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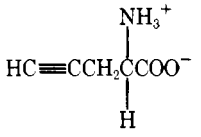
Sequence of Reactions Which Follows Enzymatic Oxidation of Propargylglycine†

Patrick Marcotte‡ and Christopher Walsh*,§

ABSTRACT: The nonenzymatic reactions which follow enzymatic oxidation of the γ-δ acetylenic amino acid propargylglycine (2-amino-4-pentynoate) have been studied. The product which accumulates in solution has been identified as 2-amino-4-hydroxy-2,4-pentadienoate γ-lactone, formed by intramolecular attack of the carboxylate anion on the electrophilic fourth carbon of 2-iminium-3,4-pentadienoate. This previously unknown substance was characterized by its reactions in acid and base and by its nuclear magnetic resonance spectrum. The lactone is preceded in the pathway by 2-amino-2-penten-4-ynoate, a transient electron-rich species which binds tightly to D-amino-acid oxidase and induces a charge-transfer complex with the electron-deficient bound flavin coenzyme. The aminediene lactone is converted by base treatment to 2-amino-4-keto-2-pentenoate, which is also a strong inhibitor of D-amino-acid oxidase and induces a charge-transfer complex.

We have recently described the properties of D-amino-acid oxidase covalently modified upon its oxidation of the acetylenic amino acid D-propargylglycine (**1**) (Marcotte and Walsh, 1978a). Since the enzyme has been demonstrated to carry out a large number of catalytic oxidations before suffering alkylation (Marcotte and Walsh, 1976), the identification of the species produced in such incubations was undertaken. Study of the interaction of D-amino-acid oxidase with D-propargylglycine has been found to be complicated by the presence of two noncovalent inhibitors, which are formed following enzymatic oxidation. Evidence as to the probable structures of these inhibitors is presented in this work, as well as the identification of the oxidation product which accumulates in solution.



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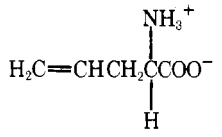

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In the following paper, the sequence of reactions following enzymatic oxidation of allylglycine (**2**) is described, studies providing a comparison of the reactivities of the olefinic and acetylenic functionalities. Two species produced in that pathway also act as strong noncovalent inhibitors of D-amino-acid oxidase and have been characterized. Although similar in some aspects to the propargylglycine pathway, the substitution of the olefin function (in allylglycine) for the acetylene induces several striking differences in the various reactions of the enzymatic oxidation products.

Experimental Section

Materials

Enzymes. D-Amino-acid oxidase from frozen hog kidneys (purchased from Pel-Freez biologicals) was initially purified as described by Brumby and Massey (1968) and then passed through DEAE[1]-Sephadex as described by Curti et al. (1973). Final purification to homogeneity was effected by chromatography on Sephadex G-100 (Pharmacia). D-Amino-acid transaminase from *Bacillus sphaericus* was purified by the procedure of Soda et al. (1974). L-Amino-acid oxidase (*Crotalus adamanteus* venom) and catalase (beef liver) were purchased from Sigma.

Reagents. DL-4-Ketonorvaline was synthesized as reported by Wiss and Fuchs (1952). Propargylglycine (2-amino-4-pentynoic acid) was synthesized and resolved by the method of Jansen et al. (1969). Hepes,[1] phenylpyruvic acid, and acetopyruvic acid were purchased from Sigma. Buffer salts and solvents were commercially available reagent-grade materials.

Chemical Synthesis of 2-Amino-4-keto-2-pentenoate. The ammonium salt of ethyl acetopyruvate was prepared by the addition of 2.5 mL of 15 N NH_4OH to 3.5 g of ethyl acetopyruvate (Marvel and Dreger, 1941) dissolved in 100 mL of tetrahydrofuran. After 30 min of stirring, the precipitate was

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[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; NMR, nuclear magnetic resonance; DEAE, diethylaminoethyl.

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filtered and washed with tetrahydrofuran and diethyl ether and then dried under the vacuum of an oil pump. The salt (3 g) was slurried in 20 mL of $CHCl_3$. Upon heating to reflux for 8 min, the salt dissolved, yielding a brown solution, which was dried with anhydrous Na_2SO_4 and evaporated to an oil. The crude product was layered onto a 100-mL silica gel 60 column, which was developed with $CHCl_3$. Fractions which exhibited a λ_{max} of 325 nm (which follow fractions containing much greater A_{300} due to ethyl acetopyruvate) were combined and evaporated. The product was rechromatographed in the same way on a 20-mL silica gel column, after which the purified product was crystallized and recrystallized from hexane: yield 75 mg; mp 38 °C; UV λ_{max} 325 nm, ϵ 13 000 (in ethanol); NMR (in $CDCl_3$) δ 1.4 (3 H, t), 2.2 (3 H, s), 4.3 (2 H, q), 5.9 (1 H, s); (the $-NH_2$ protons were not observed at room temperature). Anal. Calcd for $C_7H_{11}NO_3$: C, 53.49; H, 7.05; N, 8.91; O, 30.54. Found: C, 53.58; H, 7.00; N, 8.91; O, 30.79. A solution of the sodium salt of the enamine was prepared by dissolving the ethyl ester in a twofold excess of 1 mM NaOH. After neutralization to pH 8.0, the solution exhibited λ_{max} 321 nm, ϵ 13 000.

Methods

Characterization of the Accumulated Product Following Oxidation of Propargylglycine and 4-Ketonorvaline. At room temperature, 50 nmol of L-propargylglycine or 100 nmol of DL-4-ketonorvaline was mixed with 25 μg of catalase and 50 μg of L-amino-acid oxidase in 1.0 mL of 0.1 M Hepes (pH 8.0). Complete oxidation of the amino acid required 3–4 h. Derivatization of the enzymatic oxidation products (and also of authentic acetopyruvate and chemically synthesized 2-amino-4-keto-2-pentenoate) was accomplished by the addition of 10 μL of 1.0 M hydroxylamine hydrochloride (pH 8) or 1.0 M hydrazine hydrochloride (pH 8) to 1.0 mL of product solution. Reduction with sodium borohydride was done by the addition of a crystal of solid borohydride to a solution of product.

Acid and base titrations of the products were performed by the addition of aliquots of HCl or NaOH stock solutions to the product solution. pH values were measured with a Beckman Model 2500 pH meter, and spectra were recorded using a Perkin-Elmer 200 spectrophotometer.

To test for the solubility of the reaction products in organic solvents, a 3-mL solution of 150 nmol of the oxidation products from propargylglycine or 4-ketonorvaline was mixed with 3 mL of diethyl ether or chloroform. After vigorous mixing, the aqueous and organic layers were separated, and the ultraviolet spectrum of each was recorded.

NMR Spectrum of the Product of Propargylglycine Oxidation. A solution of 6 mg of L-propargylglycine in 10 mL of 20 mM Hepes (pH 8) was allowed to react with 1 mg of L-amino-acid oxidase and 0.1 mg of catalase for 3 h at room temperature. The solution was extracted three times with 10 mL of $CDCl_3$, and the organic extracts were combined, dried with anhydrous Na_2SO_4, and carefully evaporated to 0.5 mL by passing a stream of dry argon over the surface. The 90-MHz NMR spectrum was recorded using a Bruker WH-90 spectrometer equipped for Fourier transform NMR.

Charge-Transfer Complexes between D-Amino-acid Oxidase and Amino Acid Oxidation Products. A solution of D-amino-acid oxidase in 0.5 mL of 0.05 M sodium pyrophosphate (pH 8.5) (a solution with A_{455} = 0.2–0.4 OD/cm) was placed in a micro silica cell at 10 °C. A 1–5-μL aliquot of the amino acid to be oxidized (D-propargylglycine, DL-4-ketonorvaline) was added to the solution. When only a slight molar excess of amino acid is used, reaction is complete in seconds and the spectrum is recorded as soon as practical. To a D-amino-acid oxidase solution (10 nmol) was added 1 μL of 25 mM L-propargylglycine followed by 5 μL of L-amino-acid oxidase (0.15 nmol) to record the charge-transfer complex formed in that manner.

Direct Spectroscopic Observation of a Transient Oxidation Product of Propargylglycine. D-Amino acid oxidase (9 nmol) and 25 μg of catalase were dissolved in 2 mL of 0.1 M Hepes (pH 8.0) or 0.1 M Hepes/0.1 M butylamine hydrochloride (pH 8.0). The solution (0.9 mL) was added to both sample and reference cell at 8 °C. An aliquot of 0.1 M sodium benzoate (10 μL) was added to the reference cell. D-Propargylglycine (1 μL of 0.05 M) was added to the sample followed after 30 s with 10 μL 0.1 M benzoate. The difference spectrum was then repeatedly recorded as rapidly as possible (240-nm scan/min; 1 spectrum/min).

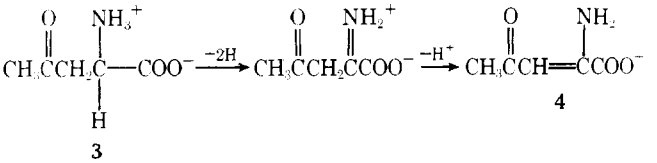
Oxidation of Propargylglycine via Catalytic Transamination. D-Propargylglycine (0.1 μmol) was oxidized in a cuvette containing 0.1 mg of protein (~10% D-amino-acid transaminase by specific activity) and 1.0 μmol of α-ketoglutarate in 1.0 mL of 0.05 M pyrophosphate buffer (pH 8.5) at 30 °C. The reference cuvette contained all species except propargylglycine. Complete oxidation required 20–30 min.

Results

Identification of the Accumulating Product of Propargylglycine Oxidation

Upon incubation of D- or L-propargylglycine with the corresponding flavoenzyme amino-acid oxidase, a product accumulates in solution which exhibits intense absorbance in the near ultraviolet (λ_{max} 300 nm, ϵ ~11 000; Marcotte and Walsh, 1976). Evidence as to the probable structure of that product is presented here.

Model Compounds, 2-Amino-4-keto-2-pentenoate, and Acetopyruvate. 2-Amino-4-keto-2-pentenoate was prepared by both enzymatic and chemical syntheses, the chemical synthesis modeled after that of a similar enamine reported by Tagaki et al. (1968). After heating a chloroform suspension of the ammonium salt of ethyl acetopyruvate to reflux for 8 min, a condensation product was isolated (in 2.5% yield) by silica gel chromatography (most of the salt reverts to ethyl acetopyruvate and volatile ammonia). The NMR spectrum of the product exhibited only a single resonance assigned to a methyl group, evidence that only one of the two possible enamine products (ethyl 2-amino-4-keto-2-pentenoate or ethyl 2-keto-4-amino-3-pentenoate) is produced in the reaction. Attempts to resolve the material into two components by silica gel or reverse-phase chromatography were unsuccessful. After saponification, the properties of the salt in buffered solution were identical to those of the product of L-amino-acid oxidase catalyzed oxidation of 4-ketonorvaline (**3**), probable evidence that the chemical condensation yields exclusively 2-amino-4-keto-2-pentenoate (**4**), although we have no direct evidence ruling out the presence of the other isomer.



This compound was found to exhibit absorption with λ_{max} 321 nm, ϵ ~13 000 at pH 8, and is quite stable to hydrolysis: no spectral change is observed over 20 h at pH 8 or 4 h at pH 12. However, 10 min at pH 2 quantitatively yields acetopy-

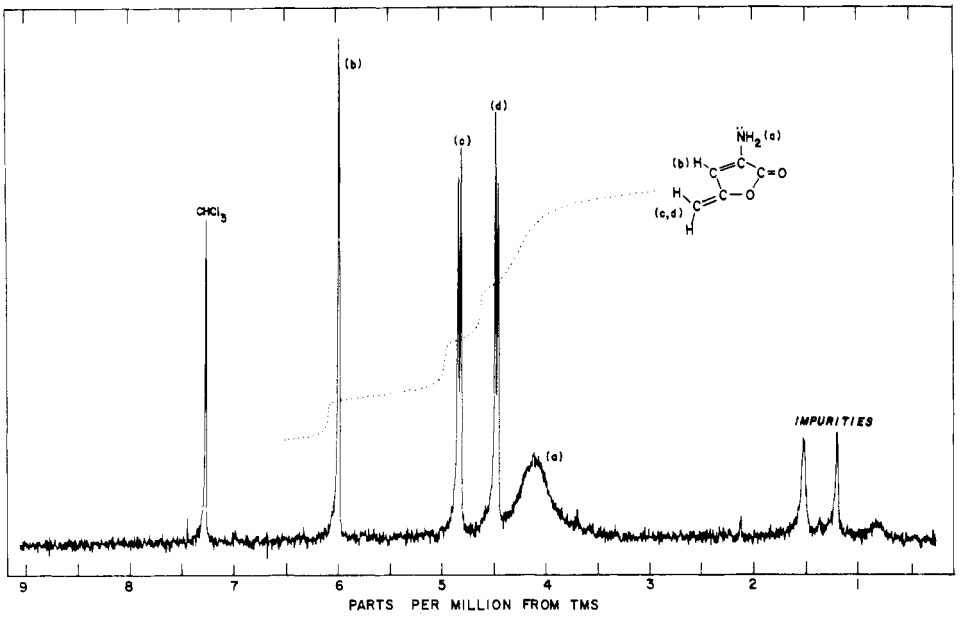
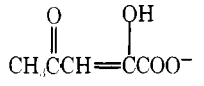


FIGURE 1: 90-MHz nuclear magnetic resonance spectrum (derived from 212 Fourier transform pulses) of the $CDCl_3$-extractable product of propargylglycine oxidation. The resonances at 0.5–2.0 ppm are due to impurities in the reagents which were also present in a $CDCl_3$ extract of a control incubation which lacked L-amino-acid oxidase.

ruvate (**5**). A solution of the ketoenamine (50 μM) is inert to the addition of solid sodium borohydride, a reflection of the electron-rich nature of the species. The enamine slowly reacts with 10 mM hydrazine or 10 mM hydroxylamine with bleaching of the absorbance,[2] the observed pseudo-first order rate constants at room temperature being 0.87 h^{-1} for hydrazine, 0.53 h^{-1} for hydroxylamine.

In contrast, acetopyruvate (**5**) reacts rapidly with borohydride (addition of solid), hydrazine (10 mM), or hydroxylamine (10 mM). Addition of any of these three agents to a pH 8 solution of acetopyruvate (0.1 mM) results in complete bleaching of the absorbance in less than 10 s.



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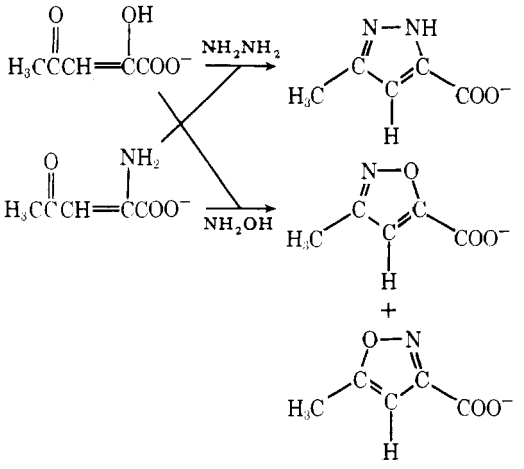
Borohydride Reducibility of the 300-nm Product. As reported earlier (Marcotte and Walsh, 1976), a solution of the oxidation product of propargylglycine can be prepared by exhaustive oxidation of L-propargylglycine (0.1 mM) by L-amino-acid oxidase in 0.1 M Hepes buffer (pH 8.0). This species exhibits λ_{max} 300 nm, $\epsilon \sim 11\,000$. We had proposed that the major contributor to this absorbance was acetopyruvate (λ_{max} 295 nm, ϵ 12 000). However, addition of solid sodium borohydride to this solution results in the rapid bleaching of approximately 10% of the absorbance—no further reduction is observed in a 90-min incubation. This datum rules out acetopyruvate as the major contributor to the absorbance.

Acid and Base Labilities of the 300-nm Product. Titration of the enzymatic product solution to pH 2 with HCl resulted in spectral changes which were time dependent and irreversible. After 10 min, neutralization with NaOH yielded a material whose spectrum was identical to that of acetopyruvate in its pH dependence (Meister and Greenstein, 1948), as well as its ready and complete reduction by borohydride.

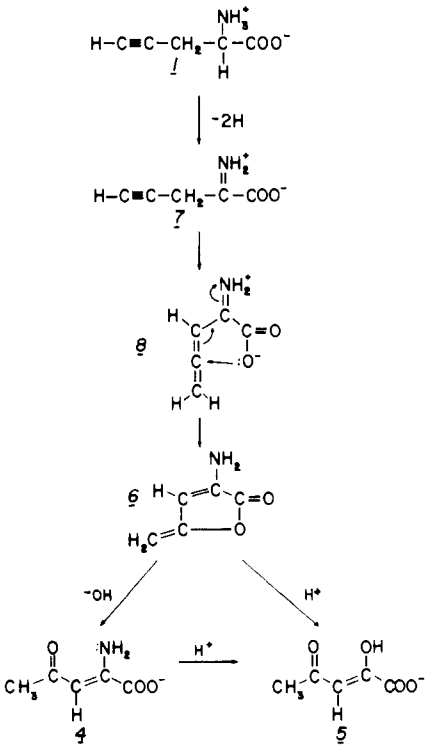
Titration of the solution of the product to pH 12 caused a rapid shift (complete in 1 min) of the absorbance maximum to 317 nm. This new absorbance was unchanged when the solution was returned to pH 8. The major contributor to this absorbance was identified as 2-amino-4-keto-2-pentenoate by the following criteria: (1) Addition of 10 mM hydroxylamine or 10 mM hydrazine caused the rapid (in s) bleaching of about 10% of the absorbance (presumably due to acetopyruvate content); the λ_{max} was shifted by this treatment from 317 to 321 nm. The remainder of the absorbance bleached with the same first-order rate constants reported above for 2-amino-4-keto-2-pentenoate. (2) Addition of solid sodium borohydride caused the bleaching of about 10% of the absorbance with a shift in λ_{max} to 321 nm. The remainder of the absorbance was stable to reduction. (3) Acidification of the 317-nm material to pH 2, followed by neutralization, yielded a spectrum with properties identical to that of acetopyruvate.

Extraction of the 300-nm Product with Organic Solvent. When a buffered (pH 8) solution of the oxidation product of propargylglycine is mixed with an equal volume of diethyl ether, approximately 70% of the absorbance partitions into the organic phase. Both acetopyruvate and 2-amino-4-keto-2-pentenoate remain totally in the aqueous phase under these conditions. The extracted product is unstable toward removal of solvent. Evaporation of the ether to dryness results in de-

[2] We believe it probable that the products of these reactions are the oxazoles or pyrazole as illustrated here, although no attempt has been made to isolate the products from these reactions. 5-Methyl-1,2-pyrazole-3-carboxylate (lithium salt) was synthesized (Elguero et al., 1966) and found to have no absorbance maximum above 240 nm, consistent with its being the product of the reaction with hydrazine.



SCHEME I.



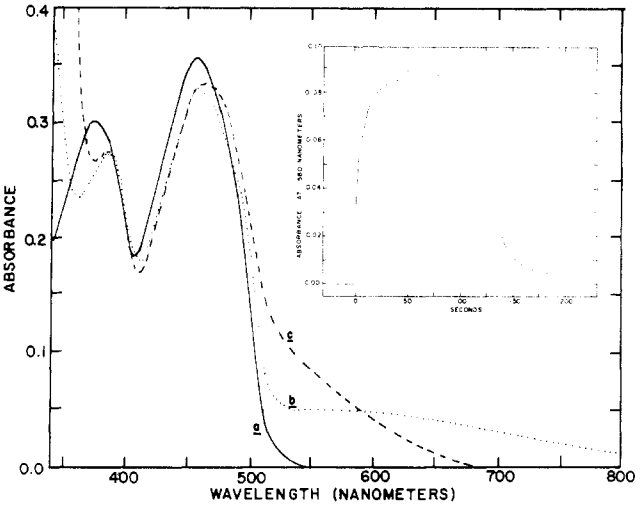


FIGURE 2: Spectra of the complexes between the oxidation products of D-propargylglycine and D-4-ketonorvaline and D-amino-acid oxidase: (a) spectrum of 13 nmol of D-amino-acid oxidase in 0.4 mL of 0.05 M pyrophosphate buffer (pH 8.5); (b) immediately after the addition of 1 μL of 0.1 M D-propargylglycine; (c) immediately after the addition of 2 μL of 0.1 M DL-4-ketonorvaline. Inset: At 0 s, 1 μL of 0.25 M D-propargylglycine was added to a solution of 21 nmol of D-amino-acid oxidase in 0.5 mL 0.05 M pyrophosphate buffer. At 120 s, 10 μL of 0.5 M sodium benzoate was added.

composition of the product; a yellowish residue is obtained which exhibits no defined absorbance maximum when subsequently dissolved in buffer. Addition of a small amount of (fresh) buffer to the ether extract followed by careful evaporation was successful in producing an aqueous solution of the purified product without evident decomposition. The recovered material was quantitatively converted by acidification to acetopyruvate and to 2-amino-4-keto-2-pentenoate (the λ_{max} now shifting directly to 321 nm) upon treatment with base.

The product was also found to be extractable with chloroform. It was found that sufficient material could be extracted into $CDCl_3$ to allow the determination of the NMR spectrum of the product, which is presented in Figure 1. Although dilute solutions (0.1 mM) of the product are stable in organic solvents for days at room temperature, as judged by changes in ultraviolet absorbance, relatively concentrated (10 mM) solutions become yellow upon standing several hours.

Proposed Structure of the Accumulated Product. We propose the γ-lactone structure **6** as the major product of propargylglycine oxidation (Scheme I). This species would be formed by intramolecular attack of the carboxylate anion as nucleophile on the electrophilic fourth carbon of the conjugated allene **8**, which we proposed in our previous work (Marcotte and Walsh, 1976) as a key intermediate in the pathway. The intramolecular attack must be more rapid than either attack of water at C_4 or hydrolysis of the iminium moiety at C_2. The molecule is uncharged, as is required by its extractability in organic solvents, and would be expected to have the observed reactivities toward acid and base. We believe that the NMR spectrum (Figure 1) is an unambiguous confirmation of the proposed structure.

Elucidation of the Pathway Following Enzymatic Oxidation of Propargylglycine

Formation of a D-Amino-acid Oxidase Charge-Transfer Complex upon Oxidation of D-Propargylglycine. Upon addition of 1–10 equiv of D-propargylglycine to a spectroscopic quantity of D-amino-acid oxidase, intense long-wavelength absorbance characteristic of a charge-transfer complex is immediately (within seconds) evidenced (Figure 2). Addition of less than a stoichiometric quantity of D-propargylglycine to the enzyme results in the formation of an intensity of charge-transfer absorbance equivalent to the amount of propargylglycine added. This demonstrates that the species responsible for the complex is produced in high yield from propargylglycine and is bound with a K_d less than the concentration of enzyme used in the experiment (10–15 μM). The species was also shown to be in rapid, noncovalent equilibrium with the enzyme by the addition of 10 mM sodium benzoate (Figure 2, inset). Since the rate of binding of this concentration of benzoate is much faster (it is greater than 200 s^{-1}, Nishikimi et al., 1971) than the observed rate of decay of the 580-nm absorbance, this experiment demonstrates that the rate of release of the species responsible for the charge-transfer complex is approximately 0.08 s^{-1}.

Complexes Produced by 2-Amino-4-keto-2-pentenoate and Acetopyruvate. Addition of a slight stoichiometric excess of 4-ketonorvaline to D-amino-acid oxidase also results in the rapid formation of a charge-transfer complex (Figure 2). Addition of chemically synthesized 2-amino-4-keto-2-pentenoate generates an identical spectrum. This enamine is bound somewhat less tightly than that produced from propargylglycine, being replaced by 10 mM benzoate within mixing ($t_{1/2}$ < 2 s). Acetopyruvate also induces perturbations in the visible spectrum of D-amino-acid oxidase upon binding; however, the changes are much less pronounced (a shoulder in the 455-nm absorbance maximum), and it was found that acetopyruvate is bound very much less tightly than the other inhibitors (K_i = 120 μM vs. D-alanine).

Properties of the D-Amino-acid Oxidase–Propargylglycine Product Complex. The charge transfer complex of D-amino-acid oxidase and the propargylglycine product is not indefinitely stable. Over 60–90 min at 10 °C, the long-wavelength absorbance decays. It does not return to the original spectrum; the residual long-wavelength absorbance is similar to that expected from the formation in solution of a small quantity of 2-amino-4-keto-2-pentenoate.

Since addition of high concentrations of butylamine changes

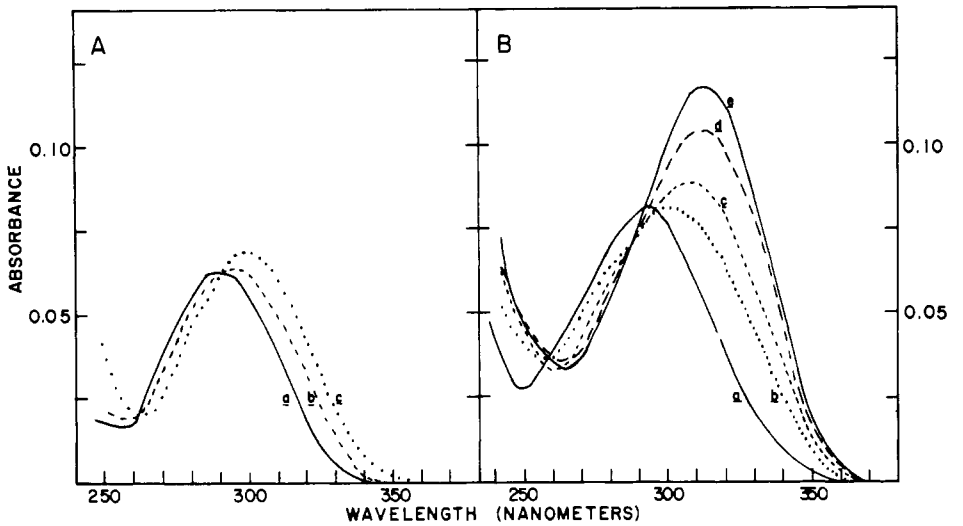


FIGURE 3: Direct observation of a transient product of propargylglycine oxidation as described under Methods. (A) In 0.1 M Hepes (pH 8.0). Spectra at the following time points are reproduced: (a) 1 min; (b) 7 min; (c) 15 min. (B) In 0.1 M Hepes/0.1 M butylamine hydrochloride (pH 8.0). Spectra at the following time points are reproduced: (a) 1 min; (b) 3 min; (c) 5 min; (d) 10 min; (e) 15 min.

the ultraviolet spectrum of the accumulating products of propargylglycine oxidation (Marcotte and Walsh, 1976), the effects of amines on the formation and decay of the charge-transfer complex were examined. Butylamine hydrochloride, 160 mM, did not have any effect on the formation of the complex; the presence of amine did cause the spectrum to return over time to its original base line, presumably by preventing the formation in solution of 2-amino-4-keto-2-pentenoate.

The production of the D-amino-acid oxidase–propargylglycine product complex does not require oxidative catalysis by D-amino-acid oxidase. This point was demonstrated by incubation of D-amino-acid oxidase with a slight molar excess of L-propargylglycine (with no effect), followed by a catalytic amount (1%) of L-amino-acid oxidase. The identical charge-transfer spectrum (involving D-amino-acid oxidase) was generated over 20 min, demonstrating that finite amounts of the product species can diffuse through solution to the D-amino acid oxidase active site.

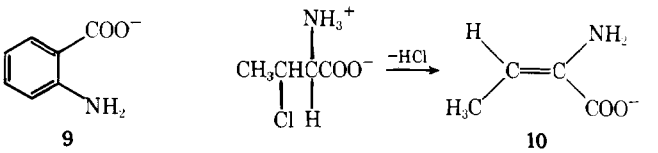
The charge-transfer complex was found *not* to be in any demonstrable way connected with the covalent modification of the enzyme discussed previously (Marcotte and Walsh, 1978a). No kinetic inactivation (by assay of withdrawn aliquots) was observed during the lifetime of the long-wavelength absorbance. An effort was made to produce covalently modified enzyme by incubation of D-amino-acid oxidase, L-propargylglycine, and L-amino-acid oxidase. Under conditions in which L-propargylglycine was oxidized continually over a 2-h period, no alkylation of D-amino-acid oxidase was observed. A strong competitive inhibitor was produced in the incubation, but, following dialysis, enzyme with native kinetic parameters was recovered.[3]

2-Amino-4-hydroxy-2,4-pentadienoate γ-lactone (**6**), the accumulating product, was shown not to be the cause of the charge-transfer complex. Addition of the ether-extracted product (recovered into buffer as previously described) to a solution of D-amino-acid oxidase resulted in no change in the visible spectrum of the enzyme. Both the complex-inducing species and the lactone are formed in high yield, evidence that both species must lie on a single pathway following oxidation of propargylglycine.

Spectroscopic Observation of a Transient Species Formed Following Oxidation of Propargylglycine. To directly observe a species produced early in the reaction, D-amino-acid oxidase was incubated at 8 °C with D-propargylglycine for 30 s. The binding of the inhibitor to the enzyme results in only a fraction of the D-propargylglycine being oxidized; therefore, the tight competitive inhibitor sodium benzoate (K_d = 3 μM, Massey and Ganther, 1965) was added. Benzoate rapidly displaces the inhibitor (Figure 2, inset), and the spectrum of the released species can then be recorded (Figure 3) as a difference spectrum vs. a reference cell containing all species except propargylglycine.

The absorbance maximum which characterizes the product of propargylglycine oxidation is dependent on the nucleophilic species present in solution (Marcotte and Walsh, 1976). As shown in Figure 3A,B, the same species appears to be the precursor of both the 300- (in Hepes buffer) and 318-nm species (in Hepes/butylamine buffer). Benzoate slows continued oxidation but does not completely stop the reaction. Therefore, the ultraviolet absorbances of Figure 3 continue to increase slowly; however it can be seen that the transient species has a λ_{max} 290 nm, $\epsilon \sim 10\,000$, and a lifetime of 5–10 min under these conditions.

Proposed Mechanism of the Reactions Following Enzymatic Oxidation of Propargylglycine. A number of stable and metastable molecules have previously been found to induce intense charge-transfer bands upon binding to D-amino-acid oxidase. Two such species are anthranilate (**9**) (Massey and Ganther, 1965), a stable molecule, and 2-aminocrotonate (**10**)



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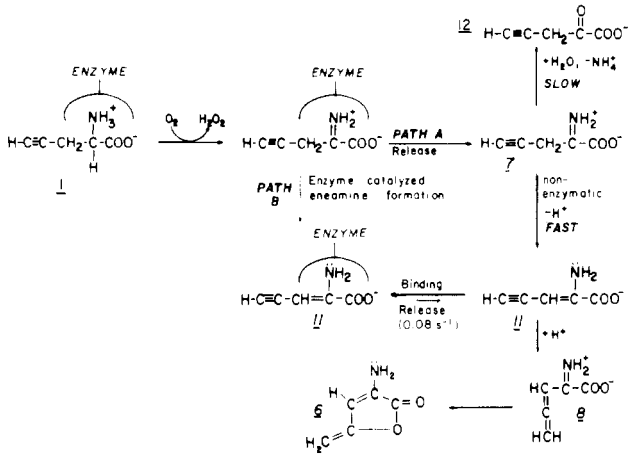
(Walsh et al., 1973), a transient species produced from D-amino-acid oxidase catalyzed elimination of HCl from β-chloro-α-aminobutyrate. These molecules have the common feature of being electron-rich enamino acids and serve as donor components to the electron-deficient, oxidized flavin coenzyme.

[3] Covalent modification of D-amino-acid oxidase is only observed when that enzyme is incubated with a large excess of D-propargylglycine. The failure to observe alkylation under milder conditions probably reflects the much lower concentration of the conjugated allene (**8**) in the vicinity of the susceptible nucleophile(s) when **8** is produced through L-amino-acid oxidase catalyzed oxidation of L-propargylglycine.

SCHEME II.



A third such enamine, 2-amino-4-keto-2-pentenoate (**4**), has been shown in this work to be the effector of a charge-transfer complex.

Since the formation of the charge-transfer complex during propargylglycine oxidation is unaffected by the presence of amines in the solution, the species responsible for the complex must precede the reactive conjugated allene (**8** of Scheme I). Furthermore, it must retain the nitrogen bound to carbon-2, because it must be a precursor of the lactone product **6**. We believe the probable identity of the species is the acetylenic enamino acid (**11** of Scheme II). It would be formed by loss of proton from C_3 of the initial product of propargylglycine oxidation, 2-iminium-4-pentynoate (**7**). The formation of this species in high yield, as well as the high-yield formation of the accumulated product lactone (**6**), implies that loss of this proton must be faster than hydrolysis of the iminium cation to 2-keto-4-pentynoate (**12**). The observed accumulation of approximately 10% acetopyruvate in incubations is an indication of the relative rates of these reactions. The acetylenic enamine must undergo protonation at C_5 in a slower step, thus forming the conjugated allene **8**, which then reacts intramolecularly to yield the accumulating product **6**.

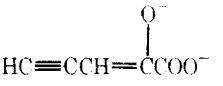
The question arises (Scheme II) as to whether the enzyme catalyzes the formation of the species responsible for the complex (path B) or whether the enzyme releases the initial oxidation product (2-iminium-4-pentynoate, **7**), followed by its rapid nonenzymatic conversion to the active species and rebinding to the enzyme (path A). This question can be answered by comparison of the maximal initial turnover number of enzyme-catalyzed propargylglycine oxidation with the rate of release of the acetylenic enamine. The former constant can be measured by direct observation of the initial rate of increase in product absorbance for a given quantity of enzyme and has been found to be 2 s^{-1} at 10 °C. The latter constant has already been determined (Figure 2, inset) and was found to be 0.08 s^{-1} at 10 °C. Therefore, path B is kinetically incompetent, and enzyme-catalyzed production of the enamine (before release) cannot be a step in the enzymatic oxidation of propargylglycine.

Properties of Products Derived from 2-Keto-4-pentynoate

Oxidation of Propargylglycine via Catalytic Transamination. We have presented evidence to show that, following flavoprotein-catalyzed oxidation of propargylglycine, formation of the acetylenic enamine **11** is kinetically favored over hydrolysis of 2-iminium-4-pentynoate (**7**) to 2-keto-4-pentynoate (**12**). Solutions of this acetylenic keto acid were prepared by employing the pyridoxal phosphate dependent enzyme, D-amino-acid transaminase, which we have found will catalyze oxidative deamination of propargylglycine with concomitant reductive amination of α-ketoglutarate (Marcotte and Walsh, unpublished observations).

The product formed in this manner was found to exhibit λ_{max} 309 nm, $\epsilon \sim 12\,000$ at pH 8.5.[4] No precursor species were detected. Over several hours at 30 °C, the spectrum shifted toward lower wavelengths. In the presence of 200 mM butylamine, the initial product exhibited λ_{max} 309 nm, but the spectrum shifted to higher wavelengths with time. The 309-nm absorbance was not extractable into diethyl ether. Upon acid titration, the product exhibited a $pK_a \simeq 6.5$; incubation at pH 5.8 for 15 min followed by neutralization yielded a spectrum identical to that of authentic acetopyruvate.

Although we have not found it possible to characterize this species as completely as others in this work, we propose the enolate anion **13** as the product derived from 2-keto-4-pentynoate. The enolate anion derived from 2-keto-4-pentenoate has been reported to have λ_{max} 305 nm (Collingsworth et al., 1973), although it rapidly decomposes in basic solution. The enolate anion of β-phenylpyruvate has been examined and found to have λ_{max} 321 nm, ϵ 18 000 (in 1 N NaOH) and exhibit a pK_a of approximately 13.



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Effect of Buffers on the Pathway Following Flavoprotein-Catalyzed Oxidation of Propargylglycine. In the following paper in this issue, the effect of the buffer on the pathway following enzymatic oxidation of allylglycine is described: 2-iminium-4-pentenoate partitions between hydrolysis and formation of an olefinic enamine in a ratio dependent on the buffer utilized in the incubation. No similar effect was observed in the pathway following oxidation of propargylglycine. In both Hepes (pH 8.0) buffer and pyrophosphate (pH 8.5) buffer, the observed product exhibited λ_{max} 300 nm, $\epsilon \sim 11\,000$ and was largely (60–70%) ether extractable in an equal volume partition. Therefore, hydrolysis at C_2 of 2-iminium-4-pentynoate to form 2-keto-4-pentynoate is not a kinetically favored reaction, and the pathway presented in Scheme II appears to describe the predominant reactions in both buffer systems.

Discussion

The identification of the products of oxidation of propargylglycine was originally pursued to produce evidence confirming the probable structure of the species responsible for the alkylation of D-amino-acid oxidase. In our first publication on the interaction of propargylglycine with the amino-acid oxidases (Marcotte and Walsh, 1976), we presented what we believed to be a reasonable speculation for the pathway following oxidation. The end product of this speculative pathway is acetopyruvate (2,4-diketovalerate). Because the expected short lifetime of the conjugated allene would make its direct observation an unlikely possibility, the identification of acetopyruvate would be evidence that there exists in the pathway a species subject to nucleophilic attack at carbon-4. We succeeded in isolating acetopyruvic acid in high yield upon work

[4] An identical spectrum was observed upon oxidation of 2-hydroxy-4-pentynoate by rat kidney L-hydroxy-acid oxidase (prepared by the method of Nakano et al., 1967). Product formation via this route was less efficient than oxidation via transamination, due to the low specific activity of the rat kidney enzyme. Therefore, the transaminase was routinely used to study products derived from 2-keto-4-pentynoate.

up of incubations of oxidase and propargylglycine; furthermore, addition of amine and thiol nucleophilies was found to have the predicted effect on the products, as characterized by their ultraviolet absorption spectra (Marcotte and Walsh, 1976).

However, several observations were reported in our previous work which could not be accounted for by the simple scheme. The kinetics of the inactivation were complicated and a number of experiments implied the formation of a reversible inhibitor in addition to the covalent modification. Acetopyruvate was demonstrated not to be the cause of this inhibition and the matter was left unresolved.

We have now demonstrated that the accumulating product is not comprised mostly of acetopyruvate. The pathway following oxidation has now been studied in detail, and the aminediene lactone structure **6** has been proposed for the isolated product. Although this structure has not been confirmed by synthesis, we believe that the simple NMR spectrum, organic solvent extractability, and demonstrated acid and base labilities to characterized substances provide sufficient evidence for the structural assignment.

In the course of this work, two species have been identified which form tightly bound "charge-transfer" complexes with D-amino-acid oxidase. The structure of one of the species, 2-amino-4-keto-2-pentenoate (**4**) is firmly established, having been confirmed by independent chemical synthesis. The second species has been assigned the structure 2-amino-2-penten-4-ynoate (**11**), based on a variety of circumstantial evidence: the responsible species must be formed in high yield, must retain the nitrogen bound to carbon-2, and must occur in the pathway preceding the conjugated allenic iminium acid (**8**). Porter and Bright (1972) have characterized 2-aminocinnamate (the enamine derived from β-phenylpyruvate) and found it to exhibit λ_{max} 300 nm, ϵ 22 500. We have identified an enamine in the pathway following oxidation of allylglycine (Marcotte and Walsh, 1978b), shown it to have λ_{max} 285 nm, $\epsilon \sim 10\,000$, and found it to be the effector of a charge-transfer complex with D-amino-acid oxidase.

After release of 2-iminium-4-pentynoate (**7**), the initial oxidation product of propargylglycine, from the enzyme, the most rapid reaction to occur is the formation of the acetylenic enamine **11**, a potent reversible inhibitor of D-amino-acid oxidase. We believe the probable explanation for the facility of this reaction is that loss of the C_3 proton occurs from the iminium carboxylate zwitterion rather than the imine carboxylate anion, a point we have illustrated in Scheme II. We have no direct evidence to support this mechanism, but the pK_a of the zwitterion would be expected to be in the physiological range (pH 6–8); therefore, an appreciable amount of the product should exist in this form.

The acetylenic enamine derived from propargylglycine oxidation subsequently reacts via a relatively slow protonation at C_5, thereby causing the formation of a conjugated allenic iminium acid. This species incorporates a highly electrophilic functional group which rapidly reacts by intramolecular attack of the carboxylate anion. The resulting aminediene lactone is relatively stable in buffered aqueous solution and must not be in significant equilibrium with its conjugated imine tautomer; for if it were, hydrolysis and borohydride reducibility would be expected to occur. Unfortunately, it was not possible to isolate the material as a pure substance, as it was found to decompose upon evaporation of an ether solution to dryness.

The work described in this paper, as well as in our characterization of the properties of propargylglycine-modified D-amino-acid oxidase published earlier (Marcotte and Walsh, 1978a), has pointed out the difficulties in using unsaturated substrate analogues as active-site-directed inactivators of enzymes. Subsequent work using molecules of this type must bear in mind the complications that can result when the reaction of the enzyme and the substrate analogue results in a partition between release of a highly reactive product and inactivation.

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