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INHIBITION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE-OXYGENASE ACTIVITIES BY HYDROXYLAMINE *

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Summary

Hydroxylamine directly and reversibly inhibits both activities of homogeneous ribulose-1,5-bisphosphate carboxylase-oxygenase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39) isolated from diverse sources. NH_2OH is an uncompetitive inhibitor of carboxylase activity with respect to ribulosebisphosphate. This reagent also reacts non-enzymically with ribulosebisphosphate to deplete this substrate. Contrary to previous reports, these results indicate that hydroxylamine directly and indirectly inhibits both activities of this bifunctional enzyme.

Ribulose-1,5-bisphosphate carboxylase-oxygenase (3-phospho-D-glycerate carboxy-lyase (dimerizing, EC 4.1.1.39) catalyzes the carboxylation of ribulose-P_2 to form 2 molecules of 3-phosphoglycerate in the first step of the C_3 photosynthetic carbon reduction cycle. In a competing reaction, this bifunctional enzyme also catalyzes the oxygenation of ribulose-P_2 to form one molecule each of 3-phosphoglycerate and 2-phosphoglycolate. This reaction comprises the first step in the C_2 photorespiratory carbon oxidation cycle [1—3]. Photorespiration is widely believed to be antithetical to net photosynthetic CO_2 fixation [1,3]. The discovery that ribulose-P_2 carboxylase-oxygenase is responsible for the initial reaction in each of these opposed metabolic pathways has led to a search for chemical modulators of this bifunctional enzyme which can alter the ratio of carboxylase to oxygenase activity in favor of net carbon fixation. Several studies of the effects of various chemicals and chloroplast

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metabolites on ribulose-P_2 carboxylase-oxygenase activity have failed to identify a modulator which can affect differentially the two competing reactions [2,4,5]. Recently, however, two research groups have reported that hydroxylamine increases the ratio of carboxylase to oxygenase activity. Bhagwat et al. [6] reported that NH_2OH specifically inhibits purified spinach ribulose-P_2 oxygenase activity while having no effect on the carboxylase reaction. Okabe et al. [7], working with partially-purified enzyme from *Anabaena cylindrica*, observed that this reagent causes a marked activation of carboxylase activity and a concomitant inhibition of the oxygenase reaction. This communication reports the results from our investigation of the effects of NH_2OH on the activities of homogeneous ribulose-P_2 carboxylase-oxygenase isolated from tobacco and spinach leaves and the procaryote *Thiobacillus intermedius*.

Experimental

Hydroxylamine · HCl was obtained from Sigma Chemical Co. and Tridom/Fluka. Solutions of NH_2OH were prepared immediately before use by dissolution of the crystalline reagent in 10 mM Tris buffer and readjusted to pH 8.0 with NaOH. Tetrasodium ribulose-P_2 was purchased from Sigma Chemical Co. and solutions were prepared in 10 mM Tris-HCl (pH 8.0) just prior to use. $NaH^{14}CO_3$ was obtained from New England Nuclear and Sepharose 6B and prepacked columns of Sephadex G-25 were purchased from Pharmacia Fine Chemicals. Ultrapure $(NH_4)_2SO_4$ was obtained from Schwarz/Mann and poly(ethylene glycol)-4000 from J.T. Baker Chemical Co.

Ribulose-P_2 carboxylase was purified to homogeneity from freshly harvested tobacco (*Nicotiana tabacum* L. cv. Xanthi) leaves by the method of Kung et al. [8]. The thrice-crystallized protein was stored at 4°C in 25 mM Tris/0.5 mM disodium EDTA, pH 7.4 (buffer A) with 0.02% (w/v) NaN_3, and, as needed, crystals were washed once in buffer A and dissolved in 0.1 M Tris/20 mM $MgCl_2$/10 mM $NaHCO_3$/0.1 M NaCl, pH 8.6. The enzyme (10 mg/ml) was then heat- and CO_2/Mg^{2+}-activated at 50°C for 20 min [4,8].

Thiobacillus intermedius was grown aerobically in a glutamate/CO_2/thiosulfate mixotrophic medium [9] and the harvested cells stored at −20°C. Ribulose-P_2 carboxylase was isolated from the crude cell homogenate by pelleting the protein from the 93 000 × *g* supernatant fluid followed by $(NH_4)_2SO_4$ fractionation and sedimentation into a discontinuous sucrose density gradient [9]. The protein was found to be homogeneous by sodium dodecyl sulfate (SDS) and non-SDS polyacrylamide gel electrophoresis [9] and was stored in 0.6 M sucrose at −20°C.

Ribulose-P_2 carboxylase was isolated at 4°C from market spinach (*Spinacia oleracea* L.) leaves by a modification of the method of Hall and Tolbert [10]. The latter procedure was followed exactly up through precipitation of the impure enzyme with 18% (w/v) poly(ethylene glycol)-4000 and 20 mM $MgCl_2$. The resultant protein pellet was then redissolved in buffer A and fractionated by $(NH_4)_2SO_4$ precipitation. The 35—55% saturation fraction was collected, resuspended in buffer A, and subjected to descending chromatography in a 5 × 45 cm column of Sepharose 6B equilibrated and eluted with buffer A. The major protein peak contained ribulose-P_2 carboxylase activity and fractions

with an A_{280nm}/A_{260nm} ratio greater than 1.2 were pooled, concentrated by precipitation between 35–55% satn. $(NH_4)_2SO_4$, and rechromatographed in the Sepharose 6B column. This procedure yields spinach ribulose-P_2 carboxylase judged to be homogeneous by SDS and non-SDS polyacrylamide gel electrophoresis and by sedimentation velocity in the analytical ultracentrifuge. Spinach enzyme prepared by this method has an A_{280nm}/A_{260nm} ratio of 1.85–1.95 and a carboxylase specific activity at 30° C of approx. 2.0 μmol $H^{14}CO_3^-$ fixed/min per mg protein. The protein was stored at 4° C as a precipitate in 55% $(NH_4)_2SO_4$, and aliquots were prepared for use by resuspension in 25 mM Tris-HCl, pH 8.0, followed by gel filtration through a small column of Sephadex G-25 equilibrated and eluted with the same buffer. The desalted enzyme preparation was diluted 5-fold into a solution sufficient to yield final concentrations of 10 mM $MgCl_2$, 20 mM $NaHCO_3$, and 25 mM Tris-HCl (pH 8.5), and was then CO_2/Mg^{2+}-activated for 20 min at 30° C.

Concentrations of the purified proteins, as mg/ml, were estimated spectrophotometrically by multiplying A^{1cm}_{280nm} by 0.70 for the tobacco enzyme, 0.84 for *T. intermedium* and 0.61 for spinach [9]. Details of the carboxylase and oxygenase assays are described in the figure and table legends and Ref. 8.

Results

This report shows that hydroxylamine acts to inhibit directly and indirectly both activities of ribulose-P_2 carboxylase-oxygenase. Fig. 1 illustrates the indirect inhibition of ribulose-P_2 carboxylase activity by hydroxylamine. This figure summarizes the results of experiments in which NH_2OH and ribulose-P_2 were mixed at several molar ratios, incubated at pH 8.0 and 25° C, and aliquots withdrawn at various times to initiate the carboxylase reaction. In these experiments, the only ribulose-P_2 theoretically present in the assays was that which was preincubated with hydroxylamine. It can be seen in Fig. 1 that preincubation of NH_2OH with ribulose-P_2 leads to carboxylase activity which progressively decreases with increasing preincubation time. In addition, this decrease in activity is increasingly pronounced at preincubation concentrations of hydroxylamine and ribulose-P_2 which have higher calculated second-order reaction rates ($r = k[NH_2OH][\text{ribulose-}P_2]$). For example, the calculated reaction rate between NH_2OH and ribulose-P_2 for curve C is 10-times that for curve A; a correspondingly increased rate of carboxylase inhibition for curve C is observed. Preincubated hydroxylamine plus ribulose-P_2 also markedly inhibits ribulose-P_2 oxygenase activity. For example, assay of spinach oxygenase activity in the presence of an aliquot of a mixture of 11 mM NH_2OH plus 5.5 mM ribulose-P_2 preincubated for 1 h at 25° C results in 20% of the activity observed with an identical NH_2OH/substrate mixture preincubated for less than 1 min. These results are in quantitative agreement with the data for the inhibition of spinach carboxylase activity (Fig. 1, curve A) where 15% of the corresponding control activity was observed using an identically preincubated sample of NH_2OH plus ribulose-P_2. Appropriate controls established that these inhibitory preincubation effects are not due to deterioration of substrate or enzyme in the absence of NH_2OH during the incubation period or to the conversion of hydroxylamine per se to a more potent inhibitory compound.

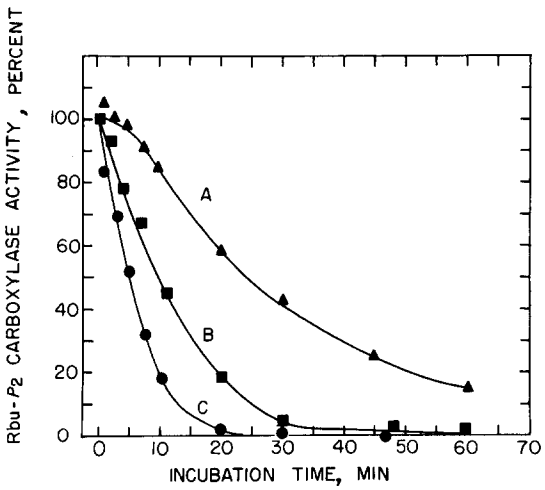

Fig. 1. Spinach ribulose-P_2 (Rbu-P_2) carboxylase activity assayed with ribulose-P_2 preincubated with hydroxylamine for differing times. NH_2OH and ribulose-P_2 were mixed in the molar ratios specified below, incubated at pH 8.0 and 25°C, and aliquots withdrawn at the indicated times to initiate the carboxylase reactions. Assay mixtures contained 0.1 M Tris, 0.1 mM Na_2EDTA, 0.5 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 25 mM $NaH^{14}CO_3$ (0.5 Ci/mol), 7 μg of CO_2/Mg^{2+}-activated enzyme, and preincubated NH_2OH plus ribulose-P_2 in a final volume of 0.55 ml at pH 8.0. Assays were run for 45 s at 30°C. Carboxylase activity at zero-time (defined as 100%) was determined without preincubating NH_2OH with ribulose-P_2, limiting contact between these compounds to the assay period. Curve A (▲———▲): preincubation concentrations were 11 mM NH_2OH and 5.5 mM ribulose-P_2, with final theoretical assay concentrations of 1 and 0.5 mM, respectively. The zero-time (100%) activity was 96% of the corresponding control rate (2.50 μmol $H^{14}CO_3^-$ fixed/min per mg protein) determined in the absence of NH_2OH. Curve B (■———■): preincubation concentrations were 40 mM NH_2OH and 2 mM ribulose-P_2, with final theoretical assay concentrations of 4 and 0.2 mM, respectively. The zero-time activity was 78% of the corresponding control (1.46 μmol $H^{14}CO_3^-$ fixed/min per mg protein). Curve C (●———●): preincubation concentrations were 110 mM NH_2OH and 5.5 mM ribulose-P_2, with final theoretical assay concentrations of 10 mM and 0.5 mM, respectively. The zero-time activity was 64% of the corresponding control (2.70 μmol $H^{14}CO_3^-$ fixed/min per mg protein).

Inhibition of carboxylase and oxygenase activity by preincubated mixtures of NH_2OH and ribulose-P_2 might be attributable to simple substrate depletion, formation of a potent reversible or irreversible inhibitory adduct, or a combination of these effects. Experiments were performed in which preincubated mixtures of NH_2OH and ribulose-P_2 were added to carboxylase assays containing 0.1 or 0.5 mM fresh ribulose-P_2; the amount of ribulose-P_2 remaining in the preincubated mixture was concurrently determined by enzymic assay [11]. We find that preincubated mixtures of NH_2OH and ribulose-P_2 become rapidly depleted in ribulose-P_2 and that preincubated mixtures which no longer contain any detectable substrate are no more inhibitory than a similar concentration of NH_2OH unexposed to ribulose-P_2. Further, the enzyme retains complete carboxylase activity when mixed with a preincubated sample of NH_2OH and ribulose-P_2, gel-filtered through Sephadex G-25 and then reassayed. From these results we conclude that the apparent inhibition of carboxylase activity depicted in Fig. 1 is due to simple substrate depletion in the preincubated mixtures of NH_2OH and ribulose-P_2 and that no irreversible enzyme inhibitor is formed. If a reversible inhibitory adduct is formed during the non-enzymic

TABLE I

DIRECT INHIBITION OF RIBULOSE-P_2 CARBOXYLASE ACTIVITY BY HYDROXYLAMINE AT LOW AND HIGH LEVELS OF BICARBONATE

The Tris-buffered assay mixtures (see Fig. 1) contained 0.5 mM ribulose-P_2, 0, 5 or 10 mM NH_2OH, 2.5 mM (2.5 Ci/mol) or 25 mM (0.5 Ci/mol) $NaH^{14}CO_3$, and 4 (spinach) or 10 (tobacco) μg of CO_2/Mg^{2+}-activated enzyme in a final volume of 0.52 ml at pH 8.0. NH_2OH was incubated with the assay mixture for 15 s followed by an additional 15 s in the presence of CO_2/Mg^{2+}-activated enzyme. The reactions were initiated with ribulose-P_2 and terminated after 30 s at 30°C.

Enzyme from:	NH_2OH (mM)	Carboxylase activity (% of control)	
		2.5 mM $NaHCO_3$	25 mM $NaHCO_3$
Spinach	0 (control)	100 *	100 *
	5	60	75
	10	49	58
Tobacco	0 (control)	100 *	100 *
	5	47	58
	10	27	37

* Control carboxylase activities at 2.5 and 25 mM $NaH^{14}CO_3$ were 0.78 and 2.7 μmol $H^{14}CO_3^-$ fixed/min per mg protein, respectively (spinach), and 0.12 and 0.45 μmol $H^{14}CO_3^-$ fixed/min per mg protein, respectively (tobacco).

reaction of NH_2OH with ribulose-P_2, it is especially impotent since its effect is undetectable at ribulose-P_2 assay concentrations of 0.1 or 0.5 mM. No similar effects were observed when NH_2OH was preincubated with CO_2 (i.e., $NaHCO_3$), the other carboxylase substrate.

Hydroxylamine also exerts a direct inhibitory effect on the carboxylase activity of the spinach, tobacco and *T. intermedius* enzymes. This direct inhibition is observed in assays initiated with ribulose-P_2 and terminated within 30 s, thus minimizing the reaction between the substrate and NH_2OH described above. For example, 10 mM hydroxylamine inhibits spinach and tobacco carboxylase activity by about 40—60% and 50—70% at 25 and 2.5 mM $NaHCO_3$, respectively (Table I). This effect is far greater than can be attributed to simple substrate depletion during 30 s at 10 mM NH_2OH and 0.5 mM ribulose-P_2 since at these concentrations the calculated second-order reaction rate is at least 12-fold lower than that employed in the substrate depletion experiments shown in Fig. 1. The higher plant-type carboxylase purified from the procaryote *T. intermedius* [9] is also strongly inhibited by NH_2OH in ribulose-P_2-initiated assays. When assayed at 20 mM $NaHCO_3$ and 0.5 mM ribulose-P_2, *T. intermedius* carboxylase activity was inhibited about 45% by 10 mM NH_2OH. These highly reproducible observations, indicative of a direct inhibition of ribulose-P_2 carboxylase activity by NH_2OH, are corroborated when the data in Fig. 1 and the corresponding minus-hydroxylamine control values are replotted as ln carboxylase specific activity vs. time. Each of the resultant plots shows a sharp upward break to the control rate at zero to 45 s time. The biphasic nature of such plots indicates two inhibitory processes with very different rate constants. We attribute the slow process to reaction of NH_2OH with substrate, while the very fast process is the direct inhibition of the enzyme by hydroxylamine. The reversible nature of this direct inhibitory effect was demonstrated by preincubating the enzyme with high concentrations of NH_2OH (10—

250 mM) for up to 90 min at 25°C. Subsequent removal of the reagent by gel filtration in Sephadex G-25 or dilution resulted in a nearly complete recovery to the control carboxylase activity.

Hydroxylamine also directly inhibits the oxygenase activity of the spinach and tobacco proteins. For example, when the assays were initiated with CO_2/Mg^{2+}-activated spinach enzyme following preincubation of 5 mM NH_2OH and ribulose-P_2 for less than 30 s in the oxygen electrode vessel, the initial rate of enzyme- and ribulose-P_2-dependent O_2 uptake at 21% O_2 was inhibited about 45 and 65% at 0.1 and 0.5 mM ribulose-P_2, respectively. The direct inhibitory effect of the reagent (3 or 5 mM) on the oxygenase and carboxylase reactions was also observed when both activities were determined by simultaneous measurement of ribulose-P_2-dependent O_2 uptake and $H^{14}CO_3^-$ fixation during a 30 s assay in the electrode vessel in the presence of 21% O_2, 1 mM total $NaH^{14}CO_3$ and 0.1 or 0.5 mM ribulose-P_2 (cf. Ref. 7). Several control experiments were performed to rule out trivial explanations of the observed inhibition of carboxylase-oxygenase activity. Hydroxylamine obtained from two different sources was equally effective in inhibiting enzyme activity, lessening the possibility that an unknown contaminant in our original source of NH_2OH was the actual inhibitor. Further, separate solutions of hydroxylamine made up in N_2-gassed buffer at pH 8.0 were vigorously bubbled with N_2, air, CO_2-free air or 21% O_2 (balance N_2) in serum-stoppered vials for 30 min immediately prior to use. None of these treatments affected the efficacy of NH_2OH as an inhibitor

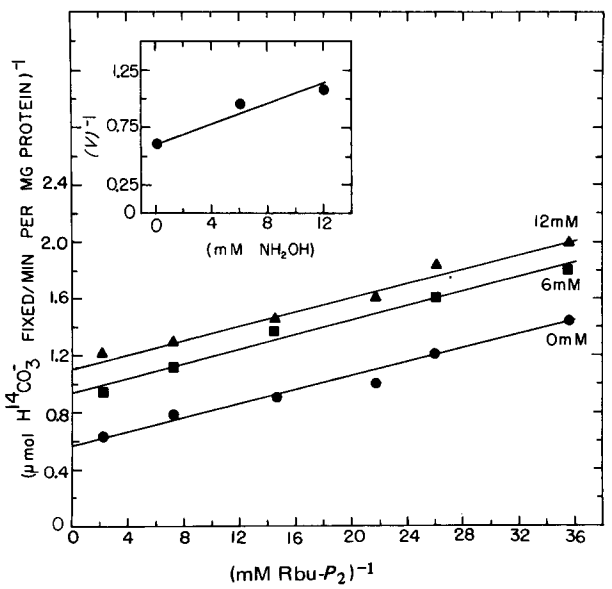

Fig. 2. Double reciprocal plots of spinach ribulose-P_2 carboxylase activity in the absence and presence of hydroxylamine vs. ribulose-P_2 (Rbu-P_2) concentration. The Tris-buffered assay mixtures (see Fig. 1) contained 22.5 mM $NaH^{14}CO_3$ (0.5 Ci/mol), 7 μg of CO_2/Mg^{2+}-activated enzyme, 0 (●———●), 6 (■———■) or 12 (▲———▲) mM NH_2OH and ribulose-P_2 as indicated in the figure in a final volume of 0.6 ml at pH 8.0. NH_2OH was added to each complete assay mixture exactly 10 min before initiation of the reaction with ribulose-P_2 and the assays were run for 30 s at 30°C. Inset: replot of $1/V$ vs. NH_2OH concentration. From this plot, an apparent K_i for NH_2OH of 10—12 mM was estimated. All plots are the result of linear regression analysis of the duplicate- or triplicate-averaged data points.

of carboxylase activity. From the results described above, we conclude that hydroxylamine per se acts in a reversible manner to directly inhibit ribulose-P_2 carboxylase-oxygenase activity.

Steady-state kinetic experiments were performed to examine the nature of the direct hydroxylamine inhibition of ribulose-P_2 carboxylase activity, and the results are presented in Fig. 2. The data for the spinach carboxylase indicate that under conditions of saturating $NaH^{14}CO_3$, NH_2OH is an uncompetitive inhibitor of the CO_2/Mg^{2+}-activated enzyme with respect to ribulose-P_2, with an apparent K_i of 10—12 mM. These results are in agreement with those of Bhagwat et al. [6] who observed uncompetitive kinetics for hydroxylamine inhibition of spinach ribulose-P_2 oxygenase activity. While several models leading to the kinetics depicted in Fig. 2 can be conceived, experimental discrimination between them is beyond the purpose and scope of this communication.

Discussion

The results summarized in this report are in partial disaccord with those recently presented by other workers [6,7]. Although our finding that ribulose-P_2 oxygenase activity is inhibited by hydroxylamine is consistent with the results presented in these previous studies, our repeated observation that NH_2OH also directly (Table I, Fig. 2) and indirectly (Fig. 1) inhibits carboxylase activity conflicts with the activation reported by Okabe et al. [7] and the lack of effect reported by Bhagwat et al. [6]. The activation of carboxylase activity by up to 10 mM hydroxylamine reported by Okabe et al. [7] might be attributable to the partially-purified state of their enzyme preparation or to a unique property of the higher plant-type [12] *Anabaena cylindrica* enzyme. However, their results are particularly difficult to interpret since the assays were initiated with heat- and CO_2/Mg^{2+}-activated enzyme, allowing an unspecified preincubation time between NH_2OH and ribulose-P_2. Bhagwat et al. [6] observed that hydroxylamine concentrations up to 6 mM had no effect on spinach ribulose-P_2 carboxylase activity. Since these workers initiated the assays with ribulose-P_2 (0.7 mM) following a 2 min preincubation of the CO_2/Mg^{2+}-activated enzyme with 25 mM $NaHCO_3$, 10 mM $MgCl_2$, $\pm NH_2OH$, we would expect the direct inhibition by hydroxylamine to have been readily observed (cf. Table I and Fig. 2). One possible explanation is that their carboxylase reaction rates became very non-linear with time and that both the control and NH_2OH-inhibited reactions went nearly to completion. This supposition is prompted by the observations that 100 μg of activated enzyme per assay were employed in their studies, 10—15-times the amount added to our carboxylase assays, and that the control specific activity was only 0.2 μmol $H^{14}CO_3^-$ fixed/min per mg protein. However, we can offer no definitive explanation for the discrepancy between these earlier studies and our observations with the homogeneous spinach, tobacco and *T. intermedius* enzymes.

An objective of many research scientists studying ribulose-bisphosphate carboxylase-oxygenase is to identify a chemical compound or physical condition which differentially modulates the activities of this bifunctional enzyme in favor of CO_2 fixation. While some workers [2] have suggested that the active

site chemistry of the enzyme precludes such a differential regulation, the large differences in size, shape and charge distribution between CO_2 and O_2 suggest that the binding of these two gaseous substrates could be differentially affected by some factor [3]. Indeed, temperature [3,13,14] and Mg^{2+}/Mn^{2+} [15—17] (Rejda, J.M. and Chollet, R., unpublished data) have been shown to affect differentially the two activities in vitro. The recent reports [6,7] that NH_2OH enhanced carboxylase activity relative to oxygenase activity in vitro prompted our examination of this system. Unfortunately, given the experimental results presented in this report, we must conclude that hydroxylamine is an inhibitor of both activities and thus is not a differential regulator of ribulose-1,5-bisphosphate carboxylase-oxygenase.

Note added in proof (Received May 29th, 1980)

We have now tested the direct effect of NH_2OH on the activity of partially-purified ribulose-P_2 carboxylase from *Anabaena cylindrica* Lemm. Carboxylase activity of the CO_2/Mg^{2+}-activated enzyme is inhibited 50--55% by 5 mM NH_2OH in ribulose-P_2-initiated assays (30 s at 30°C) (cf. Ref. 7). We conclude that ribulose-P_2 carboxylase from *A. cylindrica* is not unique in its response to hydroxylamine.

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