Inactivation of Bacterial D-Amino Acid Transaminases by the Olefinic Amino Acid D-Vinylglycine

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D-Vinylglycine (2-amino-3-buten-2-olate) functions as a transamination substrate and irreversible inactivator of the homogeneous pyridoxal phosphate-dependent D-amino acid transaminases from Bacillus subtilis and Bacillus sphaericus. In the absence of α-ketoglutarate as co-substrate, vinylglycine causes little if any inactivation of either enzyme; in the presence of excess α-ketoglutarate, both enzymes are inactivated with pseudo-first order kinetics. The limiting rate constant for inactivation of the B. sphaericus enzyme is 1.9 min⁻¹, for the B. subtilis enzyme it is 0.36 min⁻¹. The number of catalytic events before inactivation is about 450 for the B. sphaericus enzyme and about 800 for the B. subtilis enzyme; that is, about 0.2% inactivation in each catalytic cycle for the former enzyme and 0.15% for the latter. Comparisons are made with the L-aspartate aminotransferase from pig heart which is inactivated completely in one catalytic cycle and the L-alanine aminotransferase which is not inactivated in many cycles. Comparisons are also made between the likely mode of D-transaminase inactivation produced by vinylglycine and the mode of inactivation induced by β-chloro-D-alanine.

D-isomers of amino acids are important constituents of bacterial metabolism. The D-enantiomers of alanine and glutamate, for example, are found in the peptidoglycan layer of bacterial cell walls, and a variety of D-amino acids are components of peptide antibiotics. One key enzyme is the bacterial alanine racemase, which functions physiologically to provide D-alanine for cell wall biosynthesis. This enzyme is a target of novel D-amino acid transaminases. Comparisons are also made with the L-aspartate aminotransferase from pig heart which is not inactivated in many cycles. Comparisons are also made between the likely mode of D-transaminase inactivation produced by vinylglycine and the mode of inactivation induced by β-chloro-D-alanine.

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E. Wang and C. Walsh, unpublished results.
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recorded to measure the enzyme activity remaining; for controls the transaminase was incubated in the absence of vinylglycine for the same time period.

When the effect of pyruvate with vinylglycine was studied, the enzyme activity remaining was determined with n-aspartic acid as the amino acid substrate. When other α-keto acids were tested as promoters of the inactivation, both n-alanine and α-ketoglutarate were added to stop the inactivation. Correction was made for the activity or competitive inhibition (or both) by the α-keto acid on either the transaminase or lactic dehydrogenase.

Homogeneous enzyme from Bacillus sphaericus was inactivated by incubation of the enzyme at 25°C with various concentrations of n-vinylglycine in the presence of 10 mM α-ketoglutarate in a total of 1 ml of 50 mM potassium pyrophosphate buffer, pH 8.5. The incubation was quenched with 25 μl of 1 M n-alanine and the rate determined with the NADH/lactic dehydrogenase-coupled assay.

**Determination of Transamination of Vinylglycine and α-Keto[14C]Glutarate by D-Amino Acid Transaminase and Correlation with Inactivation of Enzyme—** When the d-amino acid transaminase was assayed with vinylglycine and α-ketoglutarate, but without n-alanine, a slow but measurable decrease in the absorbance at 338 nm was observed, suggestive of formation of a keto acid. To study this apparent turnover of the enzyme with vinylglycine, we measured the formation of n-[14C]glutamate from α-keto[14C]glutarate. [14C]Glutamate produced by the B. subtilis enzyme was separated from α-keto[14C]glutarate on an amino acid analyzer of the design of Spackman et al. (9), equipped with a flow cell scintillation counter (Nuclear Chicago). The protocol was designed to measure both the production of glutamate from vinylglycine and α-ketoglutarate and the percentage of active enzyme remaining as a function of time. A reaction mixture was prepared containing 0.33 mM potassium phosphate, pH 6.5, 1.0 mM vinylglycine, 2 mM α-ketoglutarate (6000 dpm/mmol) in a total volume of 2 ml at 22°C. At time zero, 30 μg of d-amino acid transaminase were added. At each interval two 0.1-ml aliquots were removed. One aliquot was added to citrate buffer, pH 2.2, and a portion of this sample was applied to the amino acid analyzer. At this pH the d-amino acid transaminase is rapidly inactivated; this aliquot measures the production of glutamate in the reaction mixture from vinylglycine and α-ketoglutarate. To the second 0.1-ml aliquot 3 μl of n-alanine (0.75 mM) were added and incubated at 22°C for 5 min. The increase in [14C]glutamate was determined as indicated above. The difference in the amounts of [14C]glutamate in the two aliquots represents the activity remaining in the reaction mixture.

The number of catalytic events before inactivation of the B. sphaericus enzyme was determined by incubation of the enzyme in 1 ml of 0.1 M glycine and 1 mM α-ketoglutarate (1000 dpm/mmol) in 50 mM potassium pyrophosphate buffer, pH 8.5, at 25°C. The 100-μl aliquots were withdrawn at intervals and injected onto 1-ml Dowex 50-H+ (400 mesh) columns. Each column was washed with water, then eluted with 4 N ammonia to determine the [14C]glutamate attached to the resin.

**RESULTS**

Inactivation of Bacillus subtilis and Bacillus sphaericus D-Amino Acid Transaminases by Vinylglycine—** The 4-carbon β,γ-unsaturated α-amino acid vinylglycine at 1 mM concentration causes only slight loss in the activity of the d-amino acid transaminases from B. subtilis or from B. sphaericus in the absence of α-keto acids (Fig. 1, left). However, initial experiments with the B. subtilis enzyme showed that addition of either α-ketoglutarate or pyruvate (Fig. 1, left) to solutions of enzyme and the same 1 mM concentration of vinylglycine caused a rapid loss of transaminase activity. Inactivation shows pseudo-first order kinetics and a half-time of about 5 min under the stated experimental conditions. Control experiments established that vinylglycine did not affect the activity of the NADH-linked dehydrogenases serving as coupling enzymes in the assay. Preliminary conformation that the time-dependent loss of B. subtilis transaminase activity represents irreversible inactivation is provided by the observation that prolonged dialysis of the enzyme does not restore activity. Other α-keto acids can also function as potentiators of vinylglycine-induced d-amino acid transaminase inactivation whereas structurally similar carboxylates and dicarboxylates cannot (Table I). In addition, pyridoxal 5'-phosphate cannot substitute for α-keto acids in potentiating the inactivation by vinylglycine.

Homogeneous d-amino acid transaminase from B. sphaericus was similarly inactivated when both vinylglycine and α-ketoglutarate were present. Fig. 2 (left) shows the pseudo-first order rate of inactivation at various concentrations of vinylglycine. Comparison of the results of Figs. 1 and 2 for the inactivation of the enzyme from B. subtilis and B. sphaericus by vinylglycine shows that the enzyme from the latter source is inactivated considerably faster than the enzyme from the former microorganism. The kinetic behavior shown in Fig. 2 (left) is consistent with inactivation proceeding only from a preformed E-I complex. The slope of each line in Fig. 2 (left) yields a first order rate constant; a double reciprocal replot of

\[
E + I \xrightleftharpoons[k_{-1}]{k_1} EI \xrightarrow{k_2} E - I
\]

these slopes in Fig. 2 (right) yields a Kᵢ of 2 mM for vinylglycine and a limiting rate constant for inactivation of k₁.
MINUTES

Inactivation of D-Amino Acid Transaminases by Vinylglycine

I/[VG] l.M-'

FIG. 2. Left, Bacillus sphaericus D-amino acid transaminase activity remaining after incubation with various concentrations of DL-vinylglycine and 10 mM α-ketoglutarate as described under "Experimental Procedures." Right, plot of reciprocal of rate constant for inactivation from left versus reciprocal of DL-vinylglycine (VG) concentration.

- 1.9 min⁻¹ (vertical intercept). Similar experiments with the transaminase from B. subtilis (Fig. 1, right) gave a Kᵦ of about 2 mM for DL-vinylglycine and a limiting rate constant for inactivation of 0.36 min⁻¹ (about 5-fold slower than the B. sphaericus enzyme). In a series of experiments with saturating vinylglycine and varied concentrations of α-ketoglutarate, the Kᵦ for the keto acid was found to be 1.1 mM. These values compare well with a reported Kᵦ value of 3.2 mM for D-alanine and 1.2 mM for α-ketoglutarate with D-alanine as co-substrate.

The pH dependence of the inactivation of the B. subtilis enzyme by vinylglycine (in the presence of α-keto acid) parallels the pH dependence of the normal catalytic transamination (10). D-Alanine at a concentration of 10 mM (a 10-fold excess over vinylglycine) affords a 70% protection against inactivation consistent with the binding of both compounds at the same active site. L-Alanine at 10 mM does not retard the rate of inactivation although substantial protection (65%) is achievable at 80 mM L-alanine, consistent with a very weak binding (Kᵦ = ~300 mM) of this enantiomer of alanine to the D-amino acid transaminase from B. subtilis. These observations are consistent with inactivation proceeding via a mechanism analogous, at least in part, to the transamination pathway.

Analysis of Vinylglycine Turnover with D-Amino Acid Transaminases—If vinylglycine is indeed partitioning between normal transamination to 2-keto-3-butenoate and enzyme inactivation, product formation should be detectable. In the absence of a specific assay for the reactive β,γ-olefinic α-amino acid, we felt that turnover should be observable by measuring concomitant conversion of α-keto[¹⁴C]glutamate into D-[¹⁴C]glutamate as described under "Experimental Procedures." This expectation was realized for the transaminase from B. subtilis (Fig. 3) as well as that from B. sphaericus (Fig. 4).

For the enzyme from the former source, Fig. 3 shows the relationship between [¹⁴C]glutamate production and remaining enzyme activity. With time both glutamate formation and enzyme activity fall off in parallel as transaminase molecules are inactivated. After 10 min, at 50% inactivation, about 20 nmol of [¹⁴C]glutamate had been generated resulting in the inactivation of 0.025 nmol of transaminase.

The data of Fig. 4 illustrate analogous behavior with B. sphaericus D-amino acid transaminase, where the formation of [¹⁴C]glutamate was measured as a function of time at three concentrations of homogeneous enzyme. The amount of [¹⁴C]glutamate at 20 min was 58 nmol for 6.7 µg of enzyme, 100 nmol for 13.3 µg, and 128 nmol for 20 µg. This is reasonable proportionality with enzyme concentration and computes to about 450 turnovers per inactivation event with the transaminase from B. sphaericus. We believe that at high concentrations of enzyme some of the enzyme is inactivated by a 2-keto-3-butenoate molecule produced by another enzyme molecule.

The data of Fig. 4 further indicate that there is a slow but real increase in the amount of [¹⁴C]glutamate formed at 10, 15,
and 20 min. This residual rate corresponds to about 1% of the estimated initial rate, i.e., the enzyme is 99% inactivated. This rate may represent the intrinsic activity of a population of alkylated transaminase molecules, i.e., the modified enzyme still catalyzes the reaction at $10^{-2}$ the rate of native enzyme.

**Effect of Thiol Nucleophile on Inactivation of D-Amino Acid Transaminase by Vinylglycine**—It is possible that the inactivation of the enzymes could occur by attack on enzyme nucleophiles not at the active site by the 2-keto-3-butenolate released into solution. To test this possibility, incubations of *B. subtilis* enzyme, L-vinylglycine, and α-ketoglutarate were performed in 5 mM dithiothreitol, which has been found to protect L-hydroxycarboxylic oxidase from inactivation by L-vinylglycolate (2-hydroxy-3-butenolate) (11).

Although this concentration of dithiothreitol for a 10-min incubation had no effect on enzyme stability or the rate of transamination of D-alanine and α-ketoglutarate, the rate of inactivation increased considerably. In experiments similar to those of Fig. 4, 25% fewer turnovers were observed from incubations containing dithiothreitol than those of controls. Overnight dialysis at 0°C against 1 mM dithiothreitol did not have a similar effect. The possibility that high concentrations of thiol cause a different enzyme nucleophile to be attacked will be examined during labeling studies.

**Effects of Vinylglycine on L-Aspartate Aminotransferase and L-Alanine Aminotransferase**—The first report of an enzymatic inactivation by vinylglycine was Rando's recent report of its effect on the L-aspartate aminotransferase from pig heart (6). We have been able to confirm that inactivation: 43% activity loss in a 15-min preincubation at 25°C with 25 mM vinylglycine; the loss of activity in 15 min at 1 mM vinylglycine was essentially negligible. But, in marked contrast to the bacterial L-amino acid transaminases reported here, there is no stimulation by α-keto acids on the rate of inactivation by vinylglycine either at pH 7.5 or at pH 8.5. This lack of stimulation by keto acids suggest that vinylglycine inactivates pig heart L-aspartate aminotransferase without significant turnover. Direct testing of this idea with dl-vinylglycine, α-keto[14C]glutarate, and 2 mg of commercial L-aspartate aminotransferase for 30 min revealed no more than 400 cpm of [14C]glutamate over a 200 to 250 cpm background, corresponding to at most 0.1 to 0.2 nmol of vinylglycine oxidized/nmol of enzyme.

We have previously reported that the other major transaminase in pig heart muscle, L-alanine aminotransferase, is not detectably inactivated by dl-vinylglycine (7). We have confirmed this insensitivity even in the presence of excess α-keto acids. One question that arises is whether L-alanine aminotransferase is inert because it cannot oxidize vinylglycine to the putative inactivator, 2-imino-3-butenolate. When vinylglycine turnover was monitored by experiments analogous to those of Fig. 4, formation of [14C]glutamate from α-keto[14C]glutarate was observed, showing that vinylglycine is transaminated at approximately 1% the rate of L-alanine.

**DISCUSSION**

A number of different possible pathways for the reaction of vinylglycine with the α-amino acid transaminases are delineated in Scheme 1. Two of these mechanisms lead to enzyme inactivation by covalent alkylation (pathways 2 and 4) while the other two (pathways 1 and 3) lead to catalytic turnover and α-keto acid production: 2-keto-3-butenolate by pathway 1 and 2-ketobutyrate by pathway 3. It is also clear from Scheme 1 that these four routes are grouped pairwise from two different product complexes. If the ES complex is converted by route A to the normal transaminase imine complex, then the conjugated imine can either be released or undergo conjugate addition by an enzyme nucleophile at carbon 4. On the other hand, if the ES complex, after abstraction of the α-H as a proton to form the stabilized α-carbanion, can undergo a 1,3-proton transfer, the product in route B will be an enzyme-bound enamine. Hydrolysis of the enamine, indicated in pathway 3, would yield the 2-ketobutyrate and pyridoxal-P form of the transaminase. This is in contrast to the keto acid production in pathway 1 where the enzyme would be left in the two-electron reduced pyridoxamine P-form. If the product-enamine complex is captured instead by an enzyme nucleophile, inactivation would again ensue. But pathway 4 is distinct from pathway 2 in that pathway 4 would predict covalent bond formation at carbon 3 of the inactivator. The situation is further complicated by the possibility that the bound enamine and bound imine might well be in equilibrium, as is shown in Scheme 1.

Mechanism B3 has been proposed to explain the production of 2-ketobutyrate from L-vinylglycine by the following pyridoxal-P-dependent enzymes: sheep liver threonine deaminase (12), *Escherichia coli* tryptophan synthetase (13), rat liver γ-cystathionase, and *Salmonella typhimurium* cystathionine γ-synthetase. However, we do not see any catalytic production of keto acid in the absence of added α-ketoglutarate, the required co-substrate for transamination. This result rules out possibility B3 as a significant path to product formation.

Vinylglycine is a transaminase substrate when α-ketoglutarate is present as measured by the conversion of radioactive α-ketoglutarate to radioactive α-glutamate. This result suggests pathway A1 is responsible for vinylglycine turnover and implies that the product is 2-keto-3-butenolate. If path A1 is responsible for catalytic turnover, then we believe it more likely that inactivation occurs by way of pathway A2 rather than B4. However, despite the fact that we have no evidence that the α-specific transaminases can isomerize vinylglycine to an enamine product, it is possible that branch B could occur occasionally, and could be responsible for inactivation. This issue must be left in doubt until the identification of the enzyme-inactivator linkage is elucidated, indicating which carbon is attached to the enzyme residue.

The production of 2-keto-3-butenolate during catalytic turn-
over leaves the enzyme molecules in the pyridoxamine-P form, unable to undergo further reaction with another molecule of vinylglycine, or any other α-amino acid, in the absence of added keto acid. These enzyme molecules are thus protected from inactivation that would proceed during many catalytic cycles, thus the insensitivity of the d-transaminase to vinylglycine in the absence of keto acid. In the presence of α-ketoglutarate, the pyridoxamine-P enzyme molecules are converted back to pyridoxal-P forms, again competent to react with vinylglycine. Further inactivation ensues, at the rate of 0.22% inactivation in any given catalytic cycle for Bacillus sphaericus enzyme or 0.15% inactivation per catalytic event with the Bacillus subtilis enzyme.

These low partitioning ratios between inactivation and normal catalytic transamination contrast with the susceptibility of pig heart L-aspartate aminotransferase to L-vinylglycine. Rando did not report whether detectable turnover accompanied inactivation of that enzyme (6), but we have now tested this point and found none. The L-aspartate transaminase must be nearly 100% inactivated in any cycle where it acts upon vinylglycine. This idea is in accord with the ability of vinylglycine to inactivate the transaminase in the absence of added α-keto acid. Any degree of normal transamination would have left that percentage of enzyme in the “protected” pyridoxamine-P form of the enzyme and refractory to complete inactivation until keto acid is added to regenerate the pyridoxal-P form. No such protection is evident in Rando’s experiments or ours with this enzyme. The basis for the different partitioning ratios are obscure. It could be a kinetic difference between rates of hydrolysis of bound imine product versus rates of nucleophilic attack by the susceptible residues of the two transaminases. Similarly unclear at the molecular level is why L-alanine transaminase utilizes vinylglycine as a transamination substrate but undergoes no detectable inactivation.5

Finally, the contrast between the behavior of d-vinylglycine and α-chloro-α-alanine with the bacterial α-specific aminotransferases should be noted. We have noted that α-chloroalanine undergoes HCl elimination but not normal transamination.3 The α-keto acid-independent HCl elimination pathway is reminiscent of putative pathway B3 of Scheme 1 whereas transamination would have been through A1. Thus the transaminases apparently will generate the product enamine with the three carbon halo substrate and not the four carbon olefinic substrate. (Chloroalanine inactivation than is most economically envisaged as proceeding by a path analogous to B4 rather than A2, since no chloropyruvate is detectable.) The routing of the enzyme essentially exclusively through path A or B of Scheme 1 then could depend on the difference in electronegativity of the chloro and vinyl β substituents in chloroalanine and vinylglycine. Or, it may reflect the inability of the enzyme to supply a proton at carbon 4 of vinylglycine necessary to capture a rearranging allylic carbanion (Scheme 2). If a protonated base is not available to provide general acid catalysis, there may be no driving force for the allylic rearrangement to the enamine (2 of Scheme 2). Instead, the α-carbanion (1 of Scheme 2) may simply undergo the usual 1,3-azallylic prototropic isomerization that is normal transamination pathway (3 of Scheme 2). In contrast, the chloride ion is a leaving group in itself and can be eliminated from β-chloroalanine without need for protonation from a BH+ group. Following this line of reasoning, one could see how inactivation from β-chloroalanine processing could be due to alklylation of an enamine intermediate while inactivation during vinylglycine processing could be from Michael attack on the bound imine product.

REFERENCES


In this connection, we have noted that propargyl glycine (2-amino-4-pentynoate) inactivates the L-alanine aminotransferase but not the L-aspartate aminotransferase. In preliminary experiments, we have found that α-propargyl glycine irreversibly inactivates the Bacillus subtilis transaminase, suggesting it may show susceptibilities intermediate to the two mammalian enzymes.
Inactivation of bacterial D-amino acid transaminases by the olefinic amino acid D-vinylglycine.
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