Vinylglycine and Propargylglycine: Complementary Suicide Substrates for L-Amino Acid Oxidase and D-Amino Acid Oxidase†

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ABSTRACT: Propargylglycine (2-amino-4-pentynoate) and vinylglycine (2-amino-3-butenoate) have been examined as substrates and possible inactivators of two flavo enzymes, D-amino acid oxidase from pig kidney and L-amino acid oxidase from *Crotalus adamanteus* venom. Vinylglycine is rapidly oxidized by both enzymes but only L-amino acid oxidase is inactivated under assay conditions. The loss of activity probably involves covalent modification of an active site residue rather than the flavin adenine dinucleotide coenzyme and occurs once every 2000 turnovers. We have confirmed the recent observation (Horiike, K., Hishina, Y., Miyake, Y., and Yamano, T. (1975) *J. Biochem. (Tokyo), 78,* 57) that D-propargylglycine is oxidized with a time-dependent loss of activity by D-amino acid oxidase and have examined some mechanistic aspects of this inactivation. The extent of residual oxidase activity, insensitive to further inactivation, is about 2%, at which point 1.7 labels/subunit have been introduced with propargyl[2-^{14}C]glycine as substrate. L-Propargylglycine is a substrate but not an inactivator of L-amino acid oxidase and the product that accumulates in the nonnucleophilic *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer is acetopyruvate. In the presence of butylamine-HCl, a species with λ_{max} 317 nm (ϵ = 15 000) accumulates that may be a conjugated eneamine adduct. The same species accumulates from D-amino acid oxidase oxidation of D-propargylglycine prior to inactivation; the inactivated apo D-amino acid oxidase has a new peak at 317 nm that is probably a similar eneamine. A likely inactivating species is 2-keto-3,4-pentadienoate arising from facile rearrangement of the expected initial product 2-keto-4-pentynoate. Vinylglycine and propargylglycine show inactivation specificity, then, for L- and D-amino acid oxidase, respectively.

The design and mechanism of active site directed enzyme inactivators are the subject of current research in this and other laboratories. Our efforts have been in the use of acetylenic and olefinic substrate analogues as chemically unreactive molecules that are converted by enzyme action into a reactive species.

We have previously used propargylglycine (2-amino-4-pentynoate) as an inactivator of rat liver γ-cystathionase (Abeles and Walsh, 1973), *S. typhimurium* cystathionine-γ-synthetase and pig heart L-alanine aminotransferase, but found pig heart L-aspartate aminotransferase unsusceptible (Marcotte and Walsh, 1975). Conversely, the β,γ-olefinic amino acid vinylglycine inactivates the L-aspartate aminotransferase (Rando, 1974), but not the L-alanine aminotransferase.[1] Recently, Horiike et al. (1975) published the observation that propargylglycine inactivates D-amino acid oxidase. They noted that, unlike the hydroxybutynoate induced inactivation of L-hydroxyacid oxidase (Cromartie and Walsh, 1975), it was the apoprotein, not the bound coenzyme, that became modified, but presented no hypothesis on the mode of inactivation. We have now examined some mechanistic features of this inactivation and also the behavior of L-amino acid oxidase with this substrate. We have also examined the oxidation of vinylglycine by D- and L-amino acid oxidases and found that only the L-amino acid oxidase becomes irreversibly inactivated.

Experimental Section

Materials. D-Amino acid oxidase (18 U/mg) was prepared from hog kidney by the method of Brumby and Massey (1968). L-Amino acid oxidase (listed as 5.2 U/mg) was purchased from Sigma Chemical Co. and used without further purification. The ratio of absorbances at 275 and 463 nm was 20 for the commercial enzyme, two times that of the crystalline enzyme (Wellner and Meister, 1960). Acetopyruvic acid (2,4-diketovaleric acid) was also purchased from Sigma. Propargylglycine (2-amino-4-pentynoic acid) was synthesized and resolved by the method of Jansen et al. (1969). D,L-Propargyl[2-^{14}C]glycine was synthesized from diethylacetamido[2-^{14}C]malonate purchased from Amersham. D,L-Vinylglycine was synthesized by the method of Rando[2] from vinylglycolate via the α-bromo acid (Rando, 1974). Other chemicals were commercially available and of reagent grade quality.

Methods. Activity of the amino acid oxidases was determined by oxygen consumption monitored with a Clark-type electrode (YSI Model 53 oxygen monitor). Kinetic assays were performed on a Gilford Model 222 uv/visible spectrophotometer. Spectra were recorded on a Beckman Model 25 uv/visible spectrophotometer.

The oxidases were preincubated with the amino acids by stirring in a small beaker in the appropriate buffer containing catalase (and FAD in the case of D-amino acid oxidase). The product of L-amino acid oxidase oxidation of propargylglycine was determined by incubation of 2.5 μmol of D,L-propargyl[2-^{14}C]glycine (500 cpm/nmol) with 95 μg of L-amino acid oxi-

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[1] P. Marcotte and C. Walsh, unpublished observations. No loss of activity was observed in a 90-min incubation with 30 mM D,L-vinylglycine. Addition of α-ketoglutarate to incubations had no effect.

[2] The synthesis was facilitated by personal communications with R. Rando, and with J. E. Baldwin of M.I.T.

dase and 20 μg of catalase in 200 μl of 100 mM Hepes[3] buffer, pH 8. After a 90-min incubation the reaction mixture was passed through two 1-ml Dowex 50 H^+ columns. The eluate was evaporated to a small volume and spotted on 12 × 4 cm Baker flex silica gel 1B plates. The plates were developed with 1-butanol–acetic acid–water (12:3:5) or water saturated diethyl ether–formic acid (7:1). The plates were sprayed with a solution of 3% vanillin–5% concentrated sulfuric acid in absolute ethanol, and heated to 120°C (Sweig and Sherma, 1972). We found that acetopyruvic acid yields a characteristic brown spot on plates developed in 1-butanol–acetic acid–water and a purple spot in the ether–formic acid system. The plates were cut into 0.5-cm sections and eluted with scintillation fluid that was counted with a Beckman LS-100C scintillation counter.

To determine the stoichiometry of enzyme labeling, 0.7 mg of D-amino acid oxidase was incubated with 6 mg of D,L-propargyl[2-^{14}C]glycine in pH 8 buffer with added catalase and FAD. After complete (≥98%) inactivation, the excess radioactivity was dialyzed from the protein and the protein was passed through a 1.2 × 50 cm Sephadex G-25 column. Tubes containing protein were found by their absorbance at 274 nm and assayed for protein by the method of Lowry et al. (1951). Radioactivity was determined by counting an aliquot of the fraction in scintillation fluid.

Results

Inactivation of L-Amino Acid Oxidase with D,L-Vinylglycine. When the FAD-dependent L-amino acid oxidase from *Crotalus adamanteus* is incubated with D,L-vinylglycine, rapid O_2 uptake ensues, indicating oxidation of the amino acid. From initial velocity data, the L isomer appears to be quite a good substrate. The measured V_{max} for vinylglycine oxidation was 11 μmol min^{-1} mg^{-1} (25°C, Tris buffer, pH 8), which we found to be near the maximal velocity of L-leucine with the same enzyme under the same conditions. The K_m for vinylglycine is, however, higher—at 8 mM compared to 1 mM reported for L-leucine. In determining these kinetic parameters, we saw no evidence of substrate inhibition by vinylglycine (up to 50 mM) reported at high concentrations of L-leucine (Massey and Curti, 1967).

Our initial objective with L-amino acid oxidase was to selectively oxidize L-vinylglycine and recover the D-isomer for use as a suicide substrate with the pyridoxal-P dependent *B. subtilis* enzyme D-amino acid aminotransferase[4] (Martinez-Carrion and Jenkins, 1965). Incomplete resolution has recently been reported using 50 mg of commercial enzyme with 2 mmol of D,L-vinylglycine (Friis et al., 1974). When we attempted this procedure with small amounts of the rattlesnake enzyme, optically pure D-vinylglycine could not be recovered.

This failure was due to a progressive, time-dependent inactivation of the enzyme during oxidation of vinylglycine (Figure 1A). The inactivation is irreversible: dialysis overnight against a large excess of buffer restores no more than 3% of the original activity. By analysis of the amount of O_2 consumed before inactivation is complete, it can be calculated that each molecule of L-amino acid oxidase oxidizes about 2000 vinyl-

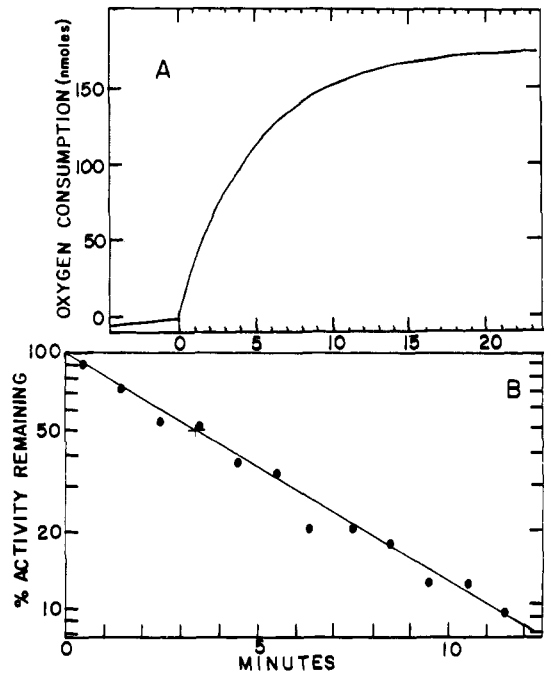
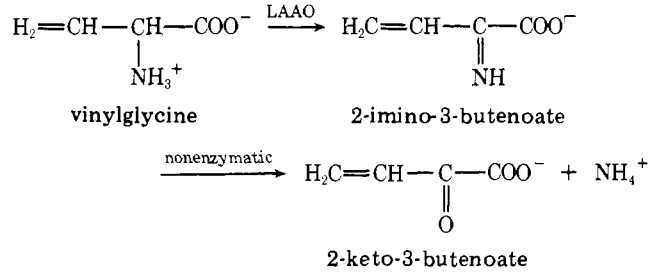


FIGURE 1: (A) Time course of reaction of L-amino acid oxidase with D,L-vinylglycine. The oxygen electrode chamber contained, in 0.4 ml of 100 mM Tris-HCl, pH 8, 6.25 mM D,L vinylglycine and 2 μg of catalase. At time zero, 8 μg of oxidase was added. (B) Relative rates derived from data in part A. The $T_{1/2}$ of inactivation was found to be 3.4 min.

glycine molecules before suffering inactivation. The data of Figure 1B suggest that inactivation follows first-order kinetics. At 6.25 mM D,L-vinylglycine, the half-time for inactivation at room temperature is 3.4 min. This rate behavior is consistent with inactivation proceeding from an enzyme–substrate (or enzyme–product) complex rather than by some collisional interaction of enzyme and inactivator. On chemical grounds, the likely inactivator is not the substrate vinylglycine but rather the product of enzymatic oxidation—the β,γ-unsaturated α-imino acid 2-imino-3-butenoate. With this expectation we



then sought to determine whether the observed inactivation occurs before release of the reactive product, i.e., at the active site, or after release of the electrophilic iminobutenoate into the medium. In the latter case, buildup of the electrophile could then result in nonspecific alkylation of available enzyme nucleophilic groups and cause loss of activity. This second alternative appears to operate in the inactivation of the FMN-dependent L-hydroxy acid oxidase by 2-vinylglycolate (Cromartie and Walsh, 1975). In that case 2-vinylglycolate is oxidized to a similar electrophile, 2-keto-3-butenoate, and causes time-dependent inactivation, but addition of exogeneous low-molecular-weight thiol nucleophiles, such as dithiothreitol, completely protects the hydroxyacid oxidase in a scavenging process.

When dithiothreitol was added to incubations of L-amino

[3] Abbreviation used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TLC, thin-layer chromatography.

[4] We have observed inactivation of the bacterial aminotransferase by both D,L-vinylglycine and by D-propargylglycine: P. Marcotte, J. Manning, C. Walsh, unpublished observations.

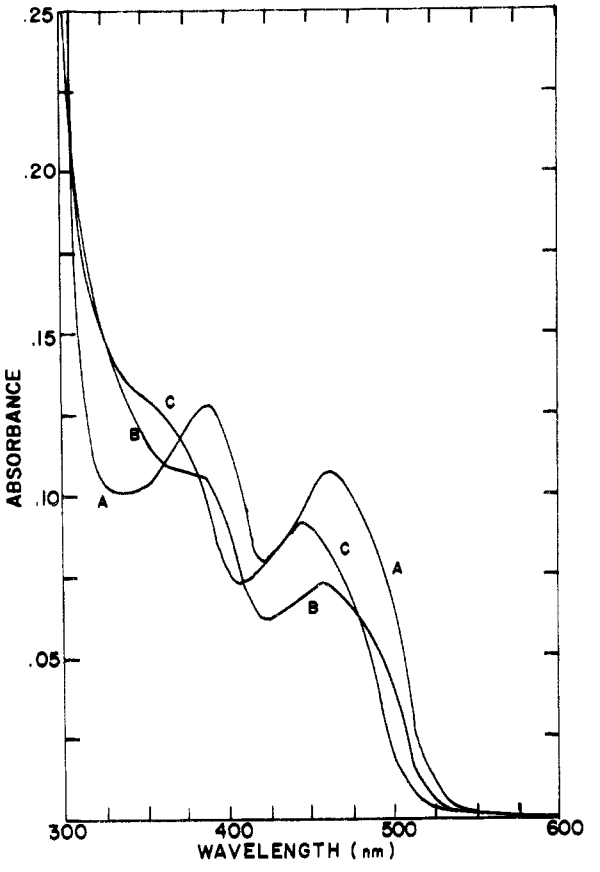


FIGURE 2: Spectral changes on inactivation of L-amino acid oxidase with D,L-vinylglycine. (A) Spectrum of 1 mg of L-amino acid oxidase in 1 ml of 100 mM Tris-HCl, pH 8. (B) After complete inactivation with 10 mg of D,L-vinylglycine followed by dialysis against Tris-HCl buffer (spectrum corrected for changes in concentration by normalizing absorbance at 275 nm). (C) Inactive enzyme of B heated to 100 °C for 5 min—protein removed by membrane filtration, and spectrum of the supernatant recorded.

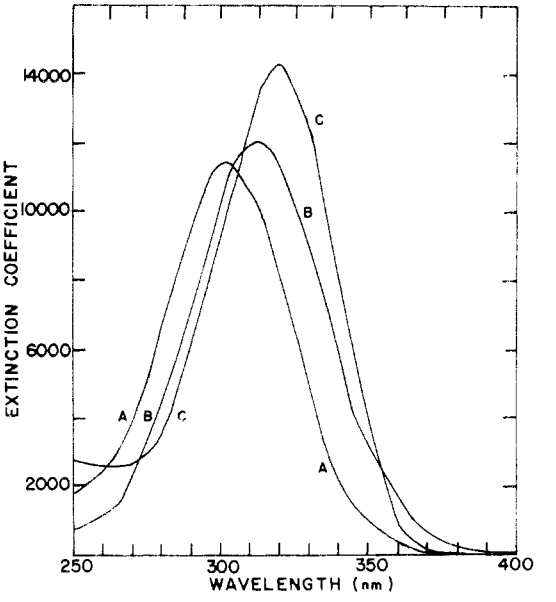


FIGURE 3: Spectrum of product of oxidation of L-propargylglycine by L-amino acid oxidase. 1 μl of 100 mM L-propargylglycine (0.1 μmol) was added to a cuvette containing 1 ml of buffer, 2 μg of catalase, and 100 μg of oxidase. After absorbance changes stabilized, the spectrum was recorded against a reference cell containing buffer, catalase, and oxidase, Buffers: (A) 10 mM Hepes, pH 8; (B) 10 mM Hepes/10 mM ethanethiol, pH 8; (C) 10 mM Hepes/100 mM butylamine, pH 8.

acid oxidase and D,L-vinylglycine, similar protection was not observed. Since a similar electrophilic inactivator is presumed in both cases, this lack of protection suggests that L-amino acid oxidase is inactivated due to covalent modification at the active site prior to release. Further proof that inactivation occurs by attachment of a stoichiometric amount of inactivator to enzyme (and is thus likely to be at the active site) awaits the synthesis of radioactively labeled vinylglycine.

A point of interest is whether inactivation involves an active site amino acid residue or involves reaction with the flavin coenzyme. Addition of FAD to incubations did not alter the kinetics of inactivation, nor did dialysis after inactivation against buffer containing 10 μM FAD lead to any significant recovery of activity. When enough enzyme was used to monitor the electronic spectrum during inactivation, the spectrum of the enzyme was somewhat changed, but importantly, the long-wavelength absorption of bound FAD (Figure 2) had not been bleached. If addition to the isoalloxazine ring of the coenzyme had occurred, major electronic perturbations would have been detected (Schonbrunn et al., 1976; Ghisla et al., 1976; Cromartie and Walsh, 1975). The flavin spectrum of vinylglycine-inactivated L-amino acid oxidase is quite similar to the bound flavin spectrum of 2-vinylglycolate inactivated L-hydroxyacid oxidase (Cromartie and Walsh, 1975). In the latter case, the released flavin was not radioactive when 2-vinyl[1-^{14}C]glycolate had been used. Therefore, we believe it likely that vinylglycine inactivates L-amino acid oxidase by an amino acid modification. This point will be further investigated with radioactive vinylglycine.

Reaction of D-Amino Acid Oxidase with D,L-Vinylglycine. With D-amino acid oxidase, D,L-vinylglycine is a good substrate. It has a K_m of 0.96 mM and a V_{max} 125% that of D-alanine. In sharp contrast to the behavior of L-amino acid oxidase, no inactivation was observed during the course of the oxidation. The enzyme reacts with vinylglycine molecules without detectable loss of enzymatic activity during a 1-hr incubation. The enzyme is clearly insensitive to whatever concentration of electrophilic product accumulates.[5] We also examined for catalytic reaction anaerobically O_2-independent conversion of vinylglycine to α-ketobutyrate, analogous to the reaction of the enzyme with β-chloroalanine (Walsh et al., 1971, 1973). No catalytic turnover under argon was detected.

Reaction of L-Amino Acid Oxidase with L-Propargylglycine. In experiments complementing those with vinylglycine, we have examined the ability of propargylglycine (2-amino-4-pentynoate) to function as substrate and possible inactivator of both D- and L-amino acid oxidases. D-Amino acid oxidase inactivation has been reported in a paper by Horiike et al. (1975) but we shall discuss our L-amino acid oxidase results first, since they provide information useful in the interpretation of the D-amino acid oxidase data.

L-Propargylglycine is an active substrate for rattlesnake venom L-amino acid oxidase with a K_m value of about 3 mM and a V_{max} of 3 μmol min^{-1} mg^{-1} (25°C, Tris buffer, pH 8). These kinetic values were obtained by polarographic oxygen electrode assay and there is substantial nonlinearity in the consumption of O_2 with time. After 6 min, the rate of O_2 consumption was only 30% that of the initial rate. However, this does not appear to be irreversible inactivation for prein-

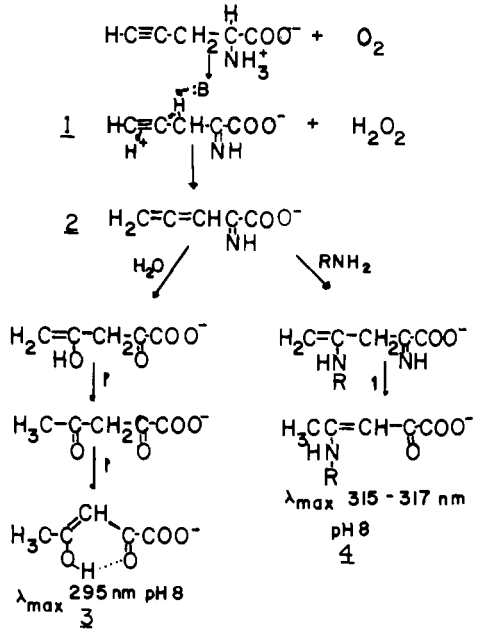
[5] Since we have not yet determined the rate of H_2O attack (or other nucleophiles) at C_4 of 2-keto-3-butenoate, we do not know its actual concentration in these incubations.

cubations of enzyme and propargylglycine for up to 2 hr did not yield fully inactive oxidase. After dialysis of the small molecules from the protein, essentially fully active oxidase was recovered. We believe the nonlinearity in initial velocity reflects a reversible product inhibition. High levels of propargylglycine, used in experiments to obtain the K_m value, prevent the inhibition—suggesting competitive inhibition by some product.

The action of L-amino acid oxidase on propargylglycine yields a product with strong absorbance in the near-ultraviolet. With a limiting amount of substrate (0.1 μmol) and excess enzyme, complete oxidation can be effected, allowing calculation of the extinction coefficient of the uv-absorbing species. The λ_{max} of the product varied with added nucleophiles in the incubation, as shown in Figure 3. In 10 mM Hepes (nonnucleophilic) or 100 mM Tris (potentially nucleophilic) λ_{max} 302 nm (ϵ = 11 000); in 10 mM Hepes, 100 mM butylamine (at pH 8), λ_{max} 315–317 nm (ϵ = 14 000–15 000); in 10 mM Hepes, 10 mM ethanethiol, λ_{max} 312 nm (ϵ = 12 000). These data suggest an initial enzymatic product is being trapped and modified by some of these amine and thiolate nucleophiles.

The probable initial oxidation product from L-propargylglycine in the presence of L-amino acid oxidase is 2-imino-4-pentynoate, compound **1**, as shown in Scheme I. The C_3-

SCHEME I



methylene hydrogens are probably acidic enough to undergo general base-catalyzed abstraction; reprotonation at C_5 would produce the conjugated allenic imino acid, **2**, 2-imino-3,4-pentadienoate. Conjugate attack of water at C_4 would yield acetopyruvate (2,4-diketovalerate), **3**. Attack by alternate nucleophiles would yield derivatives of acetopyruvate. Authentic acetopyruvate has λ_{max} 295 nm (ϵ = 12 000) at pH 8 (Meister and Greenstein, 1948). The discrepancy between these values and the 302 nm (ϵ = 11 000) could be due to a mixture of components, one of them acetopyruvate, in the enzymatic incubations in Hepes buffer. The presence of acetopyruvate accumulating in the enzymatic incubation was confirmed by thin-layer chromatographic comparison. The material showed identical R_f values with acetopyruvate, and gave the same color test with the detection reagent, in two different solvent systems. When D,L-propargyl[2-^{14}C]glycine was used, over 80% of the radioactivity spotted on the TLC plate migrated with acetopyruvate. Additionally, it was found that incubation of authentic acetopyruvate in 10 mM Hepes, 100 mM butylamine HCl (pH 8) slowly (overnight) produced a new species, λ_{max} 315 nm (ϵ = 16 000). This product was labile to treatment with beads of Dowex 50 H^+, re-forming acetopyruvate; presumably the free butylamine binds to the Dowex and the equilibrium is driven back from adduct to starting materials. We believe these results indicate reversible formation of an enamine of acetopyruvate—in all likelihood the same species trapped with butylamine when L-propargylglycine reacts with L-amino acid oxidase.

We have found monitoring of increase in absorbance at 317 nm on reaction of propargylglycine and L-amino acid oxidase in Hepes/butylamine, pH 8, buffer system to be a convenient and extremely sensitive assay of enzyme activity. The apparent extinction coefficient of the accumulating enamine (14 000–15 000) gives a tenfold increase in sensitivity over the O_2 electrode assay. In contrast to the temporal nonlinearity under conditions of O_2 electrode assay, the 317-nm absorbance increases nearly linearly through 1 absorbance unit over as much as a 15-min period. This contradiction may be resolved by the fact that the concentration of both enzyme *and* the accumulating product is an order of magnitude lower than in the O_2 electrode assay; the discrepancy further suggests reversible (not irreversible) inhibition is occurring under O_2 electrode conditions. Addition of authentic acetopyruvate or the acetopyruvate–butylamine (315 nm) adduct to O_2 electrode assay incubations does not mimic the inhibition. We suspect that the inhibitor may be the acetylenic keto acid **1** of Scheme I.

Reaction of D-Amino Acid Oxidase with D-Propargylglycine. In initial velocity determinations, we found D-propargylglycine to be an active substrate for the hog kidney D-amino acid oxidase, showing a K_m of 0.6 mM and a V_{max} of 7 μmol min^{-1} mg^{-1}. We became interested in how propargylglycine might inactivate this enzyme after the report of that fact by Horiike et al. (1975). We were interested in the nature of the inactivation, its stoichiometry, and whether the process was strictly active-site directed. Based on the above results with L-amino acid oxidase, we could determine that acetopyruvate accumulated similarly after D-amino acid oxidase action in Hepes and that butylamine addition produced the 317-nm species in this instance also. We could also confirm that this enzyme, in contrast to L-amino acid oxidase, does suffer time-dependent irreversible inactivation. Inactivation can be monitored by the O_2 uptake assay in the oxygen electrode, by direct observation of acetopyruvate formation (at 300 nm) or of the presumed butylamine–acetopyruvate adduct (317 nm), or in preincubations with aliquots diluted into an assay solution of D-alanine. As shown in Figure 4, the number of turnovers before D-amino acid oxidase is inactivated varies with conditions in the experiment. The number of turnovers is dependent on the concentration of propargylglycine, indicating the true inactivating species is competing with the amino acid. At low concentrations of propargylglycine, a substantial protection of enzyme activity is provided by 100 mM butylamine-HCl in Hepes buffer. If a second addition of enzyme is made to this solution, a pronounced lag in product formation occurs and fewer turnovers occur before activity is lost. These two experiments suggest at least a fraction of the inactivator is free in solution. Acetopyruvate or preformed acetopyruvate–butylamine did not inactivate the enzyme, nor did they significantly inhibit the enzyme at low concentration.

In contrast to the findings of Horiike et al. (1975), we found it possible to inactivate the enzyme approximately 98%, both in preincubations and by monitoring the rate of increase of 317-nm absorbance when the inactivation is carried out in the

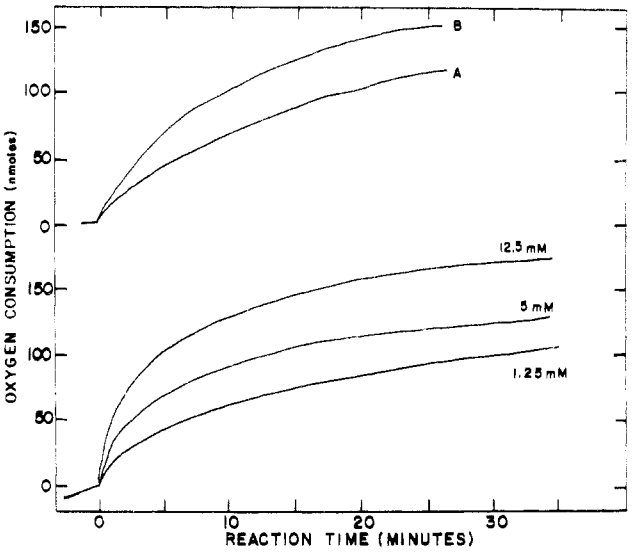


FIGURE 4: Effect of reaction conditions on inactivation rate of D-amino acid oxidase by D-propargylglycine. Incubations were performed in an 0.4 ml of oxygen electrode cell. Upper: Reaction of 2.5 mM D-propargylglycine with 30 μg of oxidase. (A) In 100 mM Hepes, pH 8. (B) In 100 mM Hepes/100 mM butylamine, pH 8. Lower: Reaction of 30 μg of oxidase and different concentrations of D-propargylglycine in 100 mM Tris-HCl, pH 8.

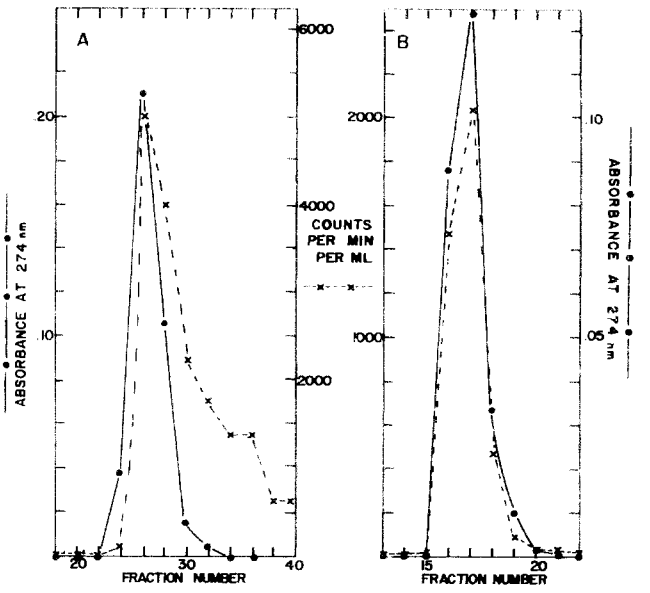


FIGURE 5: Elution profile of Sephadex G-25 column after inactivation of D-amino acid oxidase with D,L-propargyl[2-^{14}C]glycine (described in Experimental Section). (A) Inactive enzyme (in 0.5 ml) dialyzed against 2 × 500 ml of low salt buffer. 0.13 mg/ml protein assayed (Lowry) in fraction 24. (B) Inactive enzyme (in 0.5 ml) dialyzed against 2 × 500 ml of buffer containing 1 M KBr, then against 500 ml of low salt buffer. Protein (0.089 mg/ml) was assayed (Lowry) in fraction 17.

presence of butylamine. The lack of total inactivation could result from a slight lability of enzyme–inactivator covalent linkage; however, both we and the previous workers found that little activity could be restored by extensive dialysis. When the rate of inactivation vs. time is plotted, the data showed marked deviation from a simple first-order process, also noted by Horiike et al. (1975), which is consistent with some reversible inhibition accompanying the irreversible inactivation.

We next attempted to determine the stoichiometry of covalent attachment to distinguish whether multiple nonspecific alkylations occur (probably from a product attacking from solution) or whether only a small number of inactivating molecules per active site are incorporated. In our initial experiments, the enzyme was inactivated by D,L-propargyl[^{14}C]glycine, dialyzed against two changes of low-salt buffer, and then passed through a Sephadex G-25 column. As shown in Figure 5A, the radioactivity was not entirely coincident with the protein, but rather was also found in the fractions immediately following the protein. This suggests that the radioactive species is dissociating (at least in part) from the enzyme as it passes through the column. Given a subunit molecular weight of 37 000, and assuming all the radioactivity had been associated with the protein after the exhaustive dialysis, it can be calculated that there were three labels per active site in the peak tube.

Because of the inconclusive nature of these data, we then repeated the experiment—this time dialyzing the inactivated enzyme against two changes of buffer containing 1 M KBr, then against low-salt buffer. When this enzyme was passed through the Sephadex column, the radioactivity was coincident with the protein (Figure 5B) and gave a label to active site ratio of 1.7:1.

We believe this data indicates that the enzyme retains the capacity to bind substrate or product after modification, and possibly to carry out the oxidation at a much reduced rate. The capability to bind substrate or product is lost when apoenzyme is made—either by dialysis vs. high salt or passage through the Sephadex column. These results per se do not distinguish between active site directed alkylation and some postcatalytic event, but it does indicate that modification of only a small number of amino acids per subunit substantially destroys catalytic activity.

Discussion

In the short interval since the synthesis of D,L-vinylglycine was reported (Rando, 1974; Friis et al., 1974), this β,γ-olefinic α-amino acid has been tested as a substrate for a variety of pyridoxal-P dependent enzymes. Rando has reported that pig heart L-aspartate aminotransferase is irreversibly inactivated (Rando, 1974); presumably vinylglycine is transaminated to the conjugated unsaturated imino acid linked to the pyridoxamine-P form of the coenzyme. This electrophile is then attacked by a nucleophilic active site amino acid. We have recently observed the inactivation of a second transaminase by vinylglycine, namely the D-amino acid aminotransferase of *B. subtilis* (Martinez-Carrion and Jenkins, 1965), and will detail this elsewhere.[4] Three other pyridoxal-P enzymes have been reported to abstract the α-H of vinylglycine after which a 1,3-prototropic shift apparently occurs leading to the formation of the bound eneamine of α-ketobutyrate. The net result is catalytic turnover of vinylglycine to α-ketobutyrate and ammonium ion without detectable inactivation for sheep liver threonine deaminase (Kapke and Davis, 1975), *E. coli* tryptophan synthetase (Miles, 1975), rat liver γ-cystathionase (Washtien, W., and Walsh, C., unpublished observations), and *S. typhimurium* cystathionine-γ-synthetase (Donovan, J., Marcotte, P., and Walsh, C., unpublished observations).

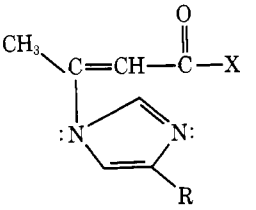
We have now shown that the two flavin-dependent enzymes hog kidney D-amino acid oxidase and snake venom L-amino acid oxidase both oxidize the appropriate enantiomer of vinylglycine. Yet only the L-amino acid oxidase is inactivated, probably by covalent reaction with the reactive product 2-keto-3-butenoate (or the imino acid). The molecular basis for specificity among the two oxidases is unclear. There is kinetic evidence with each enzyme (Massey and Curti, 1967; Massey

and Gibson, 1964) that product release is a slow step in catalysis so that the product may be retained at the active site long enough for nucleophilic attack in each case. Perhaps only the L-amino acid oxidase has an appropriately placed amino acid nucleophile. There is no detectable allylic isomerization of vinylglycine by the flavoprotein oxidases under anaerobic conditions.

The lack of protection of L-amino acid oxidase activity by added nucleophiles suggest that the inactivator is inaccessible for scavenging and implicates loss of activity as an active-site directed process. While the stoichiometry of covalent labeling remains to be determined, it is likely that only one residue per active site will be modified. It is unlikely that the isoalloxazine nucleus of the FAD coenzyme at the active site has been modified, since the chromophore remains intact on inactivation. The L-amino acid oxidase molecules catalyze approximately 2000 turnovers for every inactivating event, suggesting a low frequency of capture by an active site nucleophile. The L-amino acid oxidase inactivation can be compared with our previous studies on the FMN-dependent L-hydroxy acid oxidase from rat kidney using vinylglycolate (2-hydroxy-3-butenoate) as substrate to generate the same 2-keto-3-butenoate as a putative inactivator (Cromartie and Walsh, 1975). In that instance the keto acid is clearly released into solution and then subsequently alkylates the protein randomly, some 30–40 times before activity is lost. Scavenging nucleophiles completely protect the rat kidney enzyme, proving the ketobutenoate can be intercepted in solution and serving as control to the L-amino acid oxidase results noted above. The bound FMN of the rat kidney L-hydroxy acid oxidase is similarly unaltered on inactivation.

The reaction of the two flavoprotein amino acid oxidases with propargylglycine appeared considerably more complex at the outset. It was not clear how the actual inactivating agent was generated or what its structure might be. In our previous work with propargylglycine as a suicide substrate for rat liver γ-cystathionase, *S. typhimurium* cystathionine-γ-synthetase, and pig heart L-alanine aminotransferase, we had suggested that inactivation required the enzyme-catalyzed activation of substrate to a C_3 carbanionic intermediate that could undergo propargylic rearrangement to an electrophilic conjugated allenic ketone functionality as the actual inactivator (Abeles and Walsh, 1973; Marcotte and Walsh, 1975). No evidence exists that D-amino acid oxidase ever generates substrate β-carbanions. Horiike et al. (1975) speculated that some undefined α,β-elimination process could be responsible but there is no leaving group at the β carbon of propargylglycine. We have also checked that anaerobic turnover, a hallmark of the α,β-elimination pathway with this enzyme (Walsh et al., 1971; Walsh et al., 1973), does not occur with propargylglycine.

Thus, it seemed that some process for chemical activation of the C_3-methylene protons should be sought. Straightforward enzymatic oxidation to 2-imino-4-pentynoate (**1**, Scheme I) by the amino acid oxidases renders those hydrogens acidic, since a C_3 carbanion could now be resonance stabilized both by the C_2 carbonyl and the 4,5-acetylene group. If this anion is protonated at C_5, the resultant 2-imino-3,4-pentadienoate is formed (**2**, Scheme I). This electrophile could suffer the various fates of Scheme I; water as nucleophile yields the observed product from turnover, acetopyruvate (**3**). Attack by some enzyme nucleophile would account for the inactivation. Thirdly, addition of amine nucleophiles could lead to the 317-nm product that we suggest is the eneamine (**4** of Scheme I) shown also in Scheme I. Initial amine addition at C_4 of the allenic imino acid (or keto acid) would occur in a Michael sense to produce the nonconjugated eneamine. Subsequent isomerization to the conjugated eneamine **4** of Scheme I should be facile. This has a likely chromophore for λ_{max} 317 nm ($\epsilon \simeq$ 15 000). This hypothesis is bolstered by model studies of Morasaki and Bloch (1972) on the addition of histidine methyl ester to 2,3-butadienyl esters. Two products were isolable; one was characterized as the expected Michael adduct. The other product had λ_{max} 319 nm (ϵ = 25 000); it was ascribed the following structure, forming by prototropic shift into conjugation.



The imidazolyl–eneamine adducts were acid stable (Morasaki and Bloch, 1972), but the aliphatic eneamine we postulate is not, reverting to butylamine and acetopyruvate on treatment with Dowex 50 H^+.

Horiike et al. (1975) reported that propargylglycine induced inactivation of D-amino acid oxidase does not affect the FAD chromophore but the separated inactive apoenzyme has a new absorbance peak at 317 nm. It is quite possible that the amino acid nucleophile on the enzyme is an ϵ-NH_2 group of a lysine residue and the covalent linkage formed on inactivation is an essentially identical conjugated eneamine. Using an ϵ = 15 000 and an A_{278}/A_{317} ratio of 2.63 (from our repetition of the previous work), the number of probable labels can be calculated. This calculation depends on the value used for the extinction coefficient of apo D-amino acid oxidase. We have used the value of 1.54 for a 1 mg/ml solution of apoenzyme reported by Antonini et al. (1966) that leads to a calculated value of 1.46 labels/active site. If the enzyme nucleophile were an imidazole group of a histidine residue (ϵ = 25 000), a calculated stoichiometry of such a chromophore would be 0.87 mol/active site. These calculations are consistent with our propargyl[2-^{14}C]glycine labeling experiments that suggest a similar number of labels per enzyme active site.

Even with such encouragingly low numbers of covalent labels per active site, it is not clear when the inactivation occurs: at the active site or after release into solution or some combination. Scavenging nucleophiles can protect partially but not completely against inactivation during incubations of enzyme with propargylglycine, suggesting the third alternative. While acetopyruvate or the butylamine–acetopyruvate adduct also accumulate in solutions of L-propargylglycine and L-amino acid oxidase, in that instance no irreversible inactivation is detected. The bases for this exclusivity are unclear. It may be that D-amino acid oxidase but not L-amino acid oxidase actually catalyzes the allene-forming prototropic shift before release of product **1** (Scheme I). Or a similar adduct may form at the L-amino acid oxidase active site but the eneamine formed may be in a more labile microenvironment. It would be difficult to argue that L-amino acid oxidase does not have a reactive nucleophile at its active site in view of its inactivation by vinylglycine. Presumably, any enzyme catalytically oxidizing propargylglycine at C_2 to the acetylenic keto acid product is potentially in danger of alkylation from a rearranged electrophile.

Given the irreversible inactivation of D-amino acid oxidase during oxidation of propargylglycine, one might make comparisons with another acetylenic suicide substrate for fla-

voenzymes, namely 2-hydroxy-3-butynoate. Hydroxybutynoate inactivates all flavin-dependent hydroxy acid oxidizing enzymes examined to date, always covalently modifying the bound flavin coenzyme (Cromartie and Walsh, 1975; Schonbrunn et al., 1976; Ghisla et al., 1976). In contrast, the propargylglycine oxidation product does not react with the FAD of D-amino acid oxidase.[6] The molecular reasons for this dichotomy between the modes of inactivation of two alkynoic substrates is unclear at present but could relate to the ability of an allenic anion on the one hand to act as nucleophile and a protonated allene on the other hand to act as electrophile.

In processing vinylglycine and propargylglycine as substrates the two flavoenzymes show a reciprocal dichotomy of inactivation susceptibility. L-Amino acid oxidase is uniquely reactive in attacking the olefinic keto acid derived from vinylglycine, presumably at carbon 4. D-Amino acid oxidase shows unique reactivity for detectable combination with the putative 2-keto-3,4-pentadienoate, again most likely at carbon 4. The active site geometries, the positioning of enzymic nucleophiles, and electronic differences in addition to α,β-unsaturated carbonyls vs. conjugated allenic carbonyls may combine to provide marked rate distinctions for attack on these electrophiles. The availability of these complementary suicide substrates should aid elucidation of how these flavoenzymes carry out oxidative catalysis, their similarities and differences, as well as offering such possibilities as selection techniques for (or against) certain types of animal cells in culture (Gilbert and Migeon, 1975).

[6] V. Massey, personal communication, has informed us that 2-hydroxy-3-butynoate slowly inactivates D-amino acid oxidase *with* modification of the bound FAD inferred from drastic perturbation of the isoalloxazine chromophore.

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