Properties of D-Amino Acid Oxidase Covalently Modified upon Its Oxidation of D-Propargylglycine†

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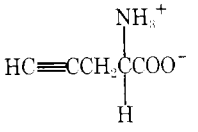
ABSTRACT: Upon oxidation of D-propargylglycine by D-amino acid oxidase, the enzyme is converted by covalent alkylation to catalytic species with different properties from those of native enzyme. At least five distinct modified enzyme species are present in the preparation, as determined by gel electrofocusing. Individual characterization of the components has not yet been attempted. The combined kinetic and spectral properties of the preparation have been studied. The modified enzymes have a marked preference for hydrophobic amino acids: the rates of oxidation decrease in the series D-phenylalanine, D-methionine, D-norleucine, D-norvaline, D-α-aminobutyrate, D-alanine. In addition, the observed K_ms of the amino acids are increased, especially those of the smaller substrates (D-alanine and D-α-aminobutyrate). A primary kinetic isotope effect is observed upon oxidation of amino acids by the modified enzymes, evidence that this catalysis exhibits a different rate-determining step from catalysis by native enzyme. The modified apoenzyme exhibits intense absorbance at 318–320 nm, not present in native enzyme. This chromophore can be partially (75%) removed by treatment of the modified enzyme with hydrazine. However, the activity of native enzyme is not substantially restored by this process, suggesting the existence of superficial alkylations in addition to the modification responsible for the observed changes in kinetic parameters.

The use of acetylenic and olefinic active-site-directed, mechanism-based inactivators (termed "suicide substrates") is the subject of ongoing research in this and other laboratories (Walsh, 1977). The flavoenzyme D-amino acid oxidase was found to be inactivated upon carrying out the oxidation of D-propargylglycine (**1**), an observation first reported by Horiike et al. (1975). In that work, the enzyme was shown to carry out at least several hundred catalytic oxidations before suffering inactivation.



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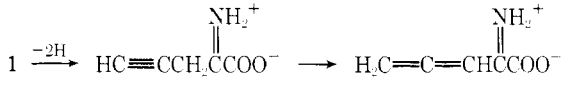
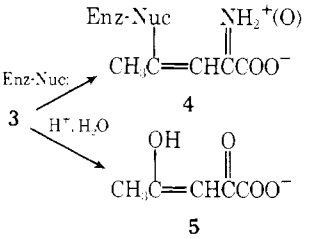
Work in this laboratory (Marcotte and Walsh, 1976) produced evidence on the mechanism of inactivation. We proposed that after oxidation of propargylglycine (**1**) to the corresponding imino acid (**2**) the β protons are rendered acidic and propargylic rearrangement leads to the conjugated allenic imino acid (**3**).



2 **3**

Attack at the electrophilic fourth carbon of the conjugated allene **3** by an enzyme nucleophile would result in covalent modification (**4**) (inactivation) of the enzyme. Attack by water molecules, we hypothesized, should after a series of prototropic shifts yield acetopyruvate (**5**), which we isolated after passage of the reaction mixture through a Dowex 50 H^+ column.



4 **5**

We now report the further characterization of the alkylated protein species produced upon oxidation of D-propargylglycine by D-amino acid oxidase. We have found that the enzyme is not truly inactivated but rather is converted by the alkylation process into a mixture of catalytic species, the combined substrate specificity and kinetic parameters of which are markedly different from those of native enzyme. These observations resolve contradictions between our earlier work (Marcotte and Walsh, 1976) and that of Horiike et al. (1975) concerning the residual activity of the enzyme upon prolonged incubation with propargylglycine.

The pathway following oxidation of propargylglycine in those catalytic events which do not result in covalent modification of the enzyme has also been further studied. Acetopyruvate has been found not to be the major product of the reaction but rather is derived from acidification of the accumulated product. In addition to the accumulated product, two other species present in the incubation have been shown to be very strong *noncovalent* inhibitors of D-amino acid oxidase. The elucidation of the probable structures of these species will be published separately.

Experimental Section

Materials

Enzymes. D-Amino acid oxidase from frozen hog kidneys (purchased from Pel-Freez Biologicals) was initially purified

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TABLE I: Substrate Specificity of Native and Propargylglycine-Modified D-Amino Acid Oxidase.

	Native enzyme		Modified enzyme	
	V_{max}[a]	K_m (mM)	V_{max}[a]	K_m (mM)
D-Phenylalanine	220	1.7	130	2
D-Methionine	120	<0.5	100	3
D-Norleucine	70	<0.5	50	2
D-Norvaline	20	<0.5	35	2
D-α-Aminobutyrate	30	0.5	30	10
D-Alanine	100	1.2	20	50
D-Proline	310	1.4	27	11
D-Valine	70	0.7	6	5
L-Phenylalanine	0		0	
L-Alanine	0		0	
D-Phenylalanine (K_i)[b]				2
Benzoate (K_i)[b]		0.006		0.3
Anthranilate (K_i)[b]		0.08		1.5

[a] Maximal velocities relative to the rate of oxidation of D-alanine by native enzyme. [b] Versus D-alanine as substrate.

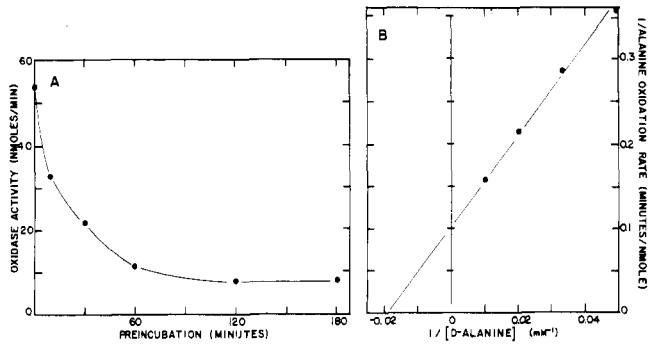

FIGURE 1: Inactivation of D-amino acid oxidase induced by D-propargylglycine (assay with D-alanine). Preincubation: in 0.25 mL of 0.05 M sodium pyrophosphate (pH 8.5), 40 mM D-propargylglycine, 2.5 μg of catalase, 0.02 mM FAD, and 2.1 nmol of D-amino acid oxidase at 25 °C. (A) Enzymatic activity of 0.01 mL of preincubation in 1.0 mL of 0.05 M pyrophosphate buffer containing 0.1 M D-alanine. (B) Apparent K_m of D-alanine after a 180-min incubation of enzyme with D-propargylglycine.

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-Amino acid oxidase (*Crotalus adamanteus* venom), catalase (beef liver), and L-lactic dehydrogenase (rabbit muscle) were purchased from Sigma.

Reagents. Propargylglycine (2-amino-4-pentynoic acid) was synthesized and resolved by the method of Jansen et al. (1969). DL-Propargyl[2-^{2}H]glycine was prepared by acid hydrolysis of diethyl acetamidopropargylmalonate (Gershon et al., 1954) in $^2HCl/^2H_2O$ purchased from Stoler isotopes. D-[^{2}H]alanine was prepared by an adaptation of the $CuSO_4$/salicyladehyde mediated exchange precedure of Ikawa and Snell (1954). DL-[2-^{2}H]Phenylalanine was purchased from MSD Isotopes. All other amino acids, FAD,[1] NADH, and Hepes were purchased from Sigma Chemical Co. Buffer salts and solvents were commercially available reagent-grade materials.

Methods

Preparation of Modified D-Amino Acid Oxidase. D-Propargylglycine (226 mg, 2 mmol) was dissolved in 5 mL of 0.1 M sodium pyrophosphate buffer, pH 8.0. FAD (0.5 μmol) and catalase (50 μg) were added. Reaction was initiated with 0.1 μmol of D-amino acid oxidase and gently stirred under an oxygen atmosphere for up to 24 h at 23–25 °C. The solution was dialyzed at 0 °C four times against 200 volumes of 1 M KBr/0.1 M pyrophosphate (pH 8.3) for 24 h each dialysis. The salt was removed by dialysis against 0.02 M pyrophosphate buffer. The enzyme was reconstituted with excess commercial FAD, and the excess was removed by dialysis against 0.02 M pyrophosphate buffer.

Assays of Oxidase Activity (Both Native and Modified Enzyme). The oxidation of all amino acids, except D-alanine and D-propargylglycine, was assayed via oxygen consumption of a solution of the amino acid in 0.05 M pyrophosphate buffer (pH 8.5) using a Clarke-type electrode in a 0.4-mL chamber thermostated at 30 °C. D-Alanine oxidation was monitored in the same buffer by coupled assay of pyruvate oxidation by NADH and L-lactic dehydrogenase. Oxidation of D-propargylglycine was measured directly from the absorbance at 318 nm of the product of propargylglycine oxidation in 0.1 M Hepes/0.1 M butylamine hydrochloride (pH 8) (Marcotte and Walsh, 1976). FAD (10 μM) and catalase (25 μg/mL) were added to all assay solutions at 30 °C.

Spectral Characterization of the Modified D-Amino Acid Oxidase. All spectra were recorded while the sample was maintained at 8–10 °C to minimize turbidity in the solutions. Enzyme in 0.5 mL of pyrophosphate buffer was used. Aliquots of 0.5 M sodium benzoate, 0.5 M sodium anthranilate, and 0.1 M D-phenylalanine were added to the modified enzyme solution to determine the effects induced by these substances.

Results

Kinetic Properties of D-Amino Acid Oxidase Modified upon Its Oxidation of D-Propargylglycine. Residual Activity. Further examination of the kinetic properties of D-amino acid oxidase after its reaction with D-propargylglycine was carried out in an effort to resolve the discrepancy between our previous work (Marcotte and Walsh, 1976) and that of Horiike et al. (1975) concerning the residual activity of the enzyme preparation upon prolonged incubation with D-propargylglycine. Horiike et al. had reported residual activity of about 20%, unsusceptible to further loss; however, we had observed 2–4% residual activity. Both groups had used D-alanine as substrate to measure the remaining oxidase activity (as in Figure 1A).

The contradiction is resolved by the fact that the residual activity observed is strongly dependent on the concentration of D-alanine used to assay the incubation. Since the concentration of D-alanine initially used in our experiments (10 mM) is well above the K_m of D-alanine oxidation by native enzyme (1.2 mM), a possible explanation was that the modified enzyme, in addition to having a much reduced V_{max}, has a greatly elevated K_m over that of native enzyme. That this is, in fact, the explanation is demonstrated in Figure 1B. Upon modification, the observed K_m has been elevated by a factor of 40.

Substrate Specificity. Because of the altered kinetic properties of the modified enzyme in its reaction with D-alanine, other amino acids were tested as substrates of the modified enzyme. The preparation (exhaustively dialyzed after incubation with propargylglycine and then reconstituted with FAD) was found to have a marked preference for hydrophobic amino acids: the rates of catalytic oxidation decreasing in the series D-phenylalanine, D-methionine, D-norleucine, D-norvaline, D-α-aminobutyrate, D-alanine. As is shown in Table I, the substrate specificity is expressed in both an increase in

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; FAD, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; DEAE, diethylaminoethyl.

TABLE II: Isotope Effect on Oxidation of Amino Acids by Native and Propargylglycine-Modified D-Amino Acid Oxidase.

	Native enzyme			Modified enzyme		
	on V_{max}[a]	on K_m[b]	on V_{max}/K_m	on V_{max}[a]	on K_m[b]	on V_{max}/K_m
D-Alanine	1.0	1.0	1.0	2.7	1.0	2.7
DL-Phenylalanine	1.6	0.5	3.2	1.9	1.0	1.9
DL-Propargylglycine	1.7	0.6	2.9	4.3	1.0	4.3

[a] Maximal velocity of enzymatic oxidation of α-[^{1}H]amino acid divided by maximal velocity of oxidation of α-[^{2}H]amino acid. [b] K_m of α-[^{1}H]amino divided by K_m of α-[^{2}H]amino acid.

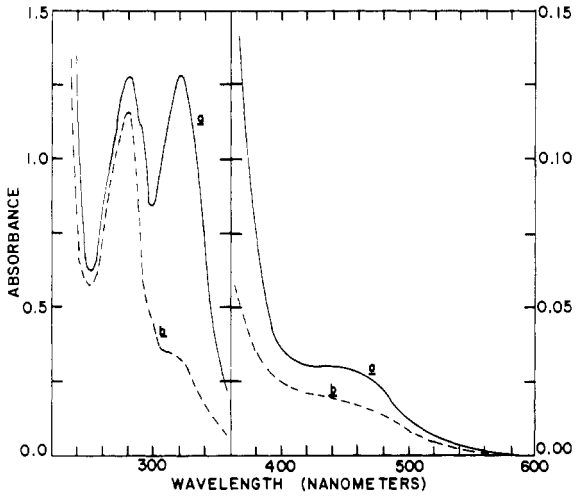

FIGURE 2: (a) Spectrum of apo-D-amino acid oxidase after a 12-h incubation with D-propargylglycine followed by exhaustive dialysis. (b) Modified enzyme of (a) after dialysis vs. 0.1 M hydrazine for 48 h.

maximal velocity and decrease in K_m as hydrophobicity is increased. D-Phenylalanine was examined as a competitive inhibitor of D-alanine oxidation by the modified enzyme. It was found to be a potent inhibitor, exhibiting a K_i of 2 mM, identical to the K_m for the oxidation by the modified enzyme. This evidence implies that the same species is catalyzing the oxidation of both amino acids (although experiments to be presented demonstrate the inhomogeneous nature of the preparation).

In addition, D-proline, the substrate with the highest V_{max} of any amino acid for native enzyme, is a poor substrate for the alkylated enzyme. The apparent K_is (vs. D-alanine) of the competitive inhibitors benzoate and anthranilate are included in Table I: the binding of these molecules is apparently severely weakened by the modification. It was found that the enzyme retains its enantiomeric specificity—L-alanine and L-phenylalanine are not detectably oxidized.

Rate-Determining Step. In Table II are summarized the results of the examination of the isotope effects (substituting deuterium for hydrogen at carbon-2) upon the oxidation of three amino acids, D-alanine, DL-propargylglycine, and DL-phenylalanine, by native and modified D-amino acid oxidase. The breakage of the C_2–H bond is clearly substantially rate determining in catalytic oxidation of these amino acids by the modified enzyme. In view of the wide variation in substrate specificity, it is of interest to note that the isotope effect on phenylalanine and propargylglycine oxidation is increased and is exhibited only on V_{max}, in contrast to the findings with native enzyme. These data support the supposition that a change in the rate-determining step is a kinetic consequence of the modification.

Spectral Properties of D-Amino Acid Oxidase Modified upon Its Oxidation of D-Propargylglycine. Apoenzyme. After incubation of D-amino acid oxidase with D-propargylglycine under an oxygen atmosphere for the desired interval, the incubation was dialyzed at 0 °C four times against 200 volumes of buffer containing 1 M KBr, followed by dialysis against low salt buffer. As had been reported by Horiike et al., the modified apoenzyme exhibits an absorbance maximum at 318–320 nm, which is not present in native apoenzyme (Figure 2). The ratio of the absorbance maxima at 318 and 278 nm is a measure of the degree of alkylation of the enzyme and is a function of the time of incubation (Table III). The A_{318}/A_{278} ratio of approximately 1.0 appears to represent the alkylation of all susceptible enzyme nucleophiles in the preparation.

TABLE III: Progress of Propargylglycine-Induced Alkylation of D-Amino Acid Oxidase as Measured by the Spectral Properties of the Apoenzyme.

Time of incub (h)	A_{318}/A_{278}	A_{450}/A_{278}
3	0.67	0.031
7	0.87	0.027
12	1.00	0.025
24	1.01	0.025

Even though the conditions of dialysis to which the modified enzyme preparation was subjected are sufficient to remove all traces of the coenzyme from the native enzyme (Massey and Curti, 1966), the modified apoenzyme retains significant visible absorbance (Figure 2). This chromophore was not reduced by addition of D-phenylalanine. After heating an aliquot of the preparation to 100 °C for 4 min, fluorescence characteristic of FAD was detected in the solution. The amount of fluorescence was approximately half that predicted by the 450-nm absorbance of the modified enzyme preparation. In reproducible experiments, the retained visible absorbance is greater in D-amino acid oxidase which had been incubated with D-propargylglycine for short intervals (2–3 h) than that incubated more exhaustively (12 h) (Table III). The reason for these observations is the inhomogeneous nature of the preparation and the presence of both active and inactive protein species in it. We have not yet attempted to separate the components, a fraction of which must have the coenzyme placed in a severely changed environment by the modification. In such enzyme molecules, the FAD has been rendered catalytically inactive and unremovable by high salt dialysis.

Another means of quantitating the extent of inactivation is a kinetic assay of the preparation with propargylglycine, monitoring the ultraviolet absorbance of the product of propargylglycine oxidation (Marcotte and Walsh, 1976). It is of interest that, even after 96 h of high salt and 24 h of low salt dialysis, enzyme which had been incubated for 12 h with D-propargylglycine remains fully modified (i.e., reincubation of a catalytic amount of enzyme with propargylglycine results in linear production of product with time), pointing out the stability of the alkylation under these conditions.

Reconstituted Modified Enzyme. Upon the addition of D-

phenylalanine to an anaerobic solution of the modified enzyme, reconstituted with stoichiometric FAD, greater than 80% of the reconstituted flavin absorbance (Figure 3A) is rapidly bleached, demonstrating the catalytic competence of most of the reconstituted enzyme molecules in the preparation. The effect of the binding of the inhibitors benzoate and anthranilate to the enzyme was also studied. In comparison with the experiments of Massey and Ganther (1965) using native enzyme (Figure 3B, inset), Figure 3B demonstrates that the manner of binding of the enzyme and inhibitors has been significantly altered upon modification.

Characterization of the Modified Enzyme Using Gel Electrofocusing. To determine the number of species produced upon the reaction of D-amino acid oxidase and propargylglycine, the protein composition of the modified enzyme preparation was analyzed by electrofocusing in polyacrylamide gels (Righetti and Drysdale, 1974). Protein was determined using a staining solution of Coomassie blue; gels were stained for activity (using DL-phenylalanine as substrate) as described by Hayes and Wellner (1969). The enzyme was homogeneous by electrofocusing analysis before incubation with D-propargylglycine.

After incubation of D-propargylglycine and D-amino acid oxidase for 3 h, only a trace of native enzyme could be detected. At least five active and several inactive bands could be observed. Native enzyme focuses near the alkaline end of gels containing pH 4–6 ampholytes; all of the modified enzymes species exhibited isoelectric points more acidic than native enzyme. When electrofocusing of a preparation which had been incubated for 7 or 12 h with D-propargylglycine was attempted, the stains did not reveal discrete bands, rather a smear of both protein and activity was present in the gel. Although not conclusive, these experiments imply the existence of a large number of protein species in the incubation.

Reaction of the Modified D-Amino Acid Oxidase with Hydrazine. We have found that acetopyruvate and its derivatives react with hydrazine in buffered solution with bleaching of their ultraviolet absorbances (Marcotte and Walsh, unpublished observations). Because we believed it probable (Marcotte and Walsh, 1976) that the modified enzyme has the residue **4** attached to an enzyme nucleophile, we felt that this group might also be labile to hydrazine. Incubation of the modified enzyme solution at 30 °C for 6 h with 0.05 M hydrazine resulted in the loss of about 75% of the 318-nm absorbance; the remainder of the absorbance was stable to this treatment over 24 h. Therefore, the reaction was conducted under milder conditions: dialysis at 0 °C vs. 0.1 M hydrazine for 48 h. A similar bleaching of the 318-nm chromophore was observed (Figure 2).

After removal of the hydrazine by dialysis, we examined the kinetic properties of this enzyme preparation. Upon reincubation of a catalytic quantity of enzyme with 0.01 M D-propargylglycine in Hepes/butylamine buffer, the rate of increase of product absorbance (318 nm) was essentially unchanged from before treatment with hydrazine. Furthermore, little kinetic inactivation was apparent: the final rate of product formation (after ~10 min) was 80% the initial rate and was unsusceptible to further reduction. Upon assay of the hydrazine-treated protein with 10 mM D-phenylalanine and 10 mM D-alanine, the rates of oxidation of these amino acids were unchanged from before treatment with hydrazine. Although the kinetic properties of this preparation were not fully characterized, these data imply that, although the chromophore has been substantially bleached, the alkylation(s) responsible for the change in kinetic parameters has, for the most part, not been removed.

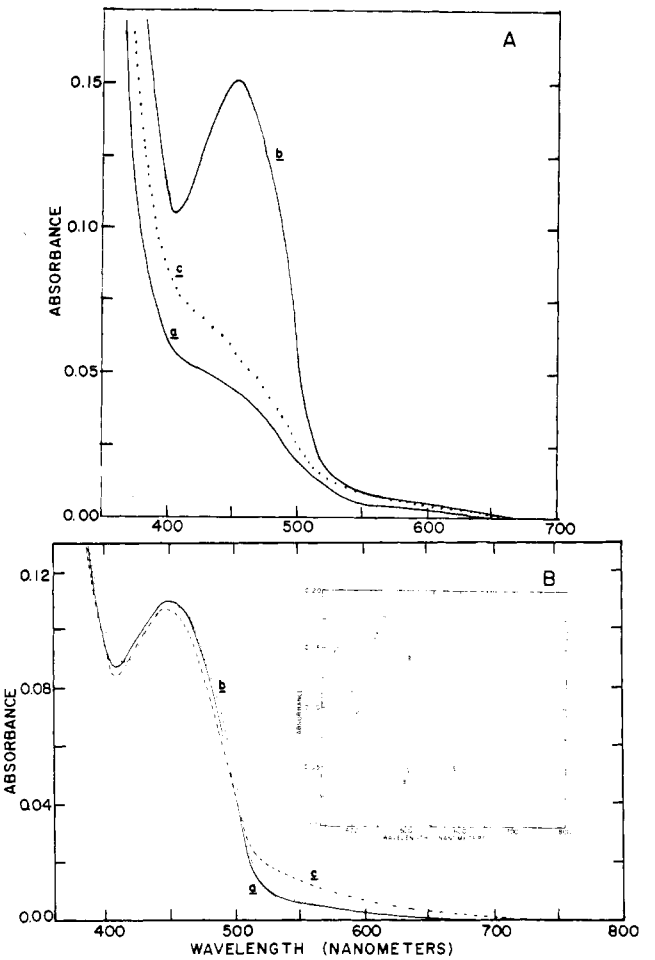

FIGURE 3: (A) (a) Modified apoenzyme spectrum; (b) modified enzyme reconstituted with FAD; (c) after addition of 2 mM D-phenylalanine to anaerobic reconstituted modified enzyme. (B) (a) Modified enzyme reconstituted with FAD; (b) upon addition of 5 mM sodium benzoate to(a); (c) upon addition of 5 mM sodium anthranilate to (a). Inset: (a) native holo-D-amino acid oxidase; (b) upon addition of 1 mM sodium benzoate to (a); (c) upon addition of 1 mM sodium anthranilate to (a).

The composition of the hydrazine-treated modified enzyme was examined using gel electrofocusing. No native D-amino acid oxidase could be detected in the gel. At least five discrete active species were present, all with isoelectric points more acidic than native enzyme.

Discussion

The modification of D-amino acid oxidase upon its oxidation of D-propargylglycine was the first active-site-directed reagent found which covalently modifies an amino acid residue of D-amino acid oxidase (Horiike et al., 1975). Recent work from the laboratory of Porter and Bright (1976) reports the modification of D-amino acid oxidase with an affinity reagent, *N*-chloro-D-leucine. Incubation of the enzyme with this substrate analogue results in the incorporation of two atoms of chlorine into the enzyme, possibly a specific tyrosine residue. Enzyme thus modified has changed kinetic parameters—the V_{max} of D-alanine oxidation is reduced to 28% and the K_m is increased from 1.2 to 6.1 mM. By using rapid kinetic techniques, Porter and Bright (1976) demonstrated that modification of one fundamental step, i.e., a 1000-fold reduction in the specific rate of flavin reduction, is the cause of the change in steady-state kinetic parameters. Such a stopped-flow apparatus was not available for the examination of propargylglycine-modified D-amino acid oxidase; therefore, indirect

methods were used to gather information on the mechanism of its catalysis.

"Wounded enzyme" species have also been produced by thiol reagents (not necessarily active-site directed); two enzymes have been so modified upon reaction with methyl methanethiolsulfonate. In the case of rabbit muscle creatine kinase (Smith and Kenyon, 1974), the modified enzyme was found to retain 20% of its initial activity with only relatively minor changes in the Michaelis constants of its substrates (Maggio et al., 1977). In contrast, pig heart lactic dehydrogenase exhibits no change in V_{max} upon modification, but the K_m of pyruvate is increased from 40 μM to 12 mM (Bloxham and Wilton, 1977). The reaction of pig heart cytoplasmic aspartate aminotransferase in syncatalytic manner with a variety of thiol reagents has been reported to yield enzyme species with a considerable fraction of its initial activity (Birchmeier et al., 1973). More detailed kinetic properties of the catalytic species produced upon these modifications were not reported.

We have not yet attempted to characterize the amino acid residue(s) which are alkylated upon reaction of D-amino acid oxidase with propargylglycine. The enzyme preparation is demonstrably heterogeneous, and only by the isolation and individual study of each of the components could meaningful experiments on the problem be performed. The observed kinetic and spectral properties of the preparation are certainly the cumulative total of the species present in the incubation; this paper is presented as preliminary work on the characterization of the bulk properties of the modified enzyme species.

The present work is the first documented case of a modified enzyme being found to have dramatically changed substrate specificity. The configuration of the active site following alkylation is likely to be more complex than simple steric interference at the active site. The introduction of five carbon, presumably negatively charged, residues induces a conformational change such that D-phenylalanine, a large and hydrophobic amino acid, is the best substrate. It is surprising that the oxidation of the smallest amino acid substrate, D-alanine, is the most severely affected. Because of the residual activity, the site of the modification cannot be exactly "at the active site" in the sense that the phrase is commonly used. However, it is clear that the modification severely alters the structure of the active site, in a manner as yet undertermined.

The rate-determining step of the enzymatic oxidation process has also been changed upon modification. The appearance of a primary kinetic isotope effect implies that a proton-transfer step is at least partially rate determining rather than product release as in native enzyme (Bright and Porter, 1975). The binding of the competitive inhibitors benzoate and anthranilate is even more severely impaired upon modification than that of any amino acid; that is consistent with enzyme recognition of these compounds as product analogues.

The reaction of the modified enzyme preparation with hydrazine was studied with the hope of gaining information on the chemical lability of the alkylation(s) responsible for the change in kinetic properties of the enzyme. However, even though evident reaction took place, little activity was restored. There are two possible explanations for these findings: either the reaction with hydrazine alters the chromophore without causing the removal of the important alkylation or there exists at least one alkylation on each enzyme molecule which is insensitive to hydrazine and which is the *primary cause* of the change in kinetic parameters.

Experiments involving the alkylation of D-amino acid oxidase with radioactively labeled propargylglycine would be useful in studying the properties of the modified enzyme species and also demonstrate the possible differential lability of the various alkylations to hydrazine and other agents. However, since the enzyme is observed to catalyze a great number of catalytic oxidations before suffering modification (Marcotte and Walsh, 1976), a large amount of labeled material would be required. We intend to attempt such experiments after the preparation has been fully characterized by other means.

The reaction of D-propargylglycine with D-amino acid oxidase has been found to be far more complicated than was evident in the original work of Horiike et al. (1975) or from our previous publication (Marcotte and Walsh, 1976). Together with the study of the products of propargylglycine oxidation which will be published subsequently, this work has demonstrated the problems that can arise in the use of presumed "active-site-directed" substrate analogues as alkylating agents of enzymes.

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