SPECIFIC INHIBITION OF THE OXYGENASE ACTIVITY OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE

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Summary Ribulose-1,5-bisphosphate oxygenase activity of ribulose-1,5-bisphosphate carboxylase was inhibited 82% by incubation with 5 mM glycidate (2,3-epoxyproprionate). Under these conditions the carboxylase was not affected. Similar results have also been obtained with the S-alkylating agent, iodoacetamide. These observations suggest the involvement of sulfhydryl-groups in the ribulose-1,5-bisphosphate oxygenase catalysis.

Furthermore, these results may explain the role of glycidate in the inhibition of glycolate synthesis and the net increase of photosynthesis in tobacco leaf discs as demonstrated by Zelitch (1).

The role of ribulose-1,5-bisphosphate (RuDP) oxygenase in the production of phosphoglycolate under photorespiration conditions is well established (2-5). At low CO_2 and high O_2 concentrations, significant rates of oxygenase activity, in addition to the diminished carboxylase activity, can be measured (5). The results of Takabe and Akazawa (6) suggest that both enzymatic activities share a common catalytic site on the large subunit of the enzyme molecule. However, the reaction mechanism of the oxygenase reaction is still uncertain. Kwok and Wildman (7) have investigated the binding of RuDP to the enzyme molecule and they have been able to show that, in the course of binding, a conformational change of the enzyme occurs. The other substrate, oxygen, is incorporated as a single atom into the carboxylic acid group of phosphoglycolate. The other

product, 3-phosphoglyceric acid, is not labeled as has been demonstrated using the ^{18}O-isotope (4).

It seems rather unlikely that oxygen reacts as a triplet molecule with RuDP. We propose, therefore, that oxygen is first activated by the conversion to the superoxide radical anion ($O_2^{\cdot-}$) and that, subsequently, a sulfhydryl-group in the catalytic center of the enzyme molecule is oxidized to a thiyl-radical. If such a mechanism is valid, it would be anticipated that substances which inactivate sulfhydryl-groups should inactivate the enzyme if they are accesible to active center sulfhydryl-groups. Thus, the effect of glycidate and iodoacetamide on the inhibition of the oxygenase activity was investigated.

Methods and Materials RuDP carboxylase was isolated from spinach leaves. The enzyme was purified to homogeneity by the following method: the leaves were ground in 100 mM Tricine-NaOH buffer (pH 8.0) containing 10 mM $MgCl_2$, 5 mM EDTA, 10 mM HCO_3^-, and 1 mM dithiothreitol. Ammonium sulfate was added to precipitate the enzyme (30 to 55% saturation). The crude enzyme preparation was passed through a Sephadex G-200 column and subjected to ultrafiltration using Amicon XM-300 filters. The final step was a sucrose gradient centrifugation according to Andersen et al (8). Fractions were analyzed for specific activity and for homogeneity by polyacrylamide gel electrophoresis (9).

The assay reaction mixture for the estimation of RuDP carboxylase activity contained in 1 ml: 50 µmoles Tricine-NaOH buffer (pH 8.0), 10 µmoles $MgCl_2$; 25 µmoles (^{14}C)-HCO_3^- (spec. act.: 1 µCi/1 µmole) and 0.6 mg of protein. After 5 min pre-incubation at 25°C, the reaction was started by the addition of 1 µmole RuDP. The reaction was stopped by acidification with glacial acetic acid and the amount of fixed carbon-^{14}C was estimated (8).

The homogenous protein fraction, which was used throughout these experiments, had a specific activity of 0.25 µmoles CO_2 fixed per mg protein per min and a $K_m(HCO_3^-)$ value of 3.8 mM.

RuDP oxygenase was determined by measuring oxygen consumption either manometrically with a Warburg apparatus or polarographically with a Gilson oxygraph. Reaction mixtures for experiments done in the Warburg apparatus contained in 2 ml: 2 mg of protein in 50 mM Tricine-NaOH buffer (pH 8.5) and 10 mM $MgCl_2$ in the main vessel and 4 µmoles RuDP in the sidearm. The gas phase was 100 % oxygen. The vessels were temperature equilibrated for 5 min at 25°C and readings were taken after the addition of RuDP.

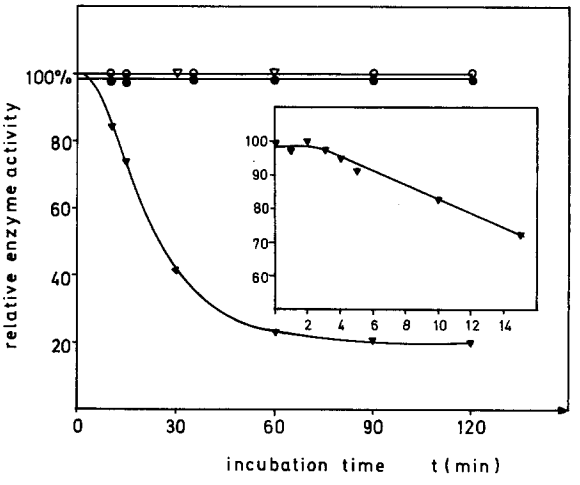

Fig.1 The influence of glycidate on the RuDP carboxylase and oxygenase activity. The oxygenase and carboxylase activity was measured as described in the Methods. The symbols represent:
▼——▼ RuDP oxygenase activity after glycidate incubation
▽——▽ RuDP oxygenase activity without gycidate incubation
●——● RuDP carboxylase activity after glycidate incubation
○——○ RuDP carboxylase activity without glycidate incubation.
The insert shows the oxygenase activity after 1 to 15 min preincubation with glycidate.

Reactions for experiments in which oxygen uptake was measured with the oxygraph were started by the addition of 1 µmole RuDP to 2 mg of protein in 1.3 ml 50 mM Tricine-NaOH buffer (pH 8.5) containing 10 mM $MgCl_2$. The buffer was equilibrated with oxygen and the reaction was measured at 20°C.

The incubation conditions for the experiments with glycidate and iodoacetamide are described in the Results.

Results The sensitivity of the RuDP carboxylase reaction to glycidate (5 mM) was measured as a function of time. As shown in Fig. 1, the enzyme was not inhibited after a short incubation time (1 to 16 min) or after a prolonged treatment of up to 2 hrs. These results are in agreement with the earlier observations by Zelitch (1), that glycidate has no effect on the RuDP carboxylase activity. In contrast, however, RuDP oxygenase activity was strongly inhibited by the incubation of the enzyme with 5 mM glycidate as shown in Fig. 1.

Table I

incubation conditions	enzyme activity in %
enzyme alone	100[1)]
enzyme with RuDP	100
enzyme with 5 mM glycidate	18
enzyme with 2 mM iodoacetamide	0
enzyme with RuDP and 2 mM iodoacetamide	0

1) The specific activity was equal to 50 nmoles/mg protein min.

In these experiments, carried out in the oxygraph, RuDP oxygenase was incubated in presence of 5 mM glycidate for 1 min to 2 hrs. RuDP was then added and the kinetics of oxygen uptake were measured. The rates were calculated and the specific activity of the oxygenase reaction without glycidate treatment was equal to 38 nmoles per mg protein and per min. and this value was arbitrarly set to 100 %.
Table I shows similar results obtained by measuring the oxygen uptake manometrically. The enzyme preparation was incubated at 20°C for 1 hr plus and minus 5 mM glycidate. In another experiment, glycidate was replaced by iodoacetamide. The enzyme was incubated in presence of 0.65 mM RuDP at 25°C. After 5 min, iodoacetamide was added (final concentration: 2 mM and the enzyme sample further incubated for 30 min. Excess iodoacetamide was removed by passing the incubation mixture through a Sephadex G-25 column equilibrated with 20 mM Tricine-NaOH buffer (pH 8.0) containing 10 mM MgCl and 1 mM RuDP. The protein fraction was collected and further used for the manometric assay. Table I shows that no oxygen uptake was observed with the alkylated protein sample. The direct addition of iodoacetamide to the reaction mixture also caused a complete loss of the oxygenase activity.

Discussion Photorespiration significantly diminishes net CO_2 assimilation. In a recent publication by Zelitch (9) concerning the improvement of the efficiency of photosynthesis, the author summarized the data in terms of the percentage of photorespiration to net photosynthesis. These values vary from 25 to 75 % for C_3-plants (9). It is, therefore, of interest to find substances which block photorespiration but that either

(i) do not effect or (ii) perhaps even stimulate the rate of photosynthesis. The physiological experiments by Zelitch show that glycidate is apparently such a photorespiration inhibitor. The formation of glycolate is severely inhibited by treatment of tobacco leaf discs with glycidate (1). We were ,therefore, interested in studying the inactivation mechanism.

Several pathways have been proposed for the synthesis of glycolate during photorespiratory events in chloroplasts. It appears that the oxidative splitting of RuDP by RuDP oxygenase is the main pathway for phosphoglycolate production with subsequent dephosphorylation to glycolate (2). Despite this well documented reaction, other pathways of glycolate formation are still in discussion, e.g. the oxidation of the activated glycolaldehyde of the fructose-6-phosphate-transketolase complex (10,11) and the reduction of glyoxylate to glycolate by the glyoxylate NADPH reductase (12). In addition to these reactions, Zelitch (13,14) proposed an alternative mechanism for glycolate production from C_1-units.

Most of the recent publications (2-5) confirm the proposal by Bassham and Kirk (14) that glycolate is derived from an intermediate of the reductive pentosephosphate cycle, most probably RuDP. Therefore, we investigated the action of glycidate on both the RuDP oxygenase and RuDP carboxylase activity of the fraction-1-protein. We have demonstrated that glycidate specifically inhibits the oxygenase activity without affecting the carboxylase activity. Similar experiments have been carried out with the alkylating reagent iodoacetamide. In presence of RuDP, the carboxylase reaction is not inhibited during the incubation with iodoacetamide as has been shown by Rabin and Trown (15). Under the same conditions, the

oxygenase activity is completly inhibited with similar kinetics as earlier observed for the inactivation of the unprotected carboxylase (15).

These results lead us to the assumption that a sulfhydryl-group is involved in the oxygenase reaction mechanism. The alkylation of this group by iodoacetamide or glycidate causes an irreversible inactivation of the enzyme activity. These observation are in agreement with the earlier results of Zelitch (1), who also observed an irreversible inhibition of photorespiration by glycidate treatment of leaf discs. Since there is no effect on the carboxylation of RuDP by glycidate or iodoacetamide, it can be speculated that this sulfhydryl-group may be necessary for the activation of the oxygen.

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