Vol. 83, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1978 Pages 954-962

SPECIFIC INHIBITION OF OXYGENASE ACTIVITY OF RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE BY HYDROXYLAMINE

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Received June 5, 1978

Summary Ribulose-1,5-diphosphate oxygenase activity of ribulose-1,5-diphosphate carboxylase was completely inhibited by preincubation of the enzyme with 5mM hydroxylamine in presence of the substrate ribulose-1,5-diphosphate. Inhibition by hydroxylamine was uncompetitive with respect to ribulose-1,5-diphosphate and noncompetitive with respect to magnesium. Carboxylase activity was not affected by hydroxylamine. These results suggest that the two activities of the enzyme can be regulated differentially and that inhibiting the oxygenase activity does not stimulate the carboxylase activity of the enzyme. The data further suggest that the inhibition by hydroxylamine may be through its interaction with carbonyl groups of the enzyme exposed on the binding of ribulose-1,5-diphosphate to the protein.

Ribulose-1,5-diphosphate oxygenase activity is associated with ribulose-1,5-diphosphate carboxylase (EC 4.1.1.39) and this catalyses the oxygenative cleavage of ribulose-1,5-diphosphate (RuDP) to yield phosphoglycolate and phosphoglycerate (1-3). Results of Takabe and Akazawa (4) and also Badger and Andrews (5) suggested that both enzyme activities share a common catalytic site on the large subunit of the enzyme molecule. Wildner (6) has proposed a reaction mechanism for oxygenase in which a cysteine residue of the active centre is 'activated' to thiyl radical and oxygen is reduced to superoxide anion. There have been a number of studies on the differential regulation of RuDP carboxylase-oxygenase activity (7-9). Chollet and Anderson (7) examined different chloroplastic metabolites for their action on these two enzyme activities, however, none of the metabolites studied produced any differential regulation of RuDP carboxylase-oxygenase reactions. Ryan and Tolbert (8) observed that several plastid metabolites including fructose-1,6-diphosphate (FDP) ribulose-5-phosphate, fructose-6-phosphate and glucose-6-

0006-291X/78/0833-0954$01.00/0

phosphate acted as effectors of RuDP oxygenase activity opposite to their previously reported effects on RuDP carboxylase. We have earlier presented evidence to suggest that the two activities of the enzyme can be modulated differently by at least one chloroplastic metabolite viz. FDP (9). It was found that FDP preferentially inhibited oxygenase function of the enzyme. In this report, we describe the effect of hydroxylamine on RuDP carboxylase-oxygenase enzyme. Oxygenase reaction was specifically inhibited by hydroxylamine without affecting the carboxylase activity of the enzyme. As it is believed that both carboxylase and oxygenase share the same catalytic site and also that O_2 and CO_2 are competitive inhibitors in carboxylation and oxygenation reactions respectively (5), it is surprising that only one of the reactions is inhibited by hydroxylamine. An attempt has been made to explain the inhibitory effect of hydroxylamine on the oxygenase reaction.

Materials and Methods RuDP carboxylase was isolated from spinach leaves. The enzyme was purified according to the method of Paulsen and Lane (10). The purity was adjudged by polyacrylamide gel electrophoresis.

RuDP carboxylase and oxygenase were assayed under identical conditions of pH, Mg^{++} and temperature after fully activating the enzyme with 20mM $MgCl_2$ and 10mM $NaHCO_3$ at pH 8.5 for about 10 minutes.

The assay mixture for estimation of RuDP carboxylase activity in 0.5 ml contained 50mM Tricine-NaOH (pH 8.5), 10mM $MgCl_2$, 25mM $NaHCO_3$ (2 µCi of $NaH^{14}CO_3$) and 100 µg of activated enzyme protein. The reaction was started by the addition of RuDP to a final concentration of 0.7mM. The reaction was stopped by acidification with glacial acetic acid and the amount of carbon ^{14}C estimated. The protein fraction used throughout these experiments had a specific activity of 200 nmoles CO_2 fixed per mg protein per minute.

RuDP oxygenase was determined by measuring the oxygen consumption polarographically using Gilson oxygraph. The reaction mixture in a total volume of 1.5 ml contained 50mM Tricine-NaOH (pH 8.5) 10mM $MgCl_2$ and 250 µg of activated enzyme protein. The reaction was started by injecting RuDP into the reaction vessel equilibrated at 30 C to a final concentration of 0.7 mM. Protein fraction used throughout these experiments had a specific activity of 80 nmoles O_2 consumed per minute per mg protein.

Results The sensitivity of RuDP carboxylase-oxygenase reaction to hydroxylamine was measured as a function of hydroxylamine concentration. As shown in Fig. 1 the carboxylase activity was not inhibited even at a concentration of 6mM hydroxylamine, whereas the oxygenase activity was inhibited to about 53% at 3mM

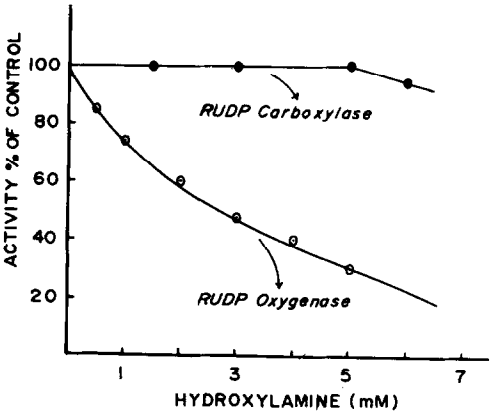

Fig. 1 - Effect of hydroxylamine on carboxylation and oxygenation reaction. Both the activities were assayed as described in Materials and Methods. The levels of hydroxylamine indicated were added to the standard assay mixture 2 minutes before the reaction was initiated by addition of RuDP.

concentration of hydroxylamine. Wishnick and Lane (11) had also reported no inhibition of carboxylase activity at 5-10mM of hydroxylamine. In order to determine the optimum conditions for hydroxylamine inhibition, the effect of pH on hydroxylamine inhibition was studied. The data in Table I show that maximum inhibition was observed at pH values of 8.0 and 8.5, this also corresponds to the broad pH optima for the enzyme activity. At and above pH 9.0, eventhough the control enzyme was about 70% active, the hydroxylamine treated enzyme did not show appreciable inhibition.

Characteristics of hydroxylamine inhibition The plots of 1/v versus series of inhibitor concentrations at two different RuDP concentrations as illustrated in Fig. 2 yielded parallel lines. Hence inhibition by hydroxylamine seems to be uncompetitive with respect to RuDP. Kinetic analysis of the inhibition of RuDP oxygenase by hydroxylamine suggests that the enzyme RuDP complex reacts with hydroxylamine to form an inactive ternary complex. A similar mechanism has been reported in case of cyanide inhibition of both carboxylase and oxygenase activities (3,11). It is likely that the inhibitor binds with the enzyme substrate complex. This will be supported by the preincubation data to be presented later. Inhibition by hydroxylamine with respect to Mg^{++} was of noncompetitive nature having a Ki value of 4.3mM (Fig. 3).

Table I

Effect of pH on hydroxylamine inhibition of oxygenase activity

pH	Hydroxylamine (mM)	Activity percent of control
7.5	3	65
8.0	3	40
8.5	3	38
9.0	3	72
9.5	3	92

Oxygenase activity was measured as described in the Materials and Methods. The activity at each of the pH values in the absence of hydroxylamine was considered as control.

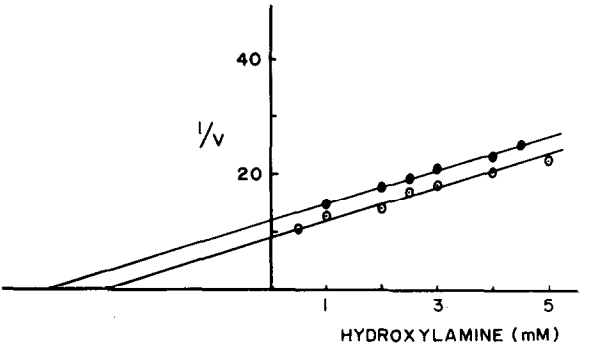

Fig. 2 - Dixon plot for inhibition of oxygenase by hydroxylamine at two different RuDP concentrations
⊙-----⊙ 0.7mM RuDP, ●-----● 0.35mM RuDP

Preincubation studies It was observed that hydroxylamine inhibition can be reversed by dilution if the enzyme was preincubated with hydroxylamine but in the absence of substrate, however, in the presence of the substrate (RuDP) the inhibitor inactivates the enzyme substrate complex which is not reversed upon dilution (Table II). The carried over hydroxylamine as such had no effect on oxygenase reaction in the preincubation studies.

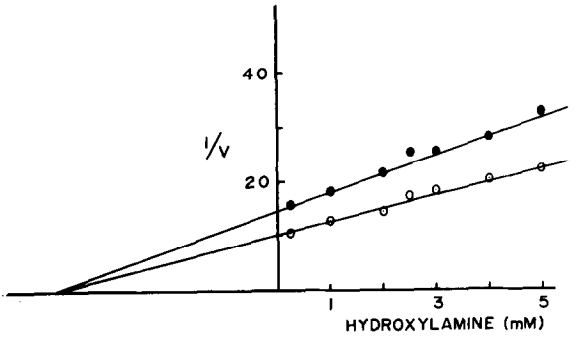

Fig. 3 - Dixon plot for inhibition of oxygenase by hydroxylamine at two different Mg^{++} concentrations
⊙-----⊙10mM $MgCl_2$, ●-----● 5mM $MgCl_2$

Table II

Effect of preincubation of the enzyme with hydroxylamine and RuDP on its oxygenase activity

Additions to the preliminary[a] reaction mixture	NH_2OH in[b] the assay (mM)	% Inhibition
1) None	0.5	Nil
2) None	5.0	73
3) 5mM NH_2OH	0.5	Nil
4) 5mM NH_2OH + RuDP	0.5	100
5) 5mM NH_2OH + RuDP + Mg	0.5	100

a) The preliminary incubation mixture consisted of 50mM Tricine-NaOH buffer (pH 8.5) and 2mg of enzyme protein in a total volume of 1ml. The variable additions were 5mM NH_2OH, 0.5mM RuDP, and 10mM $MgCl_2$ as indicated in the table. After 10 minutes of preincubation 0.1ml of aliquote was removed and assayed for the activity.
b) The levels of hydroxylamine indicated in the table were added to the standard assay mixture 2 min before the reaction was initiated by addition of RuDP. In reactions 3-5 the concentration of hydroxylamine was due to carry over NH_2OH from the preincubation stage.

It was also seen that Mg^{++} was not necessary for hydroxylamine binding. Since the carboxylase activity was not affected by hydroxylamine, we wondered whether the presence of bicarbonate affords protection against hydroxylamine inhibition. To test this, effect of bicarbonate concentrations on hydroxylamine inhibition of carboxylase and oxygenase activity was studied. The data presented in fig. 4 show that preincubation of the enzyme with 5mM hydroxylamine at 1mM bicarbonate resulted in complete loss of oxygenase activity in less than 5 minutes in the presence of 0.5mM RuDP. When similar preincubations were done in the presence of 2.5 and 5mM bicarbonate, the oxygenase activity was inhibited to the extent of 85 and 80% respectively in 10 minutes. Carboxylase activity was not affected even in the preincubation studies at any of the bicarbonate concentrations used. The presence of bicarbonate in the reaction mixture seemed to protect the oxygenase function of the enzyme against hydroxylamine inhibition to a limited extent because the plot of rate constant versus bicarbonate concentrations (calculated from the data of Fig. 4) showed that the protection provided by bicarbonate beyond 2.5mM was negligible.

Discussion Photorespiration significantly diminishes net CO_2 assimilation. It is, therefore, of interest to find substances which block photorespiration. It has been proposed that RuDP oxygenase is the major, if not the only source of glycolate, the substrate for photorespiration (1,3). In view of this, it will be useful if oxygenase activity is specifically inhibited so that the losses due to photorespiration are considerably reduced. Further, if it is shown that the oxygenase and carboxylase activities can be regulated differentially it would be possible to search ways and means to modify the genetic makeup of the plant such that oxygenase function of the carboxylase is minimised.

Our experiments show that hydroxylamine specifically inhibits oxygenase reaction but does not affect carboxylation reaction. However, under *in vitro* conditions, inhibiting the oxygenase reaction by glycidation (12) or by hydroxylamine, as in the present case, does not stimulate carboxylation reaction.

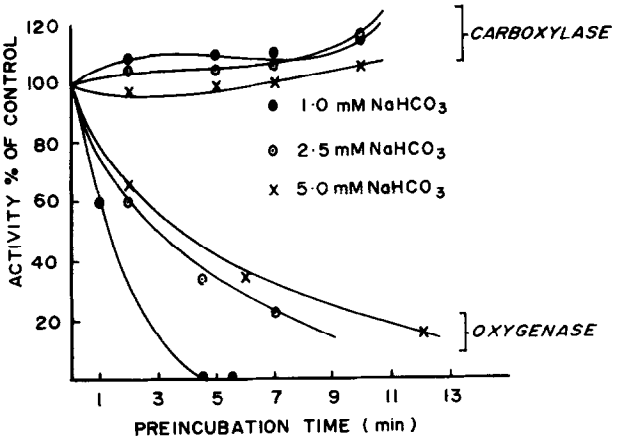

Fig. 4.- Inactivation of RuDP carboxylase-oxygenase with time as a function of bicarbonate concentration at 5mM hydroxylamine. The enzyme (2mg) was preincubated in a mixture containing 50mM Tricine-NaOH (pH 8.5), 5mM hydroxylamine, 0.5mM RuDP, 10mM $MgCl_2$ and bicarbonate concentration as indicated in a total volume of 0.5ml. Aliquots were removed at different times as indicated and assayed for enzyme activity under standard conditions.

Kinetic and preincubation studies suggest that hydroxylamine reacts with RuDP oxygenase only in the presence of the substrate RuDP to form a catalytically inert complex. A similar observation was made in case of cyanide inhibition of both carboxylase and oxygenase activities (3,11). As cyanide inhibits both the activities, the mechanism of inhibition of oxygenase activity by hydroxylamine may be different. The most plausible explanation to the specific inhibition of the oxygenase activity would seem to be that the interaction of the oxygenase molecule with the RuDP generates a site on the enzyme molecule which is susceptible to nucleophilic attack by hydroxylamine. Once hydroxylamine binds to this site oxygenation cannot proceed.

Participation of carbonyl groups associated with the protein seem to be necessary for the catalytic activity in the case of RuDP oxygenase, since carbonyl reagents like hydroxylamine and semicarbazide (unpublished observation) inhibit the activity specifically. Participation of sulfhydryl groups in the oxygenase and carboxylase reactions is suggested (12). Hydroxylamine may not be inactivating sulfhydryl groups because carboxylase activity is not inhibited. Sulfhydryl groups are implicated in RuDP binding and are essential for both carboxylase and oxygenase reactions.

Urease has previously been shown to be inhibited by hydroxylamine (13) however, the inhibition was reversed by mercaptide reagents. No such reversal was observed in the case of oxygenase inhibition by hydroxylamine. Although the SH groups may not be inactivated by NH_2OH, the possibility that NH_2OH binding to the protein induces a conformational change such that SH groups cannot participate in the reaction mechanism must be left open.

It has been recently shown that RuDP binding site apparently involves lysine (14,15). If so, the most likely binding of RuDP would be through formation of a Schiff base between e-amino groups of lysine and the keto group of RuDP. However, inactivation of the Schiff base cannot explain specific inhibition of oxygenase activity by hydroxylamine because any compound that affects RuDP binding must inhibit both the activities.

The data presented in this communication suggest that the activities of RuDP carboxylase-oxygenase can be regulated differentially when assayed under identical conditions. This perhaps indicate that either the catalytic centres for the two activities are not identical or mechanism by which these reactions are catalysed are different. The data further suggest that CO_2 and O_2 may not be merely competing for the same reaction intermediate in carboxylation and oxygenation reactions respectively.

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