants.

Regulation of the ratio of activity of RuP$_2$ carboxylase and RuP$_2$ oxygenase is equivalent to regulation of photosynthetic CO$_2$ fixation versus photorespiration (10). Regulation of RuP$_2$ carboxylase has been reviewed (11-14) and of particular concern has been the large but variable $K_m$ ($CO_2$), the effect of Mg$^{2+}$ concentration on activity, and the effect of phosphorylated intermediates of the photosynthetic carbon cycle on the carboxylase activity. With the discovery of RuP$_2$ oxygenase activity, all these factors must be considered for both activities. In this manuscript the effect of various compounds on RuP$_2$ oxygenase activity is reported and compared with their reported effects on RuP$_2$ carboxylase activity. It is concluded that both activities are subject to regulation by certain naturally occurring metabolites.

MATERIALS AND METHODS

Reagents—The sugar phosphates and other biochemicals were purchased from Sigma; the sodium salts were used as received. The trisodium salt of d-phosphogluconate and the tricyclohexylammonium salt of phosphoglycolate were obtained from General Biochemicals. The purity of phosphoglycolate has been confirmed (15). 2-Carboxyribitol 1,5-diphosphate was a gift from Dr. M. D. Lane, Johns Hopkins University.

Enzyme and Assays—RuP$_2$ carboxylase/oxygenase preparations from spinach leaves were prepared and assayed as described in the accompanying paper (16). Most results were confirmed by both assays for O$_2$ uptake, the manometric method and the oxygen electrode.

RESULTS

Inhibition by 2-Carboxyribitol 1,5-Diphosphate—This carboxylated derivative of RuP$_2$ is a potent inhibitor of RuP$_2$ oxygenase. Siegel and Lane (17, 18) showed that it inhibited RuP$_2$ carboxylase in a time-dependent irreversible fashion, that almost complete inhibition occurred at a 10:1 molar ratio of inhibitor to enzyme, and that RuP$_2$ protected the carboxylase from inhibition. They suggested that 2-carboxyribitol 1,5-diphosphate is an analogue of an intermediate or transition state in the carboxylation of RuP$_2$. We have observed that the inhibition of RuP$_2$ oxygenase by 2-carboxyribitol 1,5-diphosphate was similar to active phosphoglycolate phosphatase (5). We hypothesize that glycylate produced in this way is excreted by the chloroplast and is subsequently oxidized in the peroxisomes (6). The dual activity of isolated protein Fraction I will be referred to as RuP$_2$ carboxylase/oxygenase. Both activities have been found in enzyme preparations from spinach (2-4), soybean (2, 4), Chlamydomonas (7), Chromatium (8), and Rhodospirillum rubrum (9). The RuP$_2$ oxygenase dependent production of glycolate may accompany photosynthesis in all algae and higher plants.
its inhibition of the carboxylase. The oxygenase inhibition was time dependent (Fig. 1) and essentially irreversible. When 0.5 mg of enzyme was incubated in 1 ml of the reaction mixture which contained 125 μmol of Ammediol-Cl buffer at pH 9.3, 10 μmol of MgCl₂, 0.5 μmol of Na₂EDTA, 0.4 μmol of dithiothreitol, and 20 nmoles of 2-carboxyribitol 1,5-diphosphate, the oxygenase activity dropped to zero after 2 hours at 25°C. Dialysis for 24 hours against a 20-fold volume excess of the reaction mixture without the 2-carboxyribitol 1,5-diphosphate failed to restore activity.

Almost complete inhibition of the oxygenase occurred at a 10:1 molar ratio of inhibitor to enzyme and this corresponded to about one inhibitor molecule per RuP₂ binding site. In these analyses the oxygenase reaction rate in the presence of 2-carboxyribitol 1,5-diphosphate was plotted as a function of time at different inhibitor concentrations by Aldridge's method (19). The plot of log percent residual activity as a function of time was initially nonlinear but became linear at times longer than 5 min of incubation with the enzyme (Fig. 1). The projection of the linear portion of the curve onto the ordinate did not intersect the log 2.0. Aldridge (19) observed this phenomenon with the interaction of 2-carboxyribitol 1,5-diphosphate with RuP₂ carboxylase. He suggested that it was the result of an initially reversible combination of enzyme and inhibitor, followed by an irreversible step: \( E + I \rightarrow EI \rightarrow EI' \), where \( E \) = enzyme, and \( I \) = inhibitor. An analogous reaction pathway has been suggested by Siegel and Lane (18) for the interaction of 2-carboxyribitol 1,5-diphosphate with RubP carboxylase, and our results with the oxygenase are in agreement with that interpretation.

When RuP₂ was added simultaneously with as much as 1 mM 2-carboxyribitol 1,5-diphosphate, no change was noted in the initial rate of the oxygen uptake in comparison to the control, nor was the subsequent course of the reaction different. The RuP₂ thus served to protect the enzyme from inactivation by an excess of the inhibitor. When 0.5 mM ribose 5-phosphate or fructose 1,6-diphosphate was added to the reaction mixture containing the enzyme and 2-carboxyribitol 1,5-diphosphate, the course of inactivation of the oxygenase activity was unchanged. The protection of the enzyme can be accomplished only by RuP₂.

**Effect of 6-Phosphoglucononate on RuP₂ Oxygenase Activity—Recent reports (13, 20–23) indicate that 6-phosphoglucononate affects the activity of the enzyme for RuP₂ carboxylation. The two critical factors determining the nature of the response were the order of addition of the reactants, and the concentration of bicarbonate. 6-Phosphoglucononate was an activator of RuP₂ carboxylase when it was preincubated with the enzyme at suboptimal concentrations of Mg²⁺ and HCO₃⁻ (13, 21) but it acted as an inhibitor of the carboxylase when added to the enzyme mixture simultaneously with RuP₂, or when preincubated with the enzyme in the presence of high (10 mM) concentrations of bicarbonate (22, 23).

In our manometric assays for RuP₂ oxygenase, 6-phosphoglucononate was preincubated with the enzyme in 12 mM MgCl₂ for 18 min prior to the addition of RuP₂ and no HCO₃⁻ was added. Under these conditions the oxygenase activity was increased about 37% (Table I). In our system, 6-phosphoglucononate lowered the oxygenase \( K_m \) for Mg²⁺ without changing the \( V_{max} \) appreciably (Fig. 2). In the absence of 6-phosphoglucononate, \( K_m \) (Mg²⁺) was 5.9 × 10⁻⁵ M and in the presence of 2.5 mM 6-phosphoglucononate the \( K_m \) (Mg²⁺) was 2.4 × 10⁻⁴ M, or about 3-fold lower. On the other hand, 6-phosphoglucononate had no effect on the \( K_m \) (RuP₂) which remained approximately 4 × 10⁻⁴ M. These data were obtained manometrically with 1 atmosphere of oxygen; similar results have been observed in air with the oxygen electrode.

RuP₂ oxygenase exhibits maximum activity at Mg²⁺ concentrations between 10 and 35 mM (16). In the presence of 12 mM Mg²⁺, 1 to 3 mM 6-phosphoglucononate stimulated RuP₂ oxygenase activity in 100% oxygen about 37% (Table I) and similar results were observed in air. However, 6-phosphoglucononate concentrations greater than 10 mM inhibited RuP₂ oxygenase activity, perhaps due to chelation of the Mg²⁺. When the experiments were repeated with suboptimal Mg²⁺ concentrations (5 mM), the percentage stimulation of RuP₂ oxygenase activity by 6-phosphoglucononate was greater, but the maximum oxygenase activity was never greater than that observed with 10 mM Mg²⁺.

When 6-phosphoglucononate and RuP₂ were added simultaneously to the enzyme there was no stimulation, and in fact, 10% inhibition occurred at concentrations which would have provided maximum stimulation if 6-phosphoglucononate had been preincu-
been reported (10, 21) to inhibit the carboxylase activity under similar conditions. 6-phosphogluconate still stimulated the oxygenase, while it has presence of optimal, high concentrations of Mg2+ and substrates is somewhat similar to the stimulation of the carboxylase at sub-

Inhibition of Ribulose Diphosphate Oxygenase by Ribose 5-Phosphate—The activating effect of ribose 5-phosphate on RuP2 carboxylase has been reported (13). In contrast ribose 5-phosphate inhibited RuP2 oxygenase activity (Table II) and the apparent K, was about 1.8 mM. Ribose 5-phosphate (10 mM) inhibited the oxygenase 90%. The inhibition by ribose 5-phosphate occurred if the enzyme was preincubated with it, for example, for 18 min in the manometric assay; but if the substrate RuP2 was added simultaneously with the ribose 5-phosphate, no inhibition was observed. Ribose 5-phosphate was a less effective inhibitor of oxygenase activity in the preparations that had aged for 1 month as slurries in 50% ammonium sulfate (Table II).

The oxygenase activity as a function of RuP2 concentration in the presence and absence of 3 mM ribose 5-phosphate determined in air is depicted in Fig. 3. The apparent V max in the presence of ribose 5-phosphate is significantly lower than in its absence. There are insufficient data at low RuP2 concentrations to determine if the K m values for RuP2 are significantly different. Least squares analysis of a Lineweaver-Burk plot of the data suggests that the K m (RuP2) is not significantly different in the two cases, and the inhibition is thus of the noncompetitive type.  Effect of Hexose Phosphates on Ribulose Diphosphate Oxygenase—Fructose 1,6-diphosphate has been reported to inhibit RuP2 carboxylase (13, 21) and in contrast it stimulated RuP2 oxygenase somewhat at the relatively low concentration of 0.5 mM (Table III). At concentrations of 10 mM, fructose 1,6-diphosphate was an inhibitor of the oxygenase. Maximum stimulation at an intermediate concentration of 2 mM fructose 1,6-diphosphate was not over 30%.

<table>
<thead>
<tr>
<th>6-P gluconate</th>
<th>Rate as per cent of control</th>
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<tbody>
<tr>
<td></td>
<td>With 10 mM Mg2+</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>137</td>
</tr>
<tr>
<td>1.0</td>
<td>137</td>
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<tr>
<td>2.0</td>
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<td>10.0</td>
<td>61</td>
</tr>
<tr>
<td>12.5</td>
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*Table II*  Inhibition of ribulose diphosphate oxygenase by ribose 5-phosphate

In the manometric assay under 100% 02 atmosphere the Mg2+ concentration was 12 mM and the enzyme was preincubated with ribose 5-phosphate for 18 min before adding RuP2 from the side arm. The aged enzyme had been stored for 1 month at 4° as a slurry in 50% saturated ammonium sulfate. The enzyme was re-suspended and dialyzed against the reaction mixture for 6 hours to solubilize the enzyme before the experiment.

<table>
<thead>
<tr>
<th>RuP2-phosphate</th>
<th>Fresh enzyme</th>
<th>Aged enzyme</th>
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<tbody>
<tr>
<td></td>
<td>% Control mg protein⁻¹</td>
<td>% Control mg protein⁻¹</td>
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<tr>
<td>0</td>
<td>81</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>62</td>
<td>77</td>
</tr>
<tr>
<td>1.0</td>
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<td>5.0</td>
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<td>40</td>
</tr>
<tr>
<td>10.0</td>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Fructose 1,6-diphosphate</th>
<th>Glucose-6-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>120</td>
<td>89</td>
</tr>
<tr>
<td>1.0</td>
<td>129</td>
<td>74</td>
</tr>
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<td>5.0</td>
<td>73</td>
<td>63</td>
</tr>
<tr>
<td>10.0</td>
<td>73</td>
<td>57</td>
</tr>
</tbody>
</table>

Fructose 6-phosphate and glucose 6-phosphate were inhibitors of RuP2 oxygenase of about equal potency (Table III). The presence of 10 mM of either compound produced only 43% inhibition and neither was as effective an inhibitor as ribose 5-phosphate for which only 1 to 2 mM was needed for 50% inhibition. This behavior should be contrasted with the reported activation of the RuP2 carboxylase by these two compounds (13, 21).

*Product Inhibition of Ribulose Diphosphate Oxygenase—Neither product of the oxidation of RuP2, phosphoglycolate or 3-phosphoglycerate, was a very effective inhibitor of the oxygenase. Phosphoglycolate at 10 mM inhibited the enzyme as much as 40%, but concentrations of phosphoglycolate are extremely low in the plant because of a very active and highly specific phosphatase found in the chloroplast (15). Inhibition by 3-phosphoglycerate was hardly significant at concentrations up to 10 mM. Combinations of the two-phosphate esters were without synergetic inhibi-
tory effect. It is unlikely that the immediate products of the oxygenase reaction have any effect on this enzyme in vivo, for phosphoglycolate or glycolate do not accumulate in the chloroplast.*

*Effect of Dicarboxylic Acids, Amino Acids, and Fluoride on Ribulose Diphosphate Oxygenase—None of the dicarboxylic acids tested, malate, malonate, and L-glutamate had any effect on the RuP2 oxygenase activity at concentrations up to 10 mM (data not shown). These compounds cannot be considered to be regulators of the ratio of RuP2 carboxylase to oxygenase activity.*
Glycine and serine, products of phosphoglycolate metabolism after RuP₂ oxidation, did not inhibit RuP₂ oxygenase at concentrations up to 10 mM. Glycylglycylglycine at 10 mM was not inhibitory, but as reported previously (3), RuP₂ oxygenase in 25 mM glycylglycylglycine was only about 50% as active as in Ammediol-Cl buffer at the same pH. Glycylglycylglycine at 10 mM inhibited RuP₂ oxygenase about 50%. Little physiological significance is attached to this inhibition of the oxygenase by this tripeptide; in fact it may reflect the ability of glycylglycylglycine to chelate Mg²⁺. Avoidance of glycylglycine as a buffer for the oxygenase has already been recommended (3).

Potassium fluoride had no effect on the oxygenase activity at 1 mM, but at 10 mM it inhibited the enzyme 50%. If cupric ion is important to the functioning of the oxygenase, it is not accessible to the fluoride ion.

Effect of ATP and Pyrophosphate on Oxygenase Activity—ATP was a fairly good inhibitor of RuP₂ oxygenase. ATP (1.0 mM) reduced the activity of the enzyme to 33% of the control in the presence of 10 mM Mg²⁺ so that its chelation of Mg²⁺ could not entirely account for the inhibition. Addition of ribose 5-phosphate along with RuP₂ eliminated the inhibition by ATP. Neither AMP nor NADPH at 1 mM had any effect on the oxygenase activity.

Pyrophosphate at concentrations around 1 mM caused a small but definite stimulation of the oxygenase activity, the activity being approximately 115% of that of the control. At higher concentrations pyrophosphate inhibited the oxygenase and this inhibition could be reversed to some extent by the addition of excess Mg²⁺. The fact that the inhibition was not completely reversed by higher concentrations of Mg²⁺ suggests that the pyrophosphate may bind to a site on the enzyme. The effect of pyrophosphate on RuP₂ oxygenase activity seems complicated and may be attributed both to chelation of the Mg²⁺ and to a direct effect on the protein.

**DISCUSSION**

Since the RuP₂ carboxylase and RuP₂ oxygenase activities are functions of the same protein it is necessary to consider the regulation of both activities by various compounds which might be important in controlling photosynthesis. A tabulation of our data on the RuP₂ oxygenase activity and various data from the literature on RuP₂ carboxylase appears in Table IV.

The compounds listed in this table, with the exception of 2-carboxyribitol diphosphate, are metabolites associated with the pentose phosphate pathway, and they appear to modify the RuP₂ carboxylase and oxygenase activities when present at concentrations between 1 and 10 mM, in a solution containing 2 mg of protein ml⁻¹, approximately 4 µM enzyme. Some compounds, notably ribose 5-phosphate, fructose 6-phosphate, and ATP, have been reported to stimulate the RuP₂ carboxylase activity under certain conditions, and these compounds inhibited the oxygenase under the conditions of our assay. Conversely, fructose 1, 6-diphosphate stimulated the RuP₂ oxygenase and is reported to inhibit the carboxylase. Finally 6-phosphogluconic acid, which stimulated the RuP₂ carboxylase at suboptimal Mg²⁺ concentrations by lowering the Kₐ of the enzyme for this cofactor, brings about stimulation of RuP₂ oxygenase activity at suboptimal Mg²⁺ concentrations by the same means.

The complementarity in effect of certain of the metabolites, and the similarity in effect of 6-phosphogluconic acid, are added circumstantial evidence that the RuP₂ carboxylase and oxygenase activities are functions of the same protein. Further, these results imply that the ratio of RuP₂ carboxylase activity to RuP₂ oxygenase activity is not a fixed value but can vary within certain limits. The regulation by reductive pentose phosphate pathway intermediates is superimposed upon regulation of carbon dioxide fixation by light, Mg²⁺ concentration, pH, and availability of the substrates RuP₂ and CO₂ or O₂. There is also evidence for the idea first suggested by Pon et al. (25) and substantiated by Chu and Bassham (23), that bicarbonate may serve as an effector of RuP₂ carboxylase activity as well as a substrate. It is not unexpected that the RuP₂ carboxylase and oxygenase activities are subject to such control since the reactions catalyzed proceed with a large change in free energy. The large molecular weight and complex quaternary structure of the enzyme are indicative of a system that may be responsive to a number of controlling factors.

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2-Carboxyribitol 1,5-diphosphate inhibits the RuP₂ carboxylase and RuP₂ oxygenase at very low concentrations. In fact, effective concentrations of this compound are 1000-fold less than the concentration of the other compounds shown in Table IV. The molar ratio of inhibitor to enzyme for complete inhibition of either activity is 8 or 10 to 1. The data indicate that there are approximately the same number of catalytically active binding sites for RuP₂ for the carboxylase and for the oxygenase activities. RuDP inhibited the enzyme from inactivation, while other sugar phosphates were ineffective, and this is evidence that the site of interaction of the inhibitor with the enzyme is the RuP₂ binding site.

Insignificant effector or inhibitory activity has been found with compounds of metabolic pathways other than the pentose phosphate pathways: thus malate, malonate, and glutamate were without effect. Nor was there evidence for product inhibition by 3-P-glycerate and phosphoglycolate, alone or together, at concentrations comparable to those of the sugar phosphates.

Several points concerning the treatment of the isolated RuP₂ carboxylase and oxygenase deserve comment. The enzyme dis-
plays an age dependence in its response to effectors and in its physical properties. When the enzyme was stored as a slurry in 50% saturated ammonium sulfate for several weeks at 4°, the apparent activity of the carboxylase and oxygenase declined, although not necessarily in parallel (3). The concentration of high molecular weight polymers increases and the precipitate becomes increasingly more difficult to dissolve. The activity remaining in these solutions is increasingly less responsive to effectors, that is, higher concentrations of effectors are necessary to bring about a given amount of stimulation or inhibition.

A related phenomenon is that the redissolved enzyme requires several hours at room temperature or a brief treatment at 50° in the presence of Mg2+ and sulfhydryl reagent to display optimum activity and response to effectors. However, even though the specific activity of either RuP2 carboxylase or oxygenase in a purified preparation does not change appreciably after 2 days at room temperature, responsiveness to effectors does decline. These results are not understood but indicate that there are a large number of conformations of varying activity that the enzyme may assume. Perhaps these phenomena are related to control of the enzyme activity in vivo by environmental factors such as light and temperature.

Attention must be paid to the order of addition of substrates and effectors; failure to do so will result in confusion, as has already occurred in the literature on the effect of B-phosphogluconate on RuP2 carboxylase activity (13, 21-23). The protein responds appreciably slowly to changes in the environment and thus preincubation with effectors is necessary. Chu and Bassham (23) have explored the effects of sequence of addition of substrates and 6-phosphogluconate on the carboxylase. When the oxygenase, the effector and Mg2+ were preincubated together an increase in enzyme activity was noted, but when RuP2 and 6-phosphogluconate were added simultaneously, no effect or a slight decrease in rate were seen. Similarly, ribose 5-phosphate displayed no inhibitory effect on the RuP2 oxygenase unless it was preincubated with the enzyme, and fructose 1,6-diphosphate required a period of incubation with the enzyme to bring about stimulation of the rate of oxygen uptake. The requirement for preincubation is characteristic of a hysteretic enzyme, according to Frieden (26).

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