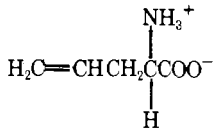
Sequence of Reactions Which Follows Enzymatic Oxidation of Allylglycine†

Patrick Marcotte‡ and Christopher Walsh*,§

ABSTRACT: The pathway following flavoprotein-catalyzed oxidation of allylglycine (2-amino-4-pentenoate) has been studied and found to be dependent on the incubation conditions. In *N*-2-hydroxyethyl-*N'*-2-ethanesulfonic acid (Hepes) buffer, the oxidation product 2-iminium-4-pentenoate predominantly reacts to form 2-amino-2,4-pentadienoate, a strong noncovalent inhibitor of D-amino-acid oxidase. However, in pyrophosphate buffer, the more rapid reaction is hydrolysis to form 2-keto-4-pentenoate, which has been found to be a substrate for L-lactic dehydrogenase. 2-Keto-4-pentenoate is in rapid equilibrium with 2-hydroxy-2,4-pentadienoate, which is also a strong noncovalent inhibitor of D-amino-acid oxidase. In both systems, these metastable intermediates react in subsequent slower steps to yield *trans*-2-keto-3-pentenoate, which accumulates in the incubation. Syntheses of *trans*-2-amino- and *trans*-2-keto-3-pentenoate are described. Comparisons between the reactivities of acetylenic and olefinic species have been made based on the differences between this pathway and that following oxidation of propargylglycine [Marcotte, P., and Walsh, C. (1978), *Biochemistry 17* (preceding paper in this issue)].

The reaction of allylglycine (**1**) with the flavoproteins D- and



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L-amino-acid oxidases has been studied for two reasons. The first was to provide a comparison of the pathway following oxidation of this γ-δ olefinic amino acid with the pathway following oxidation of the corresponding γ-δ acetylenic amino acid, propargylglycine, which is discussed in the preceding paper in this issue. Secondly, allylglycine induces seizures in higher organisms (Horton and Meldrum, 1973), and work by Orlowski et al. (1977) and Horton (1978) has established that some oxidation product of allylglycine (**1**) is the active agent rather than the amino acid itself.

The catalytic oxidation of allylglycine by L-amino-acid oxidase has previously been studied as a means of synthesis of 2-keto-4-pentenoate (Collingsworth et al., 1973), an intermediate degradation product in catechol metabolism (Bayly and Dagley, 1969). In this paper, we present a more detailed study of the reaction of allylglycine with D- and L-amino-acid oxidases, in which we demonstrate the existence of several product species in addition to 2-keto-4-pentenoate and analyze their effects on D-amino-acid oxidase.

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). L-Hydroxyacid oxidase from frozen rat kidneys was purified by the procedure of Nakano et al. (1967). L-Amino-acid oxidase (*Crotalus adamanteus* venom), catalase (beef liver), L-lactic dehydrogenase (rabbit muscle), and alcohol dehydrogenase (yeast) were purchased from Sigma.

Reagents. Allylglycine, NADH,[1] NAD^+, and Hepes were purchased from Sigma. [1-^{3}H]ethanol was purchased from New England Nuclear. 3-Methyl-1-*p*-tolyltriazine was purchased from Aldrich. Buffer salts and solvents were commercially available reagent-grade materials.

Syntheses. DL-2-Hydroxy-4-pentenoic acid was prepared by the additon of 20 mL of sodium nitrite (1 M) to 2 g of DL-allylglycine dissolved in 25 mL of water and 10 mL of 2 N acetic acid. After 2.5 h at room temperature, the solution was passed through a 100-mL Dowex 50 H^+ column and the acidic eluant evaporated to an oil. The crude product was converted to the methyl ester using diazomethane and vacuum distilled. *trans*-2-Hydroxy-3-pentenoate was prepared from hydrolysis of crotonaldehyde cyanohydrin, as described by Rossi and Schinz (1948). The product was purified by distillation of the methyl ester. *cis*-2-Hydroxy-3-pentenoate was prepared by Lindlar catalyst hydrogenation in acetone solution of methyl 2-hydroxy-3-pentynoate (Cromartie et al., 1974). Upon reaction with H_2 catalyzed by palladium on carbon, each ester absorbed 1 equiv of H_2, the product in each case being identified by GLC analysis as methyl 2-hydroxypentanoate. In the mass spectrometer, each ester exhibited a molecular ion at *m/e* 130 and a base peak at *m/e* 71 (loss of $-COCO_2CH_3$). The NMR spectra of the esters were found to be consistent with the assigned structures. Each ester was saponified by the

† From the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received July 20, 1978.* This work was supported under Grant 2011 from the National Institutes of Health.

‡ National Institutes of Health Postdoctoral Fellow, 1977-1978. Present address: Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205.

§ Camille and Henry Dreyfus Scholar, 1975-1980.

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; GLC, gas-liquid chromatography; NADH, nicotinamide adenine dinucleotide (reduced form); NAD^+, nicotinamide adenine dinucleotide (oxidized form); Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

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gradual addition of 1 equiv of lithium hydroxide to an aqueous solution followed by evaporation.

2-Keto-3-pentenoate was prepared by the addition of 20 g of activated manganese dioxide (Ventron/Alfa) in 5-g portions at 30-min intervals to a stirred solution of methyl *trans*-2-hydroxy-3-pentenoate (1 g) in 100 mL of pentane. After the addition was complete, the mixture was stirred for 30 min and then centrifuged, and the solution was filtered and evaporated to an oil. Attempts to purify the yellow oil by vacuum distillation resulted in loss of material without substantial gain in purity (as measured by GLC and NMR analysis). The lithium salt was prepared by the gradual addition of 1 equiv of lithium hydroxide to an aqueous solution of the ester followed by evaporation: λ_{max} 229 nm, ϵ 12 000 (in H_2O, pH 8.5); NMR (in D_2O) δ 2.1 (3 H, d of d), 6.0–7.5 (2 H, m).

DL-4-Hydroxynorvaline was prepared by the addition of a twofold excess of sodium borohydride to a solution of 4-ketonorvaline (Wiss and Fuchs, 1952) in 0.1 N NaOH. After a 2-h reaction, the excess borohydride was decomposed by the addition of HCl and the amino acid purified by adsorption onto a column of Dowex 50 H^+, followed by elution with 2 N NH_4OH. The product was crystallized from aqueous ethanol: mp 202 °C;[2] NMR (in D_2O) δ 1.4 (3 H, d), 2.2 (2 H, t), 4.1 (2 H, m). Anal. Calcd for $C_5H_{11}NO_3$: C, 45.09; H, 8.34; N, 10.52; O, 36.04. Found: C, 45.00; H, 8.44; N, 10.56; O, 35.91.

DL-*trans*-2-Amino-3-pentenoate was synthesized in 28% yield from *trans*-2-hydroxy-3-pentenoate with modifications of the synthesis of DL-vinylglycine described by Baldwin et al. (1977). The reaction between 2-bromo-3-pentenoate and concentrated NH_4OH was stirred at room temperature for 4 h before workup: mp 202 °C; NMR (in D_2O) δ 2.1 (3 H, d), 4.5 (1 H, d), 6.0 (2 H, m). Anal. Calcd for $C_5H_9NO_2$: C, 52.15; H, 7.89; N, 12.17; O, 27.79. Found: C, 51.95; H, 7.69; N, 12.24; O, 27.59.

Methods

Spectroscopic Characterization of the Products of Oxidation of Allylglycine, trans-2-Amino-3-pentenoate, 4-Hydroxynorvaline, 2-Hydroxy-4-pentenoate, and trans-2-Hydroxy-3-pentenoate. The products of allylglycine oxidation were characterized as follows: 1.5 mmol of L-amino-acid oxidase and 25 μg of catalase were dissolved in 2.0 mL of either 0.1 M Hepes (pH 8.0) or 0.05 M pyrophosphate (pH 8.0 or 8.5). The solution, 1.0 mL, was placed in both sample and reference cuvettes at 30 °C. The reaction was initiated by the addition of 1.0 μL of 0.1 M DL-allylglycine to the sample cuvette, after which the spectrum was recorded at intervals until no further changes were apparent. The products of oxidation (in pyrophosphate, pH 8.5) of *trans*-2-amino-3-pentenoate and 4-hydroxynorvaline were characterized in the same manner, except that 0.5 nmol of D-amino-acid oxidase was employed as oxidant. L-Hydroxyacid oxidase, 6.5 nmol, was used to produce the oxidation products of *trans*-2-hydroxy-3-pentenoate and 2-hydroxy-4-pentenoate (in pyrophosphate buffer, pH 8.5).

Reductive Trapping of the Products of Allylglycine Oxidation. To 0.2 mL of 0.1 M pyrophosphate (pH 8.0) was added 5 mg of L-allylglycine, 5 μL of [1-^{3}H]ethanol (1 mCi), 0.1 mg of catalase, 5 mg of NAD^+, 0.5 mg of L-amino-acid oxidase, 1 mg of yeast alcohol dehydrogenase, and 1 mg of L-lactic dehydrogenase. The mixture was incubated at room temperature for 120 min, after which the solution was evaporated in vacuo to dryness. The residue was dissolved in 2 mL of water containing 12.5 mg each of authentic *cis*- and *trans*-2-hydroxy-3-pentenoate and 2-hydroxy-4-pentenoate (lithium salts). The solution was passed through a Dowex 50 H^+ column, and the eluate was treated with charcoal, filtered, and evaporated to dryness. The residue was dissolved in 2 mL of diethyl ether, to which 75 mg of the methylating agent 3-methyl-1-*p*-tolyltriazine was added. After a 30-min incubation, the solution was filtered and excess triazine decomposed by the addition of glacial acetic acid. An aliquot of the solution was analyzed by injection onto a Carbowax 20M GLC column equilibrated at 100 °C. The outlet of the thermal detector was allowed to bubble into a vial of scintillation fluid, a procedure found to be >90% efficient in trapping the radioactivity eluted from the column.

The borohydride reducible products were determined by the addition of approximately 1 mg of NaB^3H_4 (100 Ci/mol) to an incubation of L-allylglycine and L-amino-acid oxidase at room temperature. The Hepes buffer (pH 8) incubation was quenched after a 60-min reaction of enzyme and allylglycine; the pyrophosphate buffer (pH 8.5), after a 15-min reaction. Any excess NaB^3H_4 was decomposed by the addition of 1 N HCl after 30-min of reaction. Incorporation of radioactivity into hydroxy acid products was determined as described above.

Charge-Transfer Complexes between D-Amino-acid Oxidase and Products of Oxidation of Allylglycine and α-Hydroxy Acids. Spectra were recorded while the samples were maintained at 10 °C to minimize turbidity in the solutions. In some experiments, NADH and L-lactic dehydrogenase were added to solutions of D-amino-acid oxidase before the addition of allylglycine. Solutions containing a catalytic amount of L-hydroxyacid oxidase (~10% the amount of D-amino-acid oxidase) were used to study the effects of the oxidation products of 2-hydroxy-4-pentenoate and 2-hydroxy-3-pentenoate.

Results

Spectroscopic Characterization of the Products of Allylglycine Oxidation. We have found that the products which are formed following the oxidation of L-allylglycine by L-amino-acid oxidase are dependent on the buffer used in the incubation. The pathways have been characterized spectrally as follows: in 0.1 M Hepes (pH 8.0) the observed product exhibits λ_{max} 285 nm (Figure 1A). This product decomposes in a first-order manner ($t_{1/2}$ = 9 min at 30 °C) yielding a new product with λ_{max} <240 nm, which was not initially characterized because of the absorbance in this region of the enzyme in solution. A very different spectrum is evidenced upon the oxidation of allylglycine in pyrophosphate buffer, at either pH 8.0 or 8.5 (Figure 1B). Little material with λ_{max} 285 nm is apparently present; however, the major product has an absorbance maximum at 265 nm which decays with a $t_{1/2}$ of 45 min at 30 °C, again to an initially uncharacterized species with absorbance at wavelengths <240 nm.

An Allylglycine Oxidation Product Which Is a Substrate for L-Lactic Dehydrogenase. An oxidation product of allylglycine is a good substrate for L-lactic dehydrogenase, as shown by the rapid oxidation of NADH when NADH and L-lactic dehydrogenase are added to incubations of L-allylglycine and L-amino-acid oxidase in pyrophosphate buffer. The identification of the reducible product provides a means of studying the pathway.

(4*R*)-[^{3}H]NADH was prepared in situ by reaction of [1-^{3}H]ethanol with NAD^+ catalyzed by yeast alcohol dehydro-

[2] Barlow and MacLeod (1964) report a mp of 205 °C for an uncharacterized mixture of isomers of 4-hydroxynorvaline synthesized by a different route.

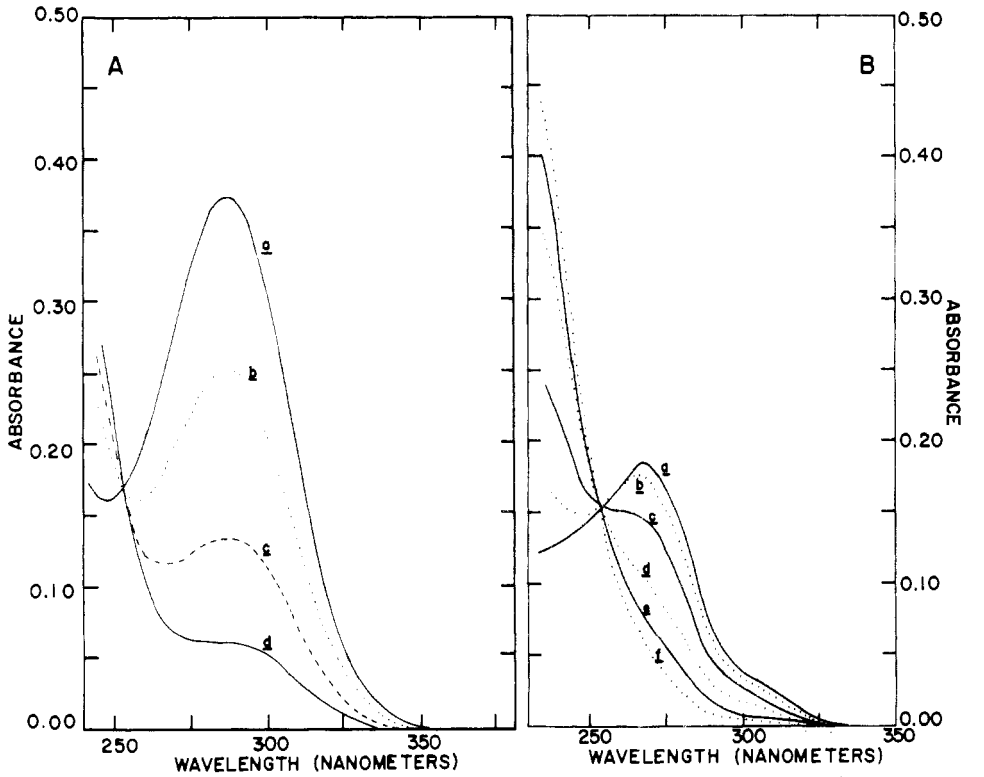
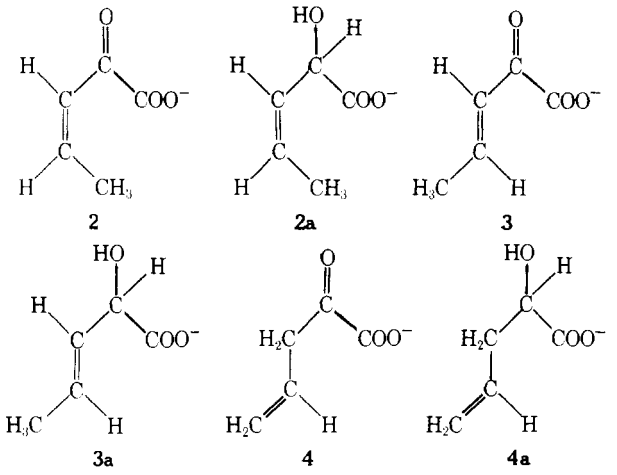


FIGURE 1: Spectroscopic characterization of the products of allylglycine (50 μM) oxidation by L-amino-acid oxidase as described under Methods. (A) In 0.1 M Hepes (pH 8.0). Spectra at the following time points are reproduced: (a) 3 min; (b) 9 min; (c) 17 min; (d) 27 min. (B) In 0.05 M pyrophosphate (pH 8.5). Spectra at the following time points are reproduced: (a) 10 min; (b) 30 min; (c) 60 min; (d) 120 min; (e) 180 min; (f) 240 min.

genase. These three materials were combined with L-allylglycine, catalase, L-amino-acid oxidase, and L-lactic dehydrogenase. After a 120-min incubation, the reaction mixture was analyzed for radioactive products as described under Methods.

Three α-keto acids were examined as the possible substrate of L-lactic dehydrogenase: *cis*-2-keto-3-pentenoate (**2**), *trans*-2-keto-3-pentenoate (**3**), and 2-keto-4-pentenoate (**4**). Reduction of these keto acids would result in formation of the corresponding α-hydroxy acids (**2a, 3a,** and **4a**). Authentic



2 **2a** **3**

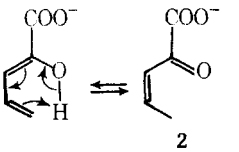
3a **4** **4a**

hydroxy acids were synthesized, and their methyl esters were found to be separable on a Carbowax 20M GLC column (Table I, retention time). As shown in Table I (buffer A) nearly all of the radioactivity applied to the GLC column was eluted with methyl 2-hydroxy-4-pentenoate. To confirm the identity of the product, 2 mg of palladium on carbon catalyst was added to the mixture, which was then incubated under a hydrogen atmosphere. Following this treatment, all of the radioactivity, as well as all of the material added as standards, was eluted from the GLC column as methyl 2-hydroxypentanoate.

Identification of Borohydride-Reducible Products. Since the pathway following oxidation of allylglycine is dependent on the incubation buffer, as determined by the observed ultraviolet spectra of the products, the reaction mixture in Hepes or pyrophosphate buffer was quenched by the addition of sodium [^{3}H]borohydride, and the incorporation of radioactivity into the products was determined by GLC analysis of the methyl esters.

The results of these experiments are presented in Table I (buffers B and C). In Hepes buffer, the major trapped product is *trans*-2-hydroxy-3-pentenoate (**3a**). In pyrophosphate buffer, the reaction was quenched after a short incubation; predominantly 2-hydroxy-4-pentenoate (**4a**) with a significant amount of **3a** were recovered. In neither buffer does any significant amount of *cis*-2-hydroxy-3-pentenoate (**2a**) appear to be formed.[3] Since the conjugated keto acid is present in incubations of the oxidation products of allylglycine, L-lactic dehydrogenase must be specific for 2-keto-4-pentenoate relative to *trans*-2-keto-3-pentenoate.

[3] We entertained the hypothesis that protonation at C_5 of 2-hydroxy-2,4-pentadienoate might be an intramolecular process, thereby yielding *cis*-2-keto-3-pentenoate, which might then more slowly isomerize to the



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thermodynamically favored trans isomer. Since no significant radioactive *cis*-2-hydroxy-3-pentenoate was recovered, intramolecular protonation is not likely to be a favored step in the pathway.

TABLE I: Reductive Trapping of the Products of Allylglycine Oxidation.

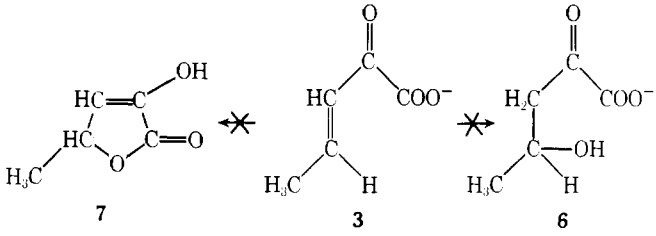
	T_R (min)[a]	cpm		
		buffer A[b]	buffer B[c]	buffer C[d]
methyl 2-hydroxypentanoate	22			
methyl 2-hydroxy-4-pentenoate	29	50 600	19 100	91 400
methyl *trans*-2-hydroxy-3-pentenoate	44	376	149 000	10 600
methyl *cis*-2-hydroxy-3-pentenoate	47	205	10 300	900

[a] Column, 6 ft × 1/8 in. 15% Carbowax 20 M on Chromosorb W at 100 °C; carrier gas, He at 30 mL/min. [b] NAD^3H/L-lactic dehydrogenase in pyrophosphate buffer (background 150–200 cpm). [c] NaB^3H_4 in Hepes buffer (background 600–800 cpm). [d] NaB^3H_4 in pyrophosphate buffer (background 600–800 cpm).

The Oxidation Products of 2-Hydroxy-4-pentenoate, 2-Hydroxy-3-pentenoate, and 2-Amino-3-pentenoate. Oxidation of 2-hydroxy-4-pentenoate by an L-hydroxyacid oxidase would yield 2-keto-4-pentenoate directly, the species which had been identified (indirectly) as that reduced by L-lactic dehydrogenase. The ultraviolet spectra of the products of oxidation of 2-hydroxy-4-pentenoate by rat kidney L-hydroxyacid oxidase are demonstrated in Figure 2A. A product with λ_{max} 265 nm is initially formed; it decays with a half-time of 45 min to a species with λ_{max} <240 nm. The spectral properties of this reaction are essentially identical to those of the oxidation product of allylglycine in pyrophosphate buffer, with the exception that less longer wavelength (290–310 nm) absorbance is present in this reaction mixture.

In Figure 2B is shown the spectrum of 2-keto-3-pentenoate, produced by enzymatic oxidation of *trans*-2-hydroxy- or *trans*-2-amino-3-pentenoate (**5**).[4] The spectrum of this product is superimposable on that of the final product (after long-term incubation) shown in Figures 1A, 1B, and 2A. An identical spectrum is observed upon dissolving chemically synthesized *trans*-2-keto-3-pentenoate in the incubation buffer.

Properties and Reactions of trans-2-Keto-3-pentenoate. Collingsworth et al. (1973) have shown that enzymes isolated from *Pseudomonas putida* will catalyze the hydration of 2-keto-4-pentenoate to (4*S*)-2-keto-4-hydroxyvalerate. Hydration of *trans*-2-keto-3-pentenoate (**3**) would result in substantial bleaching of the 229-nm absorbance maximum, as shown by the oxidation product of 4-hydroxynorvaline (Figure 2B). The chromophore of either the enzymatically or chemically synthesized *trans*-2-keto-3-pentenoate was found to decay very slowly (~10% in 24 h at 30 °C), demonstrating that hydration of this species to 2-keto-4-hydroxyvalerate (**6**) is not a facile reaction.



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In analogy to the reaction found to occur following oxidation of propargylglycine (Marcotte and Walsh, 1978b), the possible cyclization of *trans*-2-keto-3-pentenoate to α-keto-γ-methyl-γ-butyrolactone (**7**) was examined. Synthesis of authentic material (Rossi and Schinz, 1948) revealed that this material is unstable in buffered aqueous solution, presumably suffering hydrolysis to 2-keto-4-hydroxyvalerate (Dagley and Gibson, 1965). Therefore, C_4 of *trans*-2-keto-3-pentenoate is apparently not sufficiently electrophilic to cause the attack of the weakly nucleophilic carboxylate anion; rather, the conjugated keto acid accumulates in solution.

Charge-Transfer Complexes Formed upon Incubation of D-Amino-acid Oxidase and D-Allylglycine. When excess allylglycine is added to a solution of D-amino-acid oxidase in pyrophosphate buffer, a complex exhibiting intense absorbance beyond 800 nm is rapidly formed. When the solution is allowed to stand for several minutes at 10 °C, the spectrum becomes more complex, evidence that more than one species produced in the incubation is capable of forming a "charge-transfer" complex with the enzyme.

Addition of NADH and L-lactic dehydrogenase to incubations of D-amino-acid oxidase and allylglycine results in a simplification of the spectrum (Figure 3). The spectrum initially evidenced is stabilized, decaying only by a very small amount over an hour at 10 °C. However, the species responsible for this complex is not formed quantitatively from allylglycine; a fivefold excess of the amino acid must be added to observe the maximum amount of long-wavelength absorbance. By monitoring the concomitant oxidation of NADH (loss of 340-nm absorbance), it was found that 80% of the added allylglycine is converted in pyrophosphate buffer to an NADH/L-lactic dehydrogenase reducible species.

A qualitatively similar complex was observed upon incubation of D-amino-acid oxidase and allylglycine in Hepes buffer. However, only a 1.5-fold excess of allylglycine was required for formation of the maximum long-wavelength absorbance, evidence that the responsible species is produced in ~70% yield rather than the 20% observed in pyrophosphate buffer.

A Complex with D-Amino-acid Oxidase upon Oxidation of α-Hydroxy Acids. To observe complexes with D-amino-acid oxidase formed by hydroxy acid oxidation products, an aliquot of a solution of 2-hydroxy-4-pentenoate was added to a solution containing a spectroscopic amount of D-amino-acid oxidase and a catalytic amount of L-hydroxyacid oxidase. A complex, distinct from that formed directly from allylglycine, was observed as a result of such an incubation (Figure 3). As expected, addition of NADH and L-lactic dehydrogenase prevented complex formation.

No long-wavelength absorbance was observed upon incubation of D-amino-acid oxidase with *trans*-2-keto-3-pentenoate, formed by incubations of D-amino-acid oxidase, L-hydroxyacid oxidase, and *trans*-2-hydroxy-3-pentenoate. Since

[4] The specific activity of the catalytic oxidation of *trans*-2-amino-3-pentenoate by D-amino-acid oxidase is much greater than the oxidation of the amino acid by L-amino-acid oxidase or of *trans*-2-hydroxy-3-pentenoate by L-hydroxy-acid oxidase. Therefore, a very small quantity of D-amino-acid oxidase was needed to generate the spectrum of *trans*-2-keto-3-pentenoate shown in Figure 2B, allowing the observation of the spectrum to ~215 nm. Because of the absorbance of the enzyme in both the sample and reference cells, the spectra of Figures 1A, 1B, and 2A, as well as the product of oxidation of *trans*-2-hydroxy-3-pentenoate, could not be recorded below 240 nm.

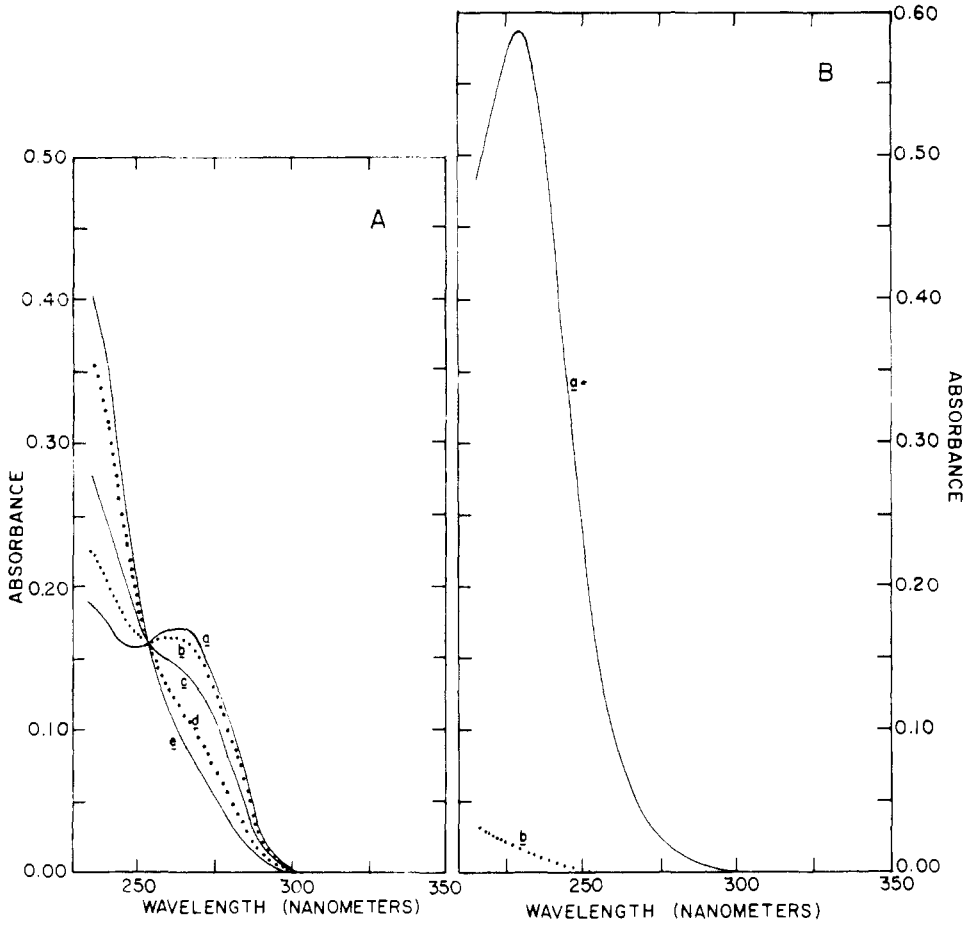
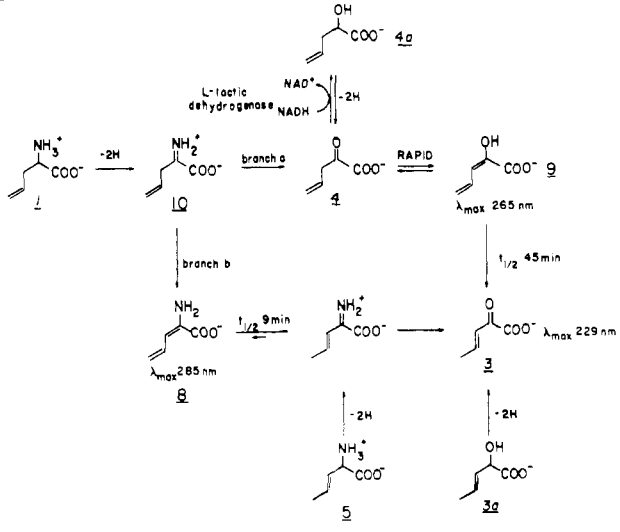


FIGURE 2: (A) Spectroscopic characterization of the products of 2-hydroxy-4-pentenoate (50 μM) oxidation by L-hydroxyacid oxidase as described under Methods. Spectra at the following time points are reproduced: (a) catalytic oxidation of the hydroxy acid was complete approximately 45 min after the start of incubation; (b) 60 min; (c) 90 min; (d) 150 min; (e) 210 min. (B) Spectroscopic characterization of the products of oxidation of *trans*-2-amino-3-pentenoate and 4-hydroxynorvaline by D-amino-acid oxidase: (a) oxidation product of *trans*-2-amino-3-pentenoate (50 μM); (b) oxidation product of 4-hydroxynorvaline (50 μM).

SCHEME I.



the species that has been shown to be the accumulated product is not responsible for a complex, two species formed in earlier steps of the pathway must be strong inhibitors of D-amino-acid oxidase.

Proposed Pathway Following Amino-acid Oxidase Catalyzed Oxidation of Allylglycine. We believe the pathway following the enzymatic oxidation of allylglycine can best be explained by the reactions of Scheme I. The species responsible for the complexes have been assigned the aminediene structure **8** and the hydroxydiene structure **9**. This assignment is based on the demonstrated position of the species in the pathway and on the precedents cited in the preceding paper of this issue concerning a similar complex produced upon enzymatic oxidation of propargylglycine (Marcotte and Walsh, 1978b).

The course of the reaction taken by the initial oxidation product 2-iminium-4-pentenoate (**10**) is dependent on the buffer used in the incubation. In Hepes buffer, branch b is predominant (~70%). However, in pyrophosphate buffer, hydrolysis of the imine to the keto acid (branch a) is four times faster than formation of the aminediene. 2-Keto-4-pentenoate (**4**) would not be expected to exhibit a λ_{max} at 265 nm. Previous workers (Bayly and Dagley, 1969; Collingsworth et al., 1973) have postulated a rapid equilibrium between 2-keto-4-pentenoate and 2-hydroxy-2,4-pentadienoate (**9**), the latter species being the major contributor to the ultraviolet absorbance.[5] Also, simple keto acids (such as pyruvate) are not observed to form long-wavelength absorbing complexes with D-amino-acid oxidase; therefore, we postulate that the hydroxydiene in equilibrium is responsible for this phenomenon. The thermo-

[5] Neither this work nor the publication of Collingsworth et al. (1973) produced direct evidence on the percentage of **4** and **9** present at equilibrium, although we believe the ultraviolet absorbance requires at least 20–30% of the product to exist in the hydroxydiene form.

dynamically favored product, *trans*-2-keto-3-pentenoate (**3**), must then be formed in a slower reaction of **9**.

In Hepes buffer, the decay of the 285-nm absorbance assigned to **8** does not result in the appearance of a new absorbance at 265 nm. This implies that the partition between formation of **8** and **4** ⇄ **9** is irreversible and the two branches of the pathway are mutually exclusive. *trans*-2-Keto-3-pentenoate (**3**) is apparently the end product in both of the buffers examined.

Stability of the Complexes between D-Amino-acid Oxidase and 2-Amino- and 2-Hydroxy-2,4-pentadienoate. As a means of demonstrating the stability of the complexes observed in Figure 3, 10 mM benzoate was added to solutions of the enzyme complexed with 2-amino-2,4-pentadienoate and 2-hydroxy-2,4-pentadienoate. In both cases, the long-wavelength absorbance decays very slowly, with a half-time of approximately 5 min at 10 °C. In contrast, a complex of D-amino-acid oxidase with anthranilate (K_d = 20 μM; Massey and Ganther, 1965) is displaced by 10 mM benzoate within the time of mixing ($t_{1/2}$ < 2 s). We have not directly measured the binding constants of these species, but these experiments suggest K_d values on the order of 10^{-8} M.

Inactivation of L-Amino-acid Oxidase upon Reaction with Allylglycine. We observed a slow loss of enzymatic activity when L-amino-acid oxidase was allowed to react with 5 mM allylglycine. Loss of activity was complete in about 3 h. However, this inactivation was completely prevented by the addition to the incubation of 5 mM dithiothreitol. Since addition of this nucleophilic scavenger prevents the inactivation, it must be concluded that the loss of activity is caused by alkylations induced by the reactive conjugated olefin, *trans*-2-keto-3-pentenoate, reacting from solution. No irreversible inctivation was observed upon reaction of D-amino-acid oxidase and allylglycine in a 3-h incubation; no essential enzyme group is apparently sensitive to the accumulating electrophile. Also, the catalytic activity of the enzyme is severely reduced by the noncovalent inhibition of D-amino-acid oxidase, slowing product accumulation. These observations are consistent with our previous findings on the differential susceptibility of D- and L-amino-acid oxidases toward inactivation upon their oxidation of vinylglycine (Marcotte and Walsh, 1976).

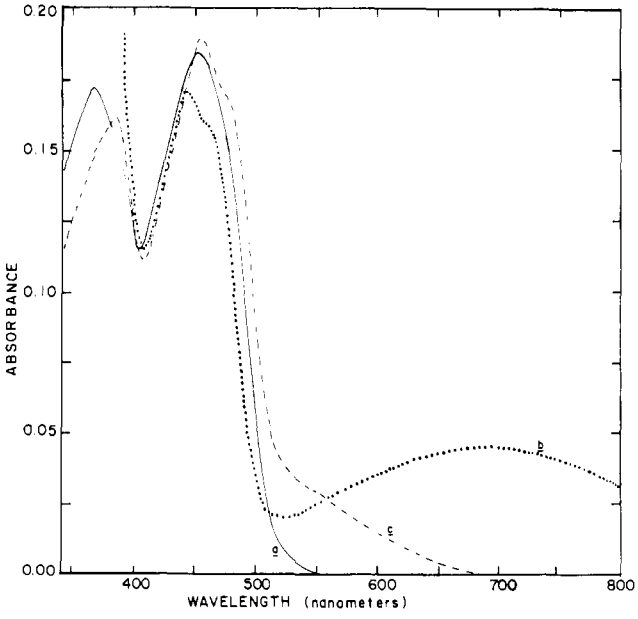


FIGURE 3: Spectra of the complexes formed between the oxidation products of allylglycine and 2-hydroxy-4-pentenoate and D-amino-acid oxidase. (a) Spectrum of oxidized D-amino acid oxidase (9 nmol) in 0.5 mL of pyrophosphate buffer (pH 8.5). (b) Spectrum obtained upon the addition of 100 nmol of DL-allylglycine; the incubation also contained 100 nmol of NADH and 0.5 mg of L-lactic dehydrogenase. (c) Spectrum of the complex derived from oxidation of 2-hydroxy-4-pentenoate. Hydroxy acid (200 nmol) was added to 0.5 mL of solution containing 9 nmol of D-amino-acid oxidase and 0.8 nmol of L-hydroxyacid oxidase.

Discussion

The pathway following oxidation of allylglycine provides an interesting contrast to the pathway following oxidation of propargylglycine. Unlike 2-iminium-4-pentynoate, which under all experimental conditions we have tested yields predominantly an acetylenic enamine (Marcotte and Walsh, 1978b), 2-iminium-4-pentenoate (**10**) partitions between formation of an olefinic enamine, 2-amino-2,4-pentadienoate (**8**), and hydrolysis. The role of the buffer in changing the course of the reaction has not been determined, nor have we pursued a systematic study of incubation conditions in an attempt to provide a cogent explanation.[6]

Both 2-amino- (**8**) and 2-hydroxy-2,4-pentadienoate (**9**) subsequently react via a relatively slow protonation at C_5. In contrast to the conjugated allene produced from propargylglycine, the conjugated olefins are not observed to lactonize, evidence of the heightened electrophilicity of the conjugated allene moiety relative to the conjugated olefin. This provides support for the mechanism invoked to explain the superiority of acetylenic substrate analogues over their olefinic counterparts as inactivators of number of enzymes (Morasaki and Bloch, 1972; Abeles and Walsh, 1973; Marcotte and Walsh, 1975).

Allylglycine has been known for some time to be a powerful convulsant, when the amino acid is administered intraperitoneally to mice (Schneider et al., 1960; McFarland and Wainer, 1965). Reduction in the rate of synthesis of γ-aminobutyrate, caused by inhibition of cerebral L-glutamate decarboxylase, has been implicated as the cause of the observed physiological reaction (Alberici et al., 1969; Horton and Meldrum, 1973). Orlowski et al. (1977) produced circumstantial evidence that an oxidation product of allylglycine, produced by oxidases in the brain, is the active agent. This point has recently been confirmed by ether extraction of an active convulsant from an acidified incubation of L-allylglycine and L-amino-acid oxidase (Horton and Meldrum, 1977; Horton, 1978).

The active species has been proposed to be 2-keto-4-pentenoate (**4**) (Orlowski et al., 1977; Horton, 1978), although this substance has proven too unstable to allow direct chemical synthesis and characterization. We believe this model system work, together with the syntheses of *trans*-2-amino- (**5**) and *trans*-2-keto-3-pentenoate (**3**), will provide new approaches toward the elucidation of the in vivo mechanism of action of allylglycine.

Acetylenic and olefinic substrate analogues have been used as agents which, after conversion by the target enzyme into active species, induce covalent modification of the enzyme. However, although D-amino-acid oxidase is severely inhibited upon oxidation of allylglycine, the complexes formed by 2-amino- (**8**) and 2-hydroxy-2,4-pentadienoate (**9**) are noncovalent and reversible. This contrasts with the reaction of D-

[6] We did examine the course of the reaction in 25 mM Tris-HCl, 35 mM KCl (pH 7.2), the buffer used by Collingsworth et al. (1973) to prepare "2-keto-4-pentenoate". The ultraviolet spectra of the products were qualitatively similar to those of the products of oxidation of allylglycine in 50 mM pyrophosphate buffer (pH 8.5) (Figure 1B), except that the subsequent isomerization reaction (decay of the 265-nm absorbance maximum) occurs much more slowly in the Tris/KCl buffer system ($t_{1/2}$ ≃ 4 h at 30 °C).

amino-acid oxidase with propargylglycine, in which both irreversible (Marcotte and Walsh, 1978a) and reversible (Marcotte and Walsh, 1978b) interactions are observed. This work has demonstrated that, when reaction between an enzyme and a substrate analogue results in release of a highly reactive product, it is necessary to characterize the pathway following the enzymatic step to determine if any species formed subsequently interacts with that enzyme or other enzymes in vivo.

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Inactivation of Ribulosebisphosphate Carboxylase by Modification of Arginyl Residues with Phenylglyoxal†

John V. Schloss,‡ I. Lucile Norton, Claude D. Stringer, and Fred C. Hartman*

ABSTRACT: Phenylglyoxal rapidly and completely inactivates spinach and *Rhodospirillum rubrum* ribulosebisphosphate carboxylases. Inactivation exhibits pseudo-first-order kinetics and a reaction order of approximately one for both enzymes, suggesting that modification of a single residue per protomeric unit suffices for inactivation. Loss of enzymic activity is directly proportional to incorporation of [^{14}C]phenylglyoxal until only 30% of the initial activity remains. For both enzymes, extrapolation of incorporation to 100% inactivation yields 4–5 mol of [^{14}C]phenylglyoxal per mol protomer. Amino acid analyses confirm the expected 2:1 stoichiometry between phenylglyoxal incorporation and arginyl modification and suggest that other kinds of amino acid residues are not modified. (Thus, inactivation correlates with modification of 2–3 arginyl residues per protomer.) The substrate ribulose bisphosphate and some competitive inhibitors reduce the rates of inactivation of the carboxylases and prevent modification of about 0.5–1.0 arginyl residue per protomer. Inactivation is therefore a consequence of modification of a small number of residues out of the 35 and 29 total arginyl residues per protomer in spinach and *R. rubrum* carboxylases, respectively.

Because of its essentiality to the photosynthetic assimilation of CO_2, Rbl-P_2[1] carboxylase (EC 4.1.1.39) has been the subject of innumerable physiological, genetic, and biochemical studies (for a review, see Jensen & Bahr, 1977). A recent surge of interest in the mechanism and regulation of catalytic activity ensued with the realization that the carboxylase has an associated oxygenase activity which accounts for photorespiration (Bowes et al., 1971; Tolbert, 1973). Photorespiration is an energy-wasteful process which reduces the net amount of CO_2 fixation; when photorespiration is decreased by cultivating plants under low oxygen tension, plant yield can increase by

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[1] Abbreviations used: Rbl-P_2, D-ribulose 1,5-bisphosphate; PGO, phenylglyoxal; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; CR-P_2, 2-carboxyribitol 1,5-bisphosphate; Fru-P_2, D-fructose 1,6-bisphosphate; 3-PGA, D-3-phosphoglyceric acid; 6-PGlu, 6-phosphogluconic acid; butanediol-P_2, butane-1,4-diol 1,4-bisphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid.

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