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Short Communication

Inhibition of Glycine Oxidation by Carboxymethoxylamine, Methoxylamine, and Acethydrazide[1]

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ABSTRACT

Carboxymethoxylamine (amino-oxyacetate), methoxylamine, and acethydrazide are shown to be effective, although not completely specific, inhibitors of glycine oxidation by the isolated glycine decarboxylase multienzyme complex, mitochondria, protoplasts, and leaf discs from peas. The inhibition probably results from a reaction between these compounds and the pyridoxal 5-phosphate cofactor of the enzyme.

The glycine decarboxylase multienzyme complex is a mitochondrial enzyme that is responsible for the photorespiratory oxidation of glycine. The enzyme complex is composed of at least four subunits: The P protein[3] (PLP binding); the H protein (lipoamide containing); the T protein (THF binding); and the L protein (dihydrolipoamide dehydrogenase) according to the nomenclature of Kikuchi (5). The enzyme complex has been solubilized from pea leaf mitochondria in an active form (11) and purified to near homogeneity (12). The complex catalyzes the release of $^{14}CO_2$ from [1-^{14}C]glycine dependent on the addition of PLP, THF, and NAD. It also catalyzes the exchange of $^{14}CO_2$ with the carboxyl carbon of glycine and the synthesis of glycine from methylene THF, NADH, CO_2, and NH_4^+. The isolated enzyme complex has a mol wt of about 290,000 and shows a strong dependence on the addition of DTT for full activity.

Because of the unique role of this enzyme in photorespiration, substantial effort has been directed towards the identification of inhibitors of this enzyme. To date, three classes of inhibitors have been found: (a) compounds that react with PLP bound to the P protein, *e.g.* INH and cyanide; (b) compounds that react with the lipoamide cofactor of the H protein, like arsenite (11); and (c) structural analogs of glycine, such as aminoacetonitrile (16) and glycine hydroxamate (6). INH was the first reported inhibitor of glycine decarboxylase (10). It inhibits glycine oxidation by reacting with the PLP cofactor of the enzyme and is reasonably specific in that it shows little inhibition of photosynthesis under nonphotorespiratory conditions (13). INH, however, has limited usefulness because of the high concentrations (13) or long incubation periods needed for inhibition. Cyanide has also been shown to inhibit glycine decarboxylase, probably by reacting with PLP (2,11), but this compound is pleiotropic in its effects. Glycine hydroxamate and aminoacetonitrile are structurally related to glycine and have been shown to inhibit glycine oxidation by cells (or leaf discs) and isolated mitochondria (6, 16). Because of the relative insensitivity of the isolated enzyme to aminoacetonitrile (11, 18), it was suggested that this compound may not inhibit the glycine decarboxylase complex directly. Arsenite inhibits a wide range of thiol containing enzyme including the lipoamide-based complexes, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, and has been shown to inhibit isolated glycine decarboxylase (11) as well as glycine oxidation by mitochondria (17).

Because of the known ability of hydroxylamine (11) and hydrazides to react with PLP and inhibit the glycine decarboxylase complex, we investigated the effects of methoxylamine, carboxymethoxylamine (amino-oxyacetate), and acethydrazide (Fig. 1) on glycine decarboxylation. These compounds are water soluble, have low mol wt, and are structurally related to glycine, the substrate of the enzyme. In addition, their small size allows for more rapid entry across the mitochondrial membrane, a factor that limits the inhibitory potential of INH (Sarojini and Oliver, unpublished). All three compounds are shown here to be potent inhibitors of glycine oxidation by the isolated enzyme, mitochondria, protoplasts, and leaf discs.

MATERIALS AND METHODS

Mitochondria were isolated from 2- to 3-week-old greenhouse-grown pea plants by techniques described earlier (11, 17). The release of $^{14}CO_2$ from [1-^{14}C]glycine was performed in 1 ml of a reaction medium composed of 300 mM sorbitol, 20 mM MOPS-NaOH (pH 7.2), 5 mM K-phosphate, and 0.1% BSA in a 10-ml side arm flask (Kontes, Vineland, NJ) fitted with a center well containing a filter paper strip dampened with 25 μl of 5 N ethanolamine. The reaction was initiated by adding 10 mM glycine (0.5 μCi/umol) and terminated by adding 100 μl of 2 N H_2SO_4. Radiocarbon released was determined by liquid scintillation counting.

Protoplasts were isolated from pea leaves, purified by flotation as assayed for photosynthetic $^{14}CO_2$ fixation and $^{14}CO_2$ release from [1-^{14}C]glycine as described earlier (19). Leaf discs (0.5 cm) were punched from mature leaves and floated on water before use. The discs were distributed to 10 ml reaction flasks (7 each) and illuminated for 30 min (750 μE/m^2·s at 27°C). The water in the flasks was then replaced with inhibitor solutions at the indicated concentrations and the illumination continued for an additional 10 min before the addition of 5.0 mM $NaH^{14}CO_3$. After 5 min, the reactions were stopped by adding 2 volumes of ethanol and boiling. The discs were homogenized and the acid-

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[3] Abbreviations: P, H, T, and L proteins, component enzymes of the glycine decarboxylase multienzyme complex; PLP, pyridoxal 5-phosphate; THF, tetrahydrofolate; INH, isonicotinic acid hydrazide; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

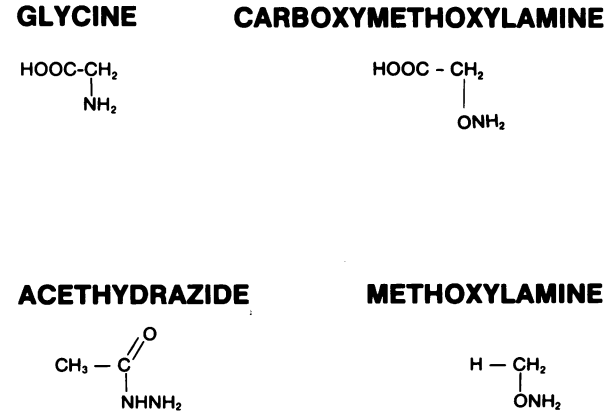

FIG. 1. Structures of carboxymethoxylamine, methoxylamine, acethydrazide, and glycine.

stable radioactivity was measured. To determine the effect of the inhibitors on glycine decarboxylase activity and the glyoxylate aminotransferases, discs were collected after the exposure to inhibitors and washed 5 times with water. The discs were then ground in 10 ml of 300 mM sorbitol, 50 mM MOPS-NaOH (pH 7.2), 2 mM EDTA, and 0.2% BSA. After filtering through Miracloth (Calbiochem), mitochondria were isolated from half the sample by differential centrifugation and assayed as above for glycine decarboxylase activity. A 1.0-ml portion of the remaining half of the sample was adjusted to contain 5 mM [1-^{14}C]glyoxylate (0.2 μCi/umol) and 5 mM each of the amino donors, alanine, glutamate, and serine. The reaction was stopped after 30 min by the addition of 2 volumes of ethanol, and the radioactive glycine formed was separated on Dowex 50 and determined by liquid scintillation counting.

The glycine decarboxylase complex was solubilized from isolated pea leaf mitochondria following acetone extraction as detailed earlier (11, 12). The enzyme was redissolved in 20 mM MOPS-NaOH (pH 7.2) and 2 mM DTT and stored at −20°C until used. The release of $^{14}CO_2$ from [1-^{14}C]glycine was assayed in a medium containing 20 mM MOPS-NaOH (pH 7.2), 20 mM glycine (0.05 μCi/μmol), 2 mM DTT, 2 mM NAD, and 0.5 mM THF. The reaction volume was 1 ml and the reaction was initiated by adding enzyme and terminated by adding 100 μl of 2 N H_2SO_4.

The three inhibitors were from Aldrich Chemical Co. Because of the toxicity of these chemicals, especially methoxylamine, caution should be exercised.

RESULTS AND DISCUSSION

Carboxymethoxylamine, methoxylamine, and acethydrazide were tested for their effect on glycine oxidation by the isolated glycine decarboxylase preparation. Because all three compounds react with free PLP, it was not added to the reactions. As a result, the maximum uninhibited rate was 43% of the rate when PLP was added. Glycine oxidation was inhibited by low concentrations of each of the inhibitors and, when the inhibitor concentrations reached sufficient levels, glycine oxidation was completely inhibited (Fig. 2). Carboxymethoxylamine was the most potent inhibitor of the isolated enzyme. The reaction was 50% inhibited by about 2 μM and completely inhibited by 20 μM carboxymethoxylamine. Methoxylamine was the next most potent inhibitor with 50% inhibition requiring 25 μM and full inhibition requiring 100 μM. Acethydrazide was the least effective of the group against the isolated enzyme complex requring 0.9 and 2.5 mM, respectively, for half and full inhibition. Other hydrazides tested, including INH, required about the same concentration for inhibition (data not shown).

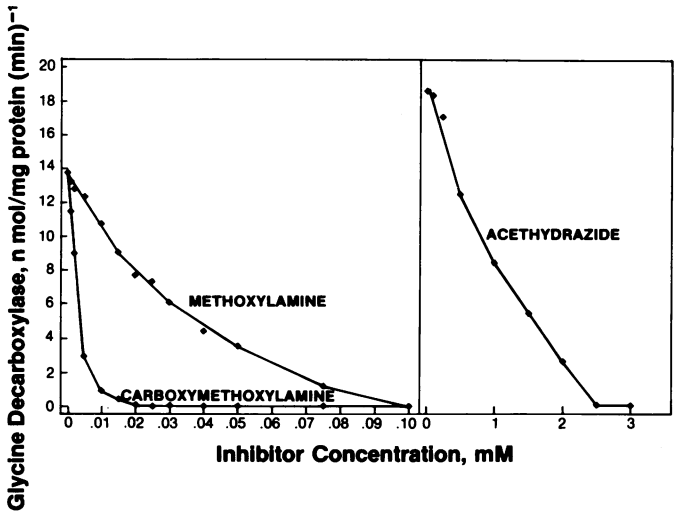

FIG. 2. Effect of carboxymethoxylamine, methoxylamine, and acethydrazide on $^{14}CO_2$ release from [1-^{14}C]glycine by isolated glycine decarboxylase. The reaction was initiated by adding 275 μg of protein and terminated after 10 min acidification.

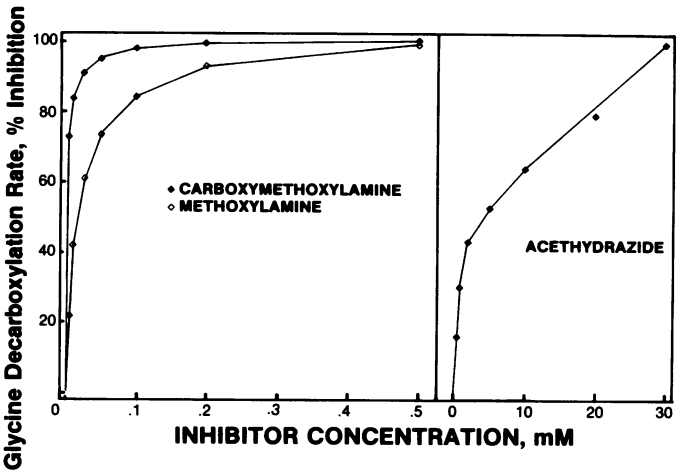

FIG. 3. The effect of carboxymethoxylamine, methoxylamine, and acethydrazide on the release of $^{14}CO_2$ from [1-^{14}C]glycine by pea leaf mitochondria. The reactions were initiated by adding mitochondria (200 μg protein) to the complete reaction medium containing the indicated concentration of inhibitor and terminated after 15 min by acidification.

The 10-fold difference in concentration needed for 50% inhibition by carboxymethoxylamine and methoxylamine may indicate that the additional carboxyl group on the former compound increases the reactivity of this chemical towards enzyme-bound PLP. As can be seen in Figure 1, the carboxymethoxylamine is a very close structural analog of glycine. The presence of the ionized carboxyl on carboxymethoxylamine may result in this compound being specifically directed to the active site for glycine binding on the enzyme complex. This should position the reactive hydroxylamine residue so that it can readily react with the aldehyde of PLP thus leading to enzyme inactivation. Methoxylamine should have the same reactivity towards PLP, but lacks the ionized carboxyl and would not be directed to the enzyme's active site.

The three compounds inhibited glycine oxidation by isolated pea leaf mitochondria (Fig. 3). At sufficiently high concentrations, each of the three inhibitors completely prevented glycine oxidation. The concentrations required for 50% and full inhibition were: carboxymethoxylamine, 5 and 100 μM; methoxylam-

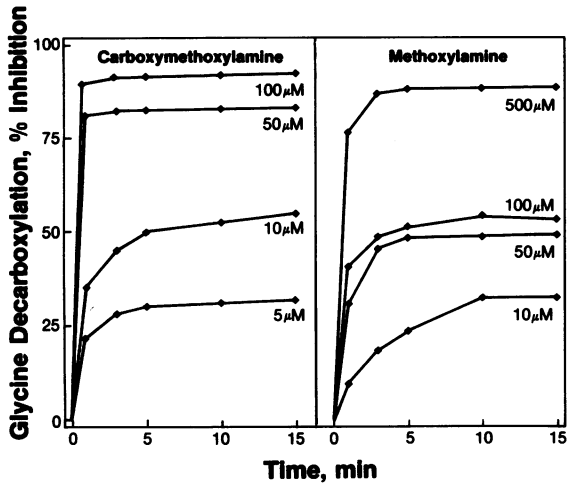

FIG. 4. Time course for inhibition by carboxymethoxylamine and methoxylamine of $^{14}CO_2$ release from [1-^{14}C]glycine by pea leaf mitochondria. The reactions were initiated by adding mitochondria (250 μg protein) to the complete reaction mixture containing the concentration of inhibitor indicated and terminated at the appropriate time by acidification. The percentage inhibition of $^{14}CO_2$ release was calculated by comparing the rate in the presence of the inhibitor with a control rate determined over the same time period without the inhibitor present. The average control rate of $^{14}CO_2$ release was 89 nmol/mg protein min^{-1}.

ine, 20 and 500 μM; and acethydrazide, 5 and 30 mM. Again, carboxymethoxylamine was the most effective of the hydroxylamine derivatives and both of these were more potent inhibitors than the hydrazide. When used against isolated mitochondria, acethydrazide was several fold more effective than INH.

One of the anticipated advantages of these compounds was that their small size might allow them to cross the mitochondrial membrane more rapidly than larger molecules like INH and, therefore, reach and inhibit the glycine decarboxylase complex more quickly. The time courses for the inhibition of mitochondrial CO_2 release from glycine by the hydroxylamine analogs is shown in Figure 4. In this experiment, the reaction was initiated by adding mitochondria to the complete reaction medium (*i.e.* the mitochondria were not preincubated with the inhibitors) and then terminated at the times indicated by acidification. At the lowest carboxymethoxylamine concentration tested (5 μM), a maximum inhibition of about 30% was observed after 5 min. At concentrations of 100 μM, nearly complete inhibition was reached by 1 min. Methoxylamine showed similar results but required longer incubation times and/or higher concentrations for equivalent inhibition.

With both of the hydroxylamines, inhibition was rapid enough to be measured with an O_2 electrode. Using 50 μM carboxymethoxylamine, 50% inhibition was observed within 30 s after adding the inhibitor; the inhibition reaching 94% within 1 min. With 100 μM carboxymethoxylamine, 100% inhibition occurred in less than 30 s. Complete inhibition was observed within 45 s after adding 100 μM methoxylamine. The inhibition of glycine oxidation was indeed very rapid. O_2 uptake linked to succinate and malate oxidation was unaffected by treatment with 150 μM of either hydroxylamine analog or 5 mM acethydrazide.

The effect of all three inhibitors on $^{14}CO_2$ release from [1-^{14}C] glycine and photosynthetic $^{14}CO_2$ fixation by pea leaf protoplasts is shown in Table I. The three compounds were effective inhibitors of glycine decarboxylation by the protoplasts. Methoxylamine was more potent than carboxymethoxylamine which in turn was a stronger inhibitor than acethydrazide. The stronger inhibition by methoxylamine suggests that the additional carboxyl group on the carboxymethoxylamine retards entrance of the latter compound across the cell membrane. The three compounds also inhibited CO_2 fixation by the protoplasts under photorespiratory conditions (0.5 mM bicarbonate) but to a lesser degree under nonphotorespiratory conditions (5.0 mM bicarbonate). Servaites and Ogren (13) have shown that chemicals that interfere with the metabolism of intermediates in the glycolate pathway inhibit photosynthesis under conditions where glycolate is actively synthesized. This probably results from the accumulation of intermediates in the glycolate pathway to toxic levels, the decrease of available amino donors (15), and the reduced flow of glycolate carbon back to glycerate and phosphoglycerate with the resulting depletion of the Calvin cycle. The decreased inhibition at high bicarbonate levels suggest that the inhibitors are causing little nonspecific inhibition of other cellular reactions at least as they relate to photosynthesis. Acethydrazide consistently stimulated CO_2 fixation at high bicarbonate concentra-

Table I. *Effect of Carboxymethoxylamine, Methoxylamine, and Acethydrazide on $^{14}CO_2$ Fixation and $^{14}CO_2$ Release from [1-^{14}C] Glycine by Pea Leaf Protoplasts*

Protoplasts were exposed to the inhibitors at the concentrations shown for 1 min before the addition of either $NaH^{14}CO_3$ in the light or 10 mM [1-^{14}C]glycine in the dark. The bicarbonate was supplied at either 0.5 mM for photorespiratory conditions or 5.0 mM for nonphotorespiratory conditions. Photosynthesis assays were terminated after 5 min and the glycine oxidation after 15 min. The values presented are percentages of the uninhibited controls (3.9 μmol/mg Chl·min^{-1} for glycine decarboxylation and 34 and 64 μmol/mg Chl·min^{-1} for CO_2 fixation for 0.5 and 5.0 mM $NaHCO_3$, respectively.

Treatment	Concn.	CO_2 from Glycine	CO_2 Fixation	
			0.5 mM $NaHCO_3$	5.0 mM $NaHCO_3$
	μM		% of control	
Water		100	100	100
Carboxymethoxylamine	10	41	84	102
	500	3	74	105
Methoxylamine	10	9	62	95
	500	0	42	98
Acethydrazide	10	55	90	93
	500	27	68	132

Table II. *Effect of Carboxymethoxylamine, Methoxylamine, and Acethydrazide on Photosynthetic $^{14}CO_2$ Fixation by Leaf Discs, Glycine Decarboxylase Activity in Mitochondria Isolated from Treated Discs, and Glyoxylate Aminotransferase Activity in Crude Extracts from Treated Discs*

Leaf discs from peas were floated on 50 μM carboxymethoxylamine, 50 μM methoxylamine, or 100 μM acethydrazide for 10 min before assaying the appropriate activities. The values in parentheses are the percentage inhibitions.

Treatment	CO_2 Fixation	Glycine Decarboxylation	Transaminase
	μmol g^{-1} fresh wt h^{-1}	nmol mg^{-1} protein min^{-1}	
Water	177	87	7.5
Carboxymethoxylamine	158 (10%)	18 (80%)	5.7 (24%)
Methoxylamine	158 (10%)	9 (90%)	6.1 (19%)
Acethydrazide	147 (16%)	22 (75%)	7.1 (5%)

tions. The basis for this stimulation is not understood but it is assumed that the effect is not mediated through inhibition of the glycolate pathway because of the limited photorespiration at these CO_2 levels.

Having shown that these three compounds were potent inhibitors of glycine oxidation by the isolated enzyme, mitochondria and protoplasts, the last portion of this study was to investigate the effects of these inhibitors on leaf discs. Pea leaf discs were floated on either water or 50 μM carboxymethoxylamine, 50 μM methoxylamine, or 100 μM acethydrazide for 10 min and their photosynthetic ability was measured along with the glycine decarboxylase activity of the mitochondria and the glyoxylate-specific transaminase activities of the cell free extracts isolated from the discs. As shown in Table II, both of the hydroxylamines inhibited CO_2 fixation about 10% while acethydrazide inhibited a little more strongly (16%). Treatment of leaf tissue with all three compounds strongly decreased the ability of mitochondria isolated from the tissues to decarboxylate glycine. Methoxylamine was more inhibitory (90%) than carboxymethoxylamine (80%) and acethydrazide (75%). All three compounds also inhibited some glyoxylate transaminases with carboxymethoxylamine showing the highest inhibition (24%) and acethydrazide the least (5%). Carboxymethoxylamine was previously used as a transaminase inhibitor in plants (1, 7) and animals (14). Jenkins *et al.* (3, 4) have shown that this compound will inhibit photosynthesis under photorespiratory conditions and suggest that serine-glyoxylate aminotransferase and glutamate-glyoxylate aminotransferase as well as a potential site in the Calvin cycle are major targets.

CONCLUSIONS

During the course of our studies on the mechanism and control of glycine oxidation and the role of this reaction in photorespiration, we were limited by the availability of compounds that could rapidly inhibit this reaction at low concentrations and show reasonable specificity for the glycine decarboxylase complex. The three compounds tested and reported here, carboxymethoxylamine, methoxylamine, and acethydrazide may help overcome some of these limitations. Carboxymethoxylamine completely inhibited glycine oxidation by isolated glycine decarboxylase at 20μM by isolated mitochondria at 50 μM, by protoplasts at 100 μM, and by intact tissue at 50 to 100 μM, following a 10-min incubation. Methoxylamine required about 5 to 10 times higher concentrations to inhibit the isolated enzyme, but had about the same efficacy as carboxymethoxylamine in the other systems. Acethydrazide was considerably less effective against the isolated enzyme, mitochondria, and protoplasts, but was only marginally less effective with leaf discs. For comparative purposes, a 1-min incubation with 100 μM methoxylamine or carboxymethoxylamine or 5 mM acethydrazide completely inhibited glycine oxidation by isolated mitochondria. Complete inhibition by INH requires 40 mM for 30 min while arsenite needs 3 mM for 5 min. Aminoacetonitrile and glycine hydroxamate are not strictly comparable because they are reversible inhibitors that are competitive with glycine, but about 3 and 5 mM are needed, respectively, to inhibit the oxidation of 10 mM glycine (data not presented).

Although these compounds are fast and effective inhibitors of glycine oxidation under a range of conditions, they are not completely specific for the glycine decarboxylase complex. All three compounds react with isolated PLP. Due to this and their small size, they would be expected to react with a range of PLP-requiring enzymes. We have noted, for example, that these compounds do inhibit the glyoxylate aminotransferases, which require PLP, in leaf discs. It has also been shown that carboxymethoxylamine reacts with these transaminases *in vitro* (3) as well as the enzymes of ammonium assimilation (7). Greening was also inhibited (4). It should be noted, however, that the inhibition of glycine decarboxylation in our system was stronger than the inhibition of transaminase activity. These compounds also showed only limited effect on photosynthetic rates in leaf discs and protoplasts under nonphotorespiratory conditions and did not inhibit mitochondrial electron transport of substrates other than glycine. Although these compounds should be useful tools for studying glycine oxidation *in vivo* and *in vitro*, potential artifacts due to limited specificity should be considered.

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