Oxygen Inhibition and Other Properties of Soybean Ribulose 1,5-Diphosphate Carboxylase*

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SUMMARY

d-Ribulose-1,5-di-P carboxylase, purified from soybeans, had $K_m$ values of 0.13 mM for CO$_2$ and 0.19 mM for ribulose-1,5-di-P under a N$_2$ atmosphere. O$_2$ inhibited $^1$CO$_2$ incorporation by the enzyme and this inhibition was rapidly reversed by N$_2$. Inhibition was competitive with respect to CO$_2$ and uncompetitive with respect to ribulose-1,5-di-P. The $K_i$ for O$_2$ was 0.8 mM. This O$_2$ inhibition, together with the ribulose-1,5-di-P carboxylase-catalyzed oxidation of ribulose-1,5-di-P to P-glycolate observed previously (Bowes, G., Ogren, W. L., and Hageman, R. H. (1971) Biochem. Biophys. Res. Commun. 45, 716-722), explains the "Warburg effect": the rapidly reversible O$_2$ inhibition of photosynthesis and stimulation of glycolate production seen in plants and isolated chloroplasts. In corn and soybean extracts, ribulose-1,5-di-P carboxylase was inhibited by O$_2$ but P-enolpyruvate carboxylase was unaffected. These data may explain the different response to O$_2$ by plants which utilize ribulose-1,5-di-P carboxylase for the initial photosynthetic carboxylation and those utilizing P-enolpyruvate carboxylase. The optimum temperature for purified ribulose-1,5-di-P carboxylase was 55°C and activation energies, in kilocalories per mole, were 18.4 in N$_2$ and 20.4 in O$_2$.

Phosphorylated compounds inhibited $^1$CO$_2$ incorporation by the enzyme. Nonphosphorylated sugars, including ribulose, did not inhibit. Fructose-1,6-di-P was a competitive inhibitor with respect to ribulose-1,5-di-P, the $K_i$ being 0.88 mM. Fructose-1,6-di-P was a more effective inhibitor than fructose-6-P, fructose-1-P, and ribulose-5-P suggesting that both phosphate groups of ribulose-1,5-di-P are involved in binding to the enzyme. HgCl$_2$ was a noncompetitive inhibitor with respect to CO$_2$, and a mixed inhibitor with respect to ribulose-1,5-di-P, suggesting that sulfhydril groups are not involved in CO$_2$ binding but may be close to the site where ribulose-1,5-di-P binds to the enzyme.

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The abbreviations used are: p-CMB, p-chloromercuribenzoic acid; IAA, iodoacetamide.
appear to have a closer relationship with the substrate ribulose-1,5-diphosphate than with CO₂ (15, 17), although whether they are an integral part of the catalytic site is still unresolved. Part of the work reported here attempts to clarify some problems associated with the role of sulphydryl groups and the mechanism of substrate binding to the enzyme.

A preliminary report of part of this work has appeared (18).

**EXPERIMENTAL PROCEDURE**

**Purification**—Ribulose-1,5-diphosphate carboxylase was purified from leaves of field- or greenhouse-grown soybeans (*Glycine max* (L.) Merril var. Wayne) by a modification of a method described for spinach (10). Extraction difficulties with soybean necessitated two homogenization steps, with the filtrates being combined. Overnight storage at the ammonium sulfate 1 M stage reduced activity, therefore purification was continued through to where the enzyme could be stored at −20°C.

The isolated enzyme was stored at 5°C as a precipitate in 55% saturated ammonium sulfate, pH 6.5, with 0.1 mM EDTA and 5.0 mM 2-mercaptoethanol. Prior to assay, an aliquot of the suspension was centrifuged at 10,000 × g for 10 min, the supernatant discarded, and the precipitated enzyme dissolved in 120 mM Tris buffer, pH 8.0, containing 0.25 mM EDTA, and 10.0 mM MgCl₂. After dissolving, enzyme activity gradually increased by up to 60%. Thus to achieve maximum activity the enzyme solution was kept at 2°C for 4 hours before use.

Stored enzyme lost activity over 2 months, so control values in different experiments varied. Although absolute values between experiments could not be compared, repetition of experiments with different preparations and enzyme ages showed that findings were comparable when the control values were taken into account. The specific activity was lower than that reported for spinach (10), being in the range of 10 to 100 nmoles of CO₂ fixed per min per mg of protein. This can be partially attributed to inactivation during isolation and to the use of lower assay concentrations of ribulose-1,5-diphosphate and NaH¹⁴CO₃.

Soluble protein in extracts was determined by the procedure of Lowry et al. (19) and in purified enzyme solutions by the method of Warburg and Christian (20).

**Assay Procedures**—Ribulose-1,5-diphosphate carboxylase activity was assayed by ¹⁴CO₂ incorporation into acid stable products. At 5.0 mM MgCl₂ the pH optimum was 8.0. The reaction vessels contained 50.0 mM Tris at pH 8.0, 5.0 mM MgCl₂, 0.1 mM ribulose-1,5-diphosphate, and 0.05 mM EDTA in a final volume of 1.0 ml. The vessels were flushed with N₂, CO₂-free air or O₂ and shaken for 15 min, then were sealed and 20.0 mM NaH¹⁴CO₃ (1.0 μCi) injected via a serum cap. CO₂-free air was used to avoid isotope dilution by atmospheric CO₂. The reaction was initiated by injection of the enzyme, or alternatively ribulose-1,5-diphosphate, and stopped with 0.1 ml of 6.0 M acetic acid after 4 min at 25°C. Aliquots were then taken and dried at 90°C, dissolved in a modified Bray’s solution (21) and the disintegrations per min determined with a scintillation counter.

In experiments with crude extracts of soybean and corn (*Zea mays* L. var. WF9 × M14), approximately 500 mg (fresh weight) of leaf discs were homogenized under N₂ at 2°C in a Ten Broeck homogenizer with 5.0 ml of 50.0 mM Tris at pH 8.0 containing 10.0 mM MgCl₂, 0.1 mM EDTA, and 5.0 mM di-isocitrate. The extracts were centrifuged at 35,000 × g for 15 min in capped tubes under N₂, and then assayed immediately for P-enolpyruvate carboxylase activity (22). The P-enolpyruvate carboxylase assay contained, in a final volume of 1.0 ml, 50.0 mM Tris at pH 8.0, 10.0 mM MgCl₂, 0.1 mM EDTA, 5.0 mM sodium glutamate, 5.0 mM NaH¹⁴CO₃ (2.0 μCi), and 2.0 mM P-enolpyruvate. The crude ribulose-1,5-diphosphate carboxylase assay mixture was similar except sodium glutamate was omitted and 0.2 mM ribulose-1,5-diphosphate substituted for P-enolpyruvate. Both the P-enolpyruvate and ribulose-1,5-diphosphate assay mixtures were initiated with 0.1 ml of the extract and stopped with 0.1 ml of 6.0 M acetic acid after 3 min at 25°C under atmospheres of N₂ or O₂.

In crude and purified enzyme assays, reaction rates were linear over the assay period employed. No prior incubation of ribulose-1,5-diphosphate carboxylase with Mg²⁺ and HCO₃⁻ was required for linear rates.

**RESULTS**

**Effect of Ribulose-1,5-Di-P on Carboxylase Activity in N₂, CO₂-Free Air, and O₂**—As reported for spinach carboxylase (2), increasing concentrations of ribulose-1,5-diphosphate up to 0.5 mM increased the reaction velocity, while higher concentrations caused inhibition (Fig. 1). O₂ inhibited ¹⁴CO₂ incorporation at all ribulose-1,5-diphosphate concentrations, 100% O₂ being more effective than 21% O₂ (CO₂-free air).

**Kinetic Studies of O₂ Inhibition**—Further investigation showed the O₂ inhibition was fully and rapidly reversible (Table I). Prior to initiation of the reaction with ribulose-1,5 di-P, flushing the reaction mixture and enzyme with N₂ for 6 min followed by O₂ for 6 min, or vice versa, produced inhibition only if the last gas treatment was O₂. Thus the inhibitory effect of O₂ was not due to permanent inactivation of the enzyme.

A double reciprocal plot of the inhibitory effect of O₂ showed that inhibition was competitive with respect to CO₂ (Fig. 2). The Kₘ value was 0.13 mM in N₂, 0.19 mM in 21% O₂ (CO₂-free air), and 0.35 mM in 100% O₂. The Kₘ in CO₂-free air is within the range of values reported for spinach carboxylase (1), when all values are calculated on the basis of CO₂ rather than HCO₃⁻ concentration. The Kₘ for O₂ was 0.8 mM, which is slightly lower than previously reported (4).
Reversible effect of O₂ on rate of ¹⁴CO₂ incorporation by soybean ribulose-1,5-di-P carboxylase

Prior to addition of 5.0 mM NaH¹⁴CO₃ and initiation of the reaction with 0.1 mM ribulose-1,5-di-P, reaction mixtures containing the enzyme were shaken at 25° and flushed with either N₂ or O₂ for 12 min. Alternatively, reaction mixtures were flushed with N₂ for 9 min followed by O₂ for 6 min or vice versa. The flasks were then sealed and enzyme activity assayed under the atmosphere of the last gas treatment.

| Treatment                     | ¹⁴CO₂ incorporation (nmol CO₂/min/mg protein) |
|-------------------------------|---------------------------------------------|
| N₂ 12 min/assay 3 min in N₂   | 43.5                                        |
| O₂ 12 min/assay 3 min in O₂   | 30.0                                        |
| N₂ 6 min/O₂ 6 min/assay 3 min in O₂ | 32.0                                    |
| O₂ 6 min/N₂ 6 min/assay 3 min in N₂ | 46.7                                      |

O₂ inhibition suggests that O₂ binds to the site that CO₂ occupies on the enzyme.

With respect to ribulose-1,5-di-P, O₂ produced mixed inhibition of the uncompetitive type (Fig. 3). The Kₘ for ribulose-1,5-di-P was 0.19 mM in N₂, which compares with reported spinach carboxylase values in air ranging from 0.12 to 0.7 mM (1). One enzyme preparation, stored in an intermediate stage of purification, exhibited variation from these Kₘ values, although in other respects its properties were unaltered.

The reduced rate of ¹⁴CO₂ incorporation observed in CO₂-free air could be largely overcome by GSH. The rate of ¹⁴CO₂ incorporation at 20 mM HCO₃⁻, measured as nanomoles per min per mg of protein, was reduced from 25.5 in N₂ to 21.9 in CO₂-free air, and the addition of 3.0 mM GSH increased the rate to 24.3. Measurements with an O₂ electrode showed that GSH reduced the O₂ concentration in a reaction mixture under CO₂-free air. Thus the protection afforded by GSH can be attributed to a decrease in the O₂ concentration of the reaction mixture, through the antioxidation of GSH, and not to an effect on enzyme sulfhydryl groups.

Effect of O₂ on Carboxylases in Corn and Soybean Leaf Extracts—¹⁴CO₂ incorporation by P-enolpyruvate carboxylase in extracts of both corn and soybean was unaffected by O₂ (Table II). In contrast, ribulose-1,5-di-P carboxylase from both plants was inhibited by 100% O₂. On a protein basis, corn showed 29% and soybean 31% inhibition of ribulose-1,5-di-P carboxylase activity at 5.0 mM NaH¹⁴CO₃ concentration. Repeating the experiment with 20.0 mM NaH¹⁴CO₃ produced similar results, except the inhibition of ribulose-1,5-di-P carboxylase was less at the higher CO₂ level.

Temperature Response of Ribulose-1,5-di-P Carboxylase Activity in N₂ and O₂—Over a 3-min assay period purified ribulose-1,5-di-P carboxylase showed maximum activity in both N₂ and O₂ at 55° (Fig. 4). Activity was greatly reduced above 60°, due to rapid inactivation of the enzyme. The percentage inhibition by O₂ when calculated on a millimolar O₂ basis, to allow for lower O₂ solubility at higher temperatures, varied only 6% over the temperature range 15–60° (Fig. 4). Between 15 and 35°, energy of activation values calculated in kilocalories per mole from the Arrhenius equation were 18.4 in N₂, 18.5 in CO₂-free air, and

![Fig. 2. Double reciprocal plot of the rate of ¹⁴CO₂ incorporation by soybean ribulose-1,5-di-P carboxylase as a function of CO₂ concentration in N₂, CO₂-free air, and O₂. The general assay conditions were as described under "Experimental Procedure" except 0.2 mM ribulose-1,5-di-P and 1.0 to 50.0 mM NaH¹⁴CO₃ were added.](image1)

![Fig. 3. Double reciprocal plot of the rate of ¹⁴CO₂ incorporation by soybean ribulose-1,5-di-P carboxylase as a function of ribulose-1,5-di-P concentration, under atmospheres of N₂, 0₂, and under N₂ with 1.0 mM fructose-1,6-di-P present. The general assay conditions were as described under "Experimental Procedure" except the addition of 0.05 to 0.60 mM ribulose-1,5-di-P initiated the reaction.](image2)

**Table II**

| Plant extract | Gas phase | P-Enolpyruvate carboxylase activity | Ribulose-1,5-di-P carboxylase activity (nmol CO₂/min/mg protein) |
|---------------|-----------|-----------------------------------|---------------------------------------------------------------|
| Corn          | N₂        | 2670                              | 190                                                          |
|               | O₂        | 2600                              | 130                                                          |
| Soybean       | N₂        | 190                               | 870                                                          |
|               | O₂        | 160                               | 600                                                          |
**Fig. 4.** Activity and O2 inhibition of soybean ribulose-1,5-di-P carboxylase as a function of temperature. The general assay conditions were as described under "Experimental Procedure." The reaction flask was incubated at temperatures ranging from 15 to 65°C while being shaken and flushed with N2 or O2 prior to sealing and initiation of the reaction with enzyme. To compensate for reduced O2 solubility at higher temperatures, the percentage inhibition of activity in O2, as compared to N2, was plotted on the basis of the calculated O2 concentration in the reaction mixture.

20.4 in O2. The figure for CO2-free air is comparable to 16.9 kcal per mole calculated for spinach carboxylase (2).

**Inhibitor Studies.**—The product of the carboxylase reaction, 3-P-glycerate, is an inhibitor of spinach carboxylase (10). The results for soybean carboxylase, in Table III, confirm the inhibitory nature of 3-P-glycerate. 2-P-Glycolate was even more inhibitory. All phosphate compounds tested produced inhibition, but fructose-1,6-di-P was the most effective. A double reciprocal plot of inhibition by 1.0 mM fructose-1,6-di-P with respect to ribulose-1,5-di-P showed competitive inhibition (Fig. 3), with a KI of 0.88 mM in N2. This supports the proposal that ribulose-1,5-di-P binds to the enzyme through one or both phosphate groups (10). An fructose-1,5-di-P inhibited to a greater extent than the monophosphates fructose-1-P, fructose-6-P, and ribulose-5-P (Table III) it is likely that both phosphate groups are involved in the binding. Sucrose, glucose, fructose, ribulose, glycolate, and glyoxylate had no appreciable inhibitory effect, thus it would seem that the phosphate groups of ribulose-1,5-di-P are the principal groups involved in its binding to the enzyme. None of the sugar phosphates tested for inhibition were able to substitute for ribulose-1,5-di-P as a 14CO2 acceptor.

NAD+, NADH, NADP+, NADPH, ADP, and ATP all caused inhibition of the reaction, probably due to the phosphate groups (Table III). The products of the photosynthetic light reactions, ATP and NADPH, were the most effective nucleotide inhibitors. Acetate, formate, and CS2, substances resembling CO2, had no inhibitory effect. The photosynthesis inhibitor a-hydroxy-2-pyridinemethanesulfonate inhibited soybean carboxylase by 50% at 10.0 mM.

Spinach ribulose-1,5-di-P carboxylase is inhibited by various compounds which are known to interact with sulfhydryl groups (2, 14, 15). Soybean ribulose-1,5-di-P carboxylase was effectively inhibited by HgCl2 (Fig. 5). Incubation with HgCl2 prior to assay increased inhibition. Incubation for 5 min with 0.01 mM HgCl2 reduced the rate of 14CO2 incorporation from 22.6 (without HgCl2) to 10.9 nmoles of CO2 per min per mg of protein, while 15-min incubation further reduced the rate to 8.3. The presence of 5 mM GSH or 6 mM diethiothreitol during the 5-min incubation period overcame inhibition, the rates being 21.1 and 21.7, respectively. This suggests that the effect of the Hg+2 ions is on sulfhydryl groups of the enzyme.

With respect to CO2, HgCl2 was a noncompetitive inhibitor of
FIG. 6. Double reciprocal plot of the rate of $^{14}$CO$_2$ incorporation by soybean ribulose-1,5-di-P carboxylase as a function of CO$_2$ concentration, in the presence and absence of 0.01 mm HgCl$_2$. The general assay conditions were as described under “Experimental Procedure.” The reaction mixture, with or without HgCl$_2$, was flushed with N$_2$ and shaken at 25°C. The enzyme and 1.0 to 5.0 mm NaHCO$_3$ were added and incubated for 10 min. The reaction was initiated with 0.2 mm ribulose-1,5-di-P.

FIG. 7. Double reciprocal plot of the rate of $^{14}$CO$_2$ incorporation by soybean ribulose-1,5-di-P carboxylase as a function of ribulose-1,5-di-P concentration, in the presence and absence of 0.01 mm HgCl$_2$. Assay conditions were as described under “Experimental Procedure” and the legend to Fig. 6. The addition of 0.01 to 0.50 mm ribulose-1,5-di-P initiated the reaction.

soybean carboxylase (Fig. 6), indicating that sulfhydryl groups are not at the site of CO$_2$ binding. With respect to ribulose-1,5-di-P, HgCl$_2$ showed mixed inhibition (Fig. 7) suggesting that Hg$^{2+}$ acts on sulfhydryl groups adjacent to the site of ribulose-1,5-di-P binding.

Discussion

Oxygen Effects—The Warburg effect is manifested by O$_2$ inhibition of photosynthesis in C$_3$ species and a concomitant O$_2$ stimulation of glycolate production. In leaves of higher plants, the glycolate produced is subsequently metabolized in part to CO$_2$ by the photorespiratory pathway (23). These O$_2$ effects are rapidly reversed by removing O$_2$ or by increasing the CO$_2$ level (5, 6, 24). Soybean ribulose-1,5-di-P carboxylase is reversibly and competitively inhibited by O$_2$ with respect to CO$_2$ (Table I, Fig. 2) and this enzyme catalyzes an oxygen-dependent oxidation of ribulose-1,5-di-P to 2-P-glycolate (25), a glycolate precursor (23). Thus the effects of O$_2$ on purified ribulose-1,5-di-P carboxylase mimic the effects of O$_2$ in the Warburg effect, strongly suggesting that ribulose-1,5-di-P carboxylase is the specific site of action of O$_2$ in the Warburg effect.

It has been suggested (5) that O$_2$ may reversibly inactive sulfhydryl groups in enzymes of the Calvin cycle, with reactivation being induced by natural reductants. In addition to ribulose-1,5-di-P carboxylase, two other enzymes of the Calvin cycle are sensitive to O$_2$: NADP$^+$-glyceraldehyde-3-P dehydrogenase (5, 26), and ribulose-5-P kinase (27). The O$_2$ inactivation of these two enzymes is probably due to sulfhydryl oxidation. There is no evidence that the oxidation of these enzymes is reversible, and sulfhydryl oxidation cannot explain the competitive nature of O$_2$ and CO$_2$ in the Warburg effect. In contrast, O$_2$ inhibition of ribulose-1,5-di-P carboxylase is not an oxidation of sulfhydryl groups. This can be deduced from the observations that Hg$^{2+}$, a sulfhydryl inhibitor, is a noncompetitive inhibitor of carboxylase with respect to CO$_2$, whereas O$_2$ is a competitive inhibitor with respect to CO$_2$. Unlike NADP$^+$-glyceraldehyde-3-P dehydrogenase and ribulose-5-P kinase, extractable ribulose-1,5-di-P carboxylase activity is not in excess of the photosynthetic rate in the plant. Any contribution to the Warburg effect by the dehydrogenase and kinase appears to be small, especially if ribulose-1,5-di-P carboxylase limits photosynthetic rate (3, 4).

In contrast to the marked inhibition of C$_3$ photosynthesis by O$_2$ in air, C$_4$ photosynthesis is affected only by O$_2$ concentrations considerably higher than atmospheric (22, 28, 29). In species with C$_4$ photosynthesis, including corn, the initial photosynthetic carboxylation is catalyzed by P-enolpyruvate carboxylase in the mesophyll. The oxalacetate produced is reduced to malate, transported to the bundle sheath, and decarboxylated. The CO$_2$ released is refixed by ribulose-1,5-di-P carboxylase and enters the C$_4$ cycle (8, 9). P-Enolpyruvate carboxylase may function as a CO$_2$ pump, increasing the CO$_2$ concentration in the bundle sheath to a level higher than atmospheric (30).

In corn extracts, P-enolpyruvate carboxylase is unaffected by oxygen while ribulose-1,5-di-P carboxylase is inhibited (Table II). Thus in C$_4$ photosynthesis, the initial carboxylation is insensitive to O$_2$, while the second carboxylation, transfer of CO$_2$ into the Calvin cycle, is inhibited by O$_2$. At atmospheric O$_2$ levels, cycling of C$_4$ photosynthesis may proceed unhindered since the initial carboxylation is not inhibited and the increased CO$_2$ level in the bundle sheath will allow CO$_2$ to compete more successfully with O$_2$ for ribulose-1,5-di-P carboxylase. At O$_2$ levels greater than atmospheric, inhibition of ribulose-1,5-di-P carboxylase would restrict the carbon flow from C$_4$ acids to the sugar phosphates of the Calvin cycle. Since P-enolpyruvate carboxylase is not affected by O$_2$, C$_4$ acids may accumulate at high O$_2$ in concentrations sufficient to inhibit P-enolpyruvate carboxylase (31), and thus inhibit photosynthesis.

Plants with C$_4$ photosynthesis also lack photoreceptor CO$_2$ evolution (3, 7). This property may also be related to the P-enolpyruvate carboxylase CO$_2$ pump. High CO$_2$ levels in the bundle sheath would inhibit the ribulose-1,5-di-P carboxylase-catalyzed oxidation of ribulose-1,5-di-P to 2-P-glycolate (25), thereby decreasing photosynthesis. Since the mesophyll layers surround the bundle sheath, P-enolpyruvate carboxylase in the mesophyll would rapidly refix any photoreceptor CO$_2$ and prevent leakage to the atmosphere (9).

In Mimulus, energy of activation values for photosynthesis of
16 to 19 kcal per mole compare closely with values for ribulose-1,5-di-P carboxylase from this plant (32). These values are similar to those obtained with purified soybean carboxylase. The agreement between activation energies for photosynthesis and the carboxylation reaction would be expected if this reaction limits the rate of light-saturated photosynthesis in C₃ species.

Inhibitor Studies—Information concerning ribulose-1,5-di-P binding to the enzyme is contradictory. Orthophosphate and 3-P-glycerate inhibit spinach carboxylase (10). In contrast, carbamyl-P and various sugar phosphates, including fructose-1,6-di-P, reportedly did not inhibit (33). In this study, fructose-1,6-di-P was an effective competitive inhibitor of soybean carboxylase with respect to ribulose-1,5-di-P (Fig. 3), and all phosphorylated compounds tested produced inhibition (Table III). The greater inhibition caused by fructose-1,6-di-P, as compared to the monophosphates, and the lack of inhibition with nonphosphorylated sugars suggests that the two phosphate groups of ribulose-1,5-di-P are the principal groups involved in binding to the enzyme.

Since the discovery that p-CMB and IAA were inhibitors of carboxylase (14), the role of sulfhydryl groups has received much attention. Reportedly IAA inhibition could be reversed if ribulose-1,5-di-P was incubated with the enzyme and inhibitor. As neither Mg²⁺ nor HCO₃⁻ were effective in this respect, it appeared that sulfhydryls were not involved in the binding of Mg²⁺ or HCO₃⁻ but may have interacted with ribulose-1,5-di-P (17). However, fructose-1,6-di-P also protected sulfhydryls against IAA alkylation but fructose-1,6-di-P was not found to be a competitive inhibitor of carboxylase with respect to ribulose-1,5-di-P in these experiments (33). This was an objection (33) to the hypothesis that the protective mechanism involved the ribulose-1,5-di-P binding site and that sulfhydryls were located there. This objection would seem to be negated by the finding that fructose-1,6-di-P is a competitive inhibitor of soybean carboxylase (Fig. 3).

p-CMB, at low concentrations, produces uncompetitive inhibition of spinach carboxylase with respect to ribulose-1,5-di-P, while higher p-CMB concentrations give mixed inhibition (16, 34). The inhibition may be due to mercurial interaction with the enzyme-ribulose-1,5-di-P complex rather than solely with the enzyme (16), which correlates with results for soybean carboxylase showing mixed inhibition by HgCl₂ with respect to ribulose-1,5-di-P and noncompetitive inhibition with respect to CO₂. These results confirm a closer relationship of sulfhydryl groups to the ribulose-1,5-di-P than to the CO₂-binding site. Although many of the sulfhydryl groups may fulfill structural requirements (16, 33, 34), possibly a small number are involved specifically at the catalytic site (35). As HgCl₂ inhibition is mixed and not competitive, this suggests that sulfhydryls are not directly involved with the binding of ribulose-1,5-di-P but may participate at the catalytic site (16) close to where ribulose-1,5-di-P binds to the enzyme.

Calvin (30) has suggested that the carboxylation of ribulose-1,5-di-P proceeds via an unstable C₄ intermediate. Support for this suggestion comes from binding studies with analogues having structures similar to that proposed for the intermediate (37, 38). The C₄ intermediate may be produced by the enediol of ribulose-1,5-di-P acting as a nucleophilic agent on CO₂ (39). A sulfhydryl group or groups could play a catalytic role, not by binding ribulose-1,5-di-P, but rather by extracting a proton from the C₅ hydroxyl of ribulose-1,5-di-P and thus acting as the initiator of the carboxylase reaction.

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