Electrophilic Amination of a Single Methionine Residue Located at the Active Site of D-Amino Acid Oxidase by *O*-(2,4-Dinitrophenyl)hydroxylamine†

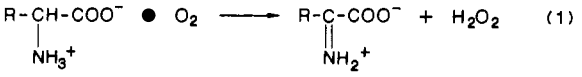
Claudius D'Silva, Charles H. Williams, Jr., and Vincent Massey*

Department of Biological Chemistry, University of Michigan, and Veterans Administration Medical Center, Ann Arbor, Michigan 48109

Received February 19, 1986; Revised Manuscript Received April 4, 1986

ABSTRACT: *O*-(2,4-Dinitrophenyl)hydroxylamine is a rapid active-site-directed inhibitor of D-amino acid oxidase: modification results in specific incorporation of an amine group into an accessible nucleophilic residue with concomitant release of 2,4-dinitrophenol. The reaction is prevented by the competitive inhibitor benzoate, indicating an active-site-directed reaction. A stoichiometry of 1–1.5 mol of amine residues per enzyme bound flavin adenine dinucleotide monomer was observed at pH 7.0. Amino acid and sequence analyses show that His-217 is not the target of the modification reaction. Dependence of the modification on pH, model studies on functional groups present on amino acids, and thiolysis studies on aminated enzyme collectively indicate that the modification is located on a methionine residue at or near the active site of the enzyme. Aminated enzyme, although spectrally similar to native enzyme, exhibits a 7–9-nm blue shift in the 455-nm flavin absorption. Benzoate perturbs the spectrum of aminated enzyme, but binding relative to native enzyme is much weaker (K_d ca. 300 times greater at pH 8.0).

D-Amino acid oxidase (EC 1.4.3.3) is a flavoprotein that catalyzes the oxidative deamination of D-amino acids to the corresponding imino acids, concomitant with the reduction of oxygen to hydrogen peroxide as summarized in eq 1. The



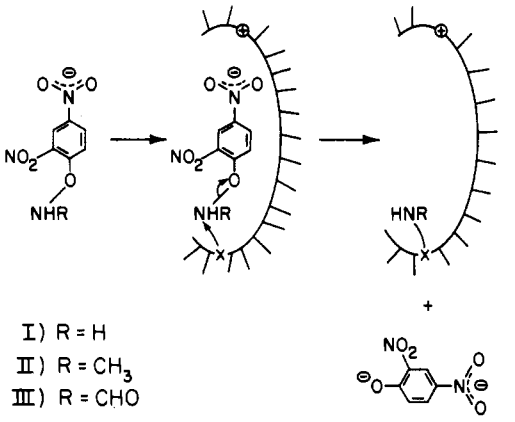
(1)

imino acid is subsequently hydrolyzed nonenzymatically to α-keto acid and ammonia (Porter & Bright, 1972). This enzyme is a typical example of a flavoprotein dehydrogenase/oxidase that undertakes catalysis by abstraction of a proton from the α-carbon of the substrate with a basic residue and subsequently transfers two electrons from the transient substrate carbanion to the flavin. Support for this mechanism comes from rapid reaction studies (Porter et al., 1977), the elimination reaction with β-chloroalanine (Walsh et al., 1971) and α-amino-β-chlorobutyrate (Walsh et al., 1973), the suicide reaction with vinyl- and D-propargylglycine (Marcotte & Walsh, 1978), and the formation of a covalent adduct during oxidation of the nitroethane anion (Porter et al., 1973) [see Bright and Porter (1975) for a review].

Several residues have been identified at or near the active site. These include Lys-204 (Swenson et al., 1982), Tyr-55 (Swenson et al., 1982), Tyr-224 (Ronchi et al., 1980), His-217 (Swenson et al., 1983, 1984a,b), and a presently unidentified arginine residue (Nishino et al., 1980; Ferti et al., 1981; Fitzpatrick & Massey, 1983). In addition, ligand binding studies indicate that groups with pK_a = 6.3 and 9.3 (in addition to the 3-imino group of FAD[1]) facilitate binding of benzoate (Quay & Massey, 1977).

Of these residues His-217 is an attractive candidate for assignment to the residue of pK_a = 6.3 implicated in ligand binding or in proton abstraction. Both dansyl chloride (Swenson et al., 1983) and methyl *p*-nitrobenzenesulfonate (MNBS) (Swenson et al., 1984a,b) modify His-217, but the latter reagent achieves a minimal modification by introduction of a methyl group (Nakagawa & Bender, 1970). Kinetic evaluation of enzyme methylated at His-217 (Swenson et al., 1984b) shows a 2-fold isotope effect for this enzyme that is not observed with native enzyme when deuterated D-alanine is used as a substrate, indicating that C–H bond cleavage becomes partially rate limiting, consistent with the perceived role for this residue. Encouraged by results obtained via the use of methyl *p*-nitrobenzenesulfonate in assigning a role to His-217, we considered further information on the roles of residues at the active site of this enzyme could be obtained by the use of a similar reagent, capable of minimal modification.

Scheme I



O-(2,4-Dinitrophenyl)hydroxylamine (DNPHA) (I) is an electrophilic aminating agent that reacts with a wide variety of nucleophiles to achieve a minimal modification in the in-

†Supported by grants from the U.S. Public Health Service, GM 11106 and GM 21444, and by the Medical Research Service, Veterans Administration.

*Address correspondence to this author at the Department of Biological Chemistry.

[1] Abbreviations: DNPHA, *O*-(2,4-dinitrophenyl)hydroxylamine; DAAO, D-amino acid oxidase; MNBS, methyl *p*-nitrobenzenesulfonate; FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; HPLC, high-performance liquid chromatography; Boc, *tert*-butyloxycarbonyl.

0006-2960/86/0425-5602$01.50/0

troduction of an amine group (Sheradsky et al., 1972; Tamura et al., 1973; Oae & Yamamoto, 1973). This reagent may be expected to be active site directed in view of its structural similarity to methyl *p*-nitrobenzenesulfonate and fluorodinitrobenzene, both of which specifically modify D-amino acid oxidase. Reaction with an appropriately positioned nucleophile would result in the introduction of a small amine residue and release of 2,4-dinitrophenol as shown in Scheme I, with subsequent impairment of the catalytic activity of the enzyme. Unlike methyl *p*-nitrobenzenesulfonate, this reagent is stable in aqueous solution, possesses a planar structure, and is slightly smaller in size. These properties suggest that this reagent will cause minimal disruption of the active site, thus making it an ideal candidate for enzyme modification.

In this paper we report the properties of this reagent, and its derivatives, and its use as a potent active-site-directed inhibitor of D-amino acid oxidase. Modification results in an enzyme similar in several respects to that obtained by modification with methyl *p*-nitrobenzenesulfonate, in which His-217 was modified. However, analysis of the nature of the modification indicates that the residue modified is not His-217 but may, in fact, be a methionine residue on the basis of pH dependence data, specificity of the reagent with amino acids, and stability of the modification to thiols.

Experimental Procedures

Materials and Methods. D-Amino acid oxidase was purified from pig kidney as described previously (Curti et al., 1973). *N*-Acetyl-L-cysteine, *N*-acetyl-L-methionine, oxidized glutathione, *N*-methylhydroxylamine, hydroxylamine-*O*-sulfonic acid, and *N*-Boc-L-histidine were obtained from Sigma Chemical Co., *N*-acetyltryptophan was from Nutritional Biochemical Corp., fluorodinitrobenzene was from Calbiochem, and *tert*-butyl *N*-hydroxycarbamate was from Aldrich.

Syntheses. (1) *O*-(2,4-Dinitrophenyl)hydroxylamine (DNPHA) (I) was synthesized from *tert*-butyl *N*-(2,4-dinitrophenoxy)carbamate or ethyl *O*-(2,4-dinitrophenyl)-acetohydroxamate by the method of Sheradsky et al. (1972) and Tamura et al. (1973). The product was further purified by column chromatography on silica (mp 105–107 °C; lit. 110 °C) and the purity ascertained by thin-layer chromatography. The ultraviolet absorption in 0.1 M potassium phosphate buffer (pH 7.5) showed a λ_{max} at 300 nm with an ϵ_{300nm} of 10 700 M^{-1} cm^{-1} (2,4-dinitrophenol showed its absorption maximum at 358 nm with an ϵ_{358nm} of 15 400 M^{-1} cm^{-1}). Proton NMR (Varian T60) ($CDCl_3$) gave δ 6.3 (2 H, s, NH_2), 8.0 (1 H, d, J = 8.8 Hz), 8.4 (1 H, dd, J = 3.0, 8.8 Hz), and 8.8 (1 H, d, J = 3.0 Hz).

(2) *O*-(2,4-Dinitrophenyl)-*N*-methylhydroxylamine (II) was synthesized from *tert*-butyl *N*-(2,4-dinitrophenoxy)-*N*-methylcarbamate by the method of Sheradsky et al. (1972). The product was further purified by column chromatography on silica (mp 71–72 °C; lit. 74 °C). The ultraviolet absorption in 0.02 M sodium pyrophosphate buffer (pH 8.5) showed a λ_{max} at 298 nm with an ϵ_{298nm} of 11 400 M^{-1} cm^{-1}. Proton NMR ($CDCl_3$) gave δ 3.0 (3 H, d, CH_3, J = 6.9 Hz), 6.7 (1 H, d, NH, exchangeable, J = 6.9 Hz), 7.8 (1 H, d, J = 8.8 Hz), 8.3 (1 H, dd, J = 3, 8.8 Hz), and 8.7 (1 H, d, J = 3.0 Hz).

(3) *O*-(2,4-Dinitrophenyl)-*N*-formylhydroxylamine (III) was synthesized by formylation of I using formic acid–acetic anhydride (mp 112–113 °C). The ultraviolet absorption in ethanol showed a λ_{max} at 286 nm with an ϵ_{286nm} of 11.6×10^3 M^{-1} cm^{-1}. ^{13}C NMR ($CDCl_3$) (Bruker) gave 116.29, 121.23, 121.8, 131.58, 132.68, 140.35 (Ar), and 159.0 (C=O). Due to potential light instability problems associated with nitro compounds all solutions and compounds were protected from light.

Enzyme Assays. Enzyme activity was determined spectrophotometrically by using D-phenylglycine as a substrate (Fonda & Anderson, 1967). A typical assay mixture contained 4.5 mM D-phenylglycine and 3.3 μM added FAD in 50 mM sodium pyrophosphate, pH 8.5, saturated with air. The change in absorbance at 252 nm was monitored for several minutes after addition of enzyme in a spectrophotometer cell thermostated at 25 °C.

Enzyme Modification. Modification was routinely undertaken at 25 °C in the pH range 6.0–8.0 in 50 mM sodium phosphate buffer. Benzoate-free D-amino acid oxidase was adjusted to a final concentration of 0.1–0.2 mM and made 0.1 mM in free FAD. Chromatographically purified DNPHA in a stock solution of 50 mM in acetonitrile was added in a 5-fold molar excess over enzyme-bound FAD to initiate the reaction. Aliquots were removed as the reaction progressed and diluted 1000-fold into enzyme assay media, and the initial rate was determined. After a total reaction time of 20–30 min, residual reagent and 2,4-dinitrophenol product were removed by gel filtration on a column of Sephadex G-25 (1 × 10 cm) equilibrated with sodium phosphate buffer.

Thiol Titration of Denatured, Aminated DAAO. A solution (0.95 mL) containing 8 M guanidine hydrochloride, 7.9 mM EDTA, 20 mM Tris, and 0.23 mM 5,5′-dithiobis(2-nitrobenzoic acid), final pH 7.4, was placed in the center compartment of an anaerobic cuvette. In the side arm was placed aminated DAAO (0.05 mL), pH 8.0, which in 1 mL would have a final concentration of 4.9 μM. After anaerobiosis was achieved by repeated evacuation and flushing with O_2-free N_2, a value for A_{412} was determined for the guanidine solution, and the contents of the cell were mixed with the contents of the side arm (final volume = 1 mL) and A_{412} was redetermined. The change in A_{412} after correction for the absorption of free FAD was used in calculations to determine the concentration of thiol present per mole of enzyme-bound FAD monomer, with a value of 13 600 M^{-1} cm^{-1} for the extinction coefficient of 5-thio-2-nitrobenzoate dianion at 412 nm (Beutler et al., 1963).

Tryptic Digestion, Peptide Mapping, Isolation, and Characterization. Modified protein was denatured under N_2 for 5 h at 25 °C in 6 M guanidine hydrochloride containing 1 mM EDTA and a 10-fold molar excess of dithioerythritol over total protein thiols at pH 8.0. Recrystallized iodoacetic acid in a 1.05-fold molar excess over total thiols was then added. After a 30-min incubation the solution was acidified and dialyzed against water. Digestion of the modified protein was performed in 2 M urea and 20 mM phosphate buffer, pH 8.0, with TPCK–trypsin (4% w/w) at 37 °C for 10–16 h. Analytical peptide mapping and peptide isolation by reverse-phase HPLC were undertaken according to procedures reported elsewhere (Swenson et al., 1982, 1983). Amino acid sequences were determined by the automated gas-phase Edman degradation procedures with an Applied Biosystem Model 470A gas-phase sequencer. Samples were dissolved in 88% formic acid and applied to polybrene-pretreated reaction cartridge filter disks and the phenylthiohydantoin amino acid derivatives identified manually on a Beckman Model 332 gradient liquid chromatography system equipped with a Hewlett-Packard 3390A integrator.

Amino Acid Analysis. The acid hydrolysates (6 N HCl, 110 °C, 24 h) of purified peptides were derivatized with phenyl isothiocyanate/triethylamine reagent (Tarr, 1986) prior to separation on a Waters Pico-Tag column eluted with a two-

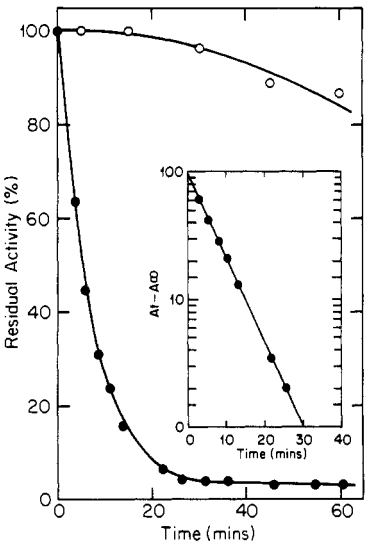


FIGURE 1: Time course for inactivation of DAAO by *O*-(2,4-dinitrophenyl)hydroxylamine (DNPHA) in the absence (closed circles) and presence (open circles) of benzoate: [DAAO] = 100 μM, [free FAD] = 0.1 mM, pH 8.5, T = 25 °C, and [DNPHA] = 5 × DAAO. Activity was assayed spectrophotometrically at pH 8.5 with 4.5 mM D-phenylglycine as substrate; $[O_2]$ = 0.24 mM. Inset: Semilog plot of residual activity (corrected for final activity of 2.9%) vs. time.

part linear gradient. The phenylthiocarbamoyl derivatives were detected and quantified with a Waters Associates Model 440 absorbance detector (λ 254 nm) interfaced to a Model 730 datamodule. Phenylthiocarbamoyl-im-aminohistidine (phenylthiocarbamoyl derivative of histidine aminated on one of the two imidazole nitrogens) was found to elute after lysine under these conditions of analysis.

Carboxypeptidase Y Sequence Analysis of T22. Peptide (8 nmol) was hydrolyzed with 1.1 μg of carboxypeptidase Y at 37 °C, in 50 mM sodium acetate, pH 5.5. Aliquots were removed periodically with time and derivatized with *o*-phthalaldehyde/mercaptoethanol reagent (Hill et al., 1979) prior to injection onto a Waters Associates μBondapak C_{18} (0.39 × 30 cm) column and eluted with a three-part linear gradient [see Swenson et al. (1984a)]. The *o*-phthalaldehyde derivative of im-aminohistidine was found to elute at 23.6 min in comparison to histidine, 9.2 min, as might be expected for an amino acid with two derivatizable amine residues. Im-aminohistidine was synthesized by amination of Boc-L-histidine with hydroxylamine-*O*-sulfonic acid (Okamoto et al., 1966; Weygand & Hilgetag, 1972). Im-aminohistidine was obtained by deprotection of the Boc-L-histidine derivative with anhydrous trifluoroacetic acid.

RESULTS

Inhibition of D-Amino Acid Oxidase by O-(2,4-Dinitrophenyl)hydroxylamine and Its Derivatives. D-Amino acid oxidase when incubated with a 5-fold molar excess of *O*-(2,4-dinitrophenyl)hydroxylamine at pH 8.5, 25 °C, undergoes a rapid time-dependent loss in activity, 96% in 30 min with D-phenylglycine as substrate (Figure 1). The rate of inactivation was found to conform to pseudo-first-order kinetic behavior for >5 half-lives (inset, Figure 1) with k_{obsd} = 0.155 min^{-1} under the conditions used. At equimolar concentrations of reagent and enzyme, the reaction was shown to obey second-order kinetics with 84% loss in activity occurring after 90 min.

The corresponding *N*-methyl derivative under the same conditions caused negligible loss in enzyme activity (4%) over the same time (30 min), and the *N*-formyl derivative, although

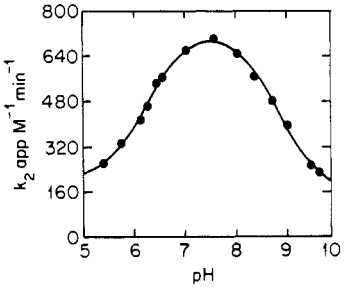


FIGURE 2: Effect of pH on rate of inactivation of DAAO by *O*-(2,4-dinitrophenyl)hydroxylamine (DNPHA): [DAAO] = 10 μM, [free FAD] = 0.1 mM, [DNPHA] = 0.2 mM, and T = 25 °C. The points are experimental; the curve is a best fit theoretical curve assuming a two pK_a model in which the values of pK_{app} were 6.3 and 8,8, with limiting values of 200 M^{-1} min^{-1} (lower left) and 150 M^{-1} min^{-1} (lower right).

comparatively stable in ethyl alcohol, was too unstable in aqueous buffer to be of any practical use.

The rapid time-dependent nature of the inactivation process with *O*-(2,4-dinitrophenyl)hydroxylamine implies a specific modification of the enzyme. Comparison of the specific activity of native and modified enzyme after passage through Sephadex G-25 showed the activity remaining to be 6%, which compared well with the high-dilution assay of 4%.

A criterion for active-site labeling is to use a competitive inhibitor such as benzoate (Yagi & Ozawa, 1962) to protect the active site. If *O*-(2,4-dinitrophenyl)hydroxylamine is active site directed, then the presence of added benzoate should decrease the inhibitory effect of the reagent. The result obtained clearly shows a much slower rate of inactivation, consistent with active-site modification (Figure 1).

Effect of Inhibitor Concentration on Inactivation Rates. The effect of *O*-(2,4-dinitrophenyl)hydroxylamine concentration on the pseudo-first-order rate of inactivation of D-amino acid oxidase was studied in detail at four pH values. Plots of k_{obsd} vs. DNPHA were linear, passing through the origin, consistent with second-order kinetics. At pH 6.2, 7.2, 8.6, and 9.4 the values of second-order rate constants (k_2) obtained were 407, 850, 515, and 319 M^{-1} min^{-1}, respectively.

A more detailed study of the effect of pH on the inactivation rate was made by the incubation of enzyme with a 20.8-fold excess of reagent over enzyme concentration (10 μM). The pH profile of the apparent second-order rate constant, k_2 (k_{obsd}/[DNPHA]), vs. pH in the range 5.4–9.6 is shown in Figure 2. The result shows that a good correlation exists between the experimental data and the computer-generated bell-shaped curve calculated by using a two pK_a model. The dependence implicates ionizable groups of pK_{app} = 6.3 in the deprotonated form and 8.8 in the protonated form to contribute to the inactivation rate.

Stoichiometry of Amination. The high degree of inactivation observed at equimolar concentrations of reagent and enzyme (86% at 90 min) suggested specific modification of a single active-site residue. Nitrogen, unlike carbon, has no long-lived β-emitting radioactive isotopes, thus precluding quantification and identification of the residue modified by this technique. The results obtained with the *N*-methyl and *N*-formyl derivatives of *O*-(2,4-dinitrophenyl)hydroxylamine, which were primarily evaluated for introduction of radioactivity in the form of a ^{14}C label, proved unsatisfactory due to the low reactivity or poor stability. In view of the above constraints, the stoichiometry of reaction was ascertained by quantifying the release of 2,4-dinitrophenol on reaction of a fixed quantity of enzyme (16 μM) with a 20-fold molar excess of reagent at pH 7.0, 25 °C, in the presence of 100 μM FAD.

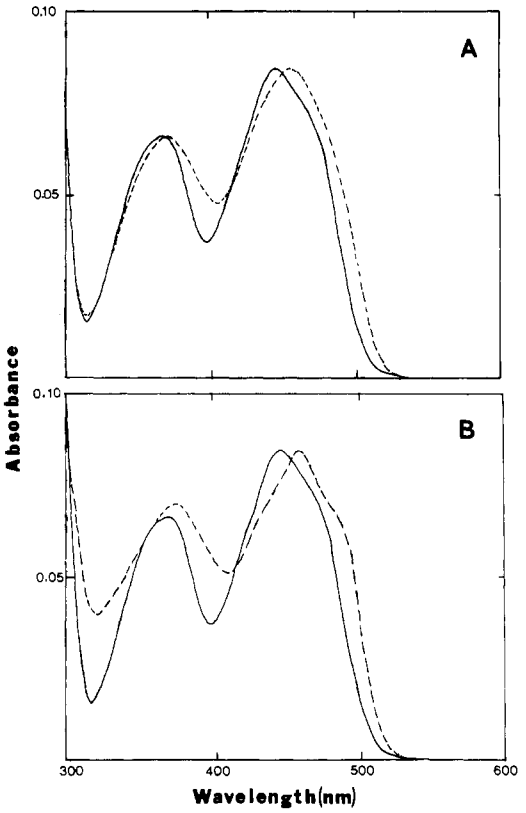


FIGURE 3: (A) Spectrum of native DAAO (dashed line) and aminated DAAO (solid line). The spectrum of aminated enzyme was obtained after Sephadex G-25 passage to remove reagents and products. (B) Spectrum of aminated DAAO (solid line) and aminated DAAO + benzoate (6.4 mM) (dashed line).

The quantity of 2,4-dinitrophenol released on complete inactivation (25–30 min) was obtained by difference spectra. The change in absorbance at 360 nm was used to calculate the concentration, with an extinction coefficient of 15 400 M^{-1} cm^{-1}. Due to a slow time-dependent formation of turbidity, the final value of A_{360} used in calculations was determined after filtration of the enzyme through a 0.45-μm Gelman HPLC disposable filter. A mean value of 1.5 mol of 2,4-dinitrophenol was calculated to be released per mole of enzyme-bound FAD at pH 7.0.

Spectroscopic Properties of Native and Aminated Enzyme. If covalent modification of D-amino acid oxidase by *O*-(2,4-dinitrophenyl)hydroxylamine is active site directed as inferred from the protection against inactivation afforded by benzoate, then it is possible that a sizable spectroscopic change may be observed due to perturbation of the flavin environment. The spectra of modified and native enzyme are shown in Figure 3A. Modified enzyme shows a 7–9-nm blue shift in the 455-nm flavin absorption relative to native enzyme, consistent with active-site modification. Furthermore, the modified enzyme is capable of binding benzoate with pronounced perturbation of the absorption spectrum (Figure 3B), but with a decreased affinity (K_d ca. 300 times higher than with native enzyme; results not shown). This is consistent with the sequence of reactions shown in Scheme I, where the modified enzyme should still be capable of binding benzoate.

Isolation of Tryptic Peptide T22 and Elimination of His-217 as the Possible Site of Modification. D-Amino acid oxidase is modified by both *O*-(2,4-dinitrophenyl)hydroxylamine and methyl *p*-nitrobenzenesulfonate, resulting in an enzyme sharing several similarities: (a) The residual activity after modification was found to be 4–6% of native enzyme. (b) A group of pK_{app} between 6.2 and 6.8 in the deprotonated state

Table I: Amino Acid Composition and Sequence Determined of T22

residue	T22 (A)[a]	T22 (B)[b]
Asp (2)	1.96	1.08
Glu (1)	0.76	0.56
His (1)	0.90	0.95
Arg (1)	1.10	1.06
Thr (1)	0.83	1.0
Ile (2)	1.99	1.93
Leu (1)	1.14	1.07
Phe (1)	1.03	1.00

location 212–221
sequence NH_2-Asn-Phe-Ile-Ile-Thr-His-Asp-Leu-Glu-Arg-COOH

[a] Isolated from tryptic digest of enzyme modified in the absence of benzoate. [b] Isolated from tryptic digest of enzyme modified in presence of 10 mM benzoate.

Table II: Carboxypeptidase Y Cleavage of *S. aureus* Protease Fragment 212–218[a]

sequence determined	Asn-Phe-Ile-Ile-Thr-His-Asp
–benzoate (A), 25 °C, 17 h	←1.3→0.710.50 0.84[b]
sequence determined	Asn-Phe-Ile-Ile-Thr-His-Asp
+benzoate (B), 25 °C, 16 h	- ←1.24→ 0.6 0.29 0.73[b]

[a] The reaction conditions are given under Experimental Procedures. [b] Mol of amino acid released/mol of peptide.

was implicated in the inactivation process. (c) The spectra of modified enzyme exhibited a 7–9-nm blue shift in the 455-nm flavin absorption, relative to native enzyme. The similarities between these two modified forms of the enzyme suggested that the residue modified in both cases may be the same, that is, His-217 as found with MNBS (Swenson et al., 1983). To ascertain whether His-217 was modified, tryptic peptide T22 was located in HPLC tryptic peptide maps of protein modified at pH 7.5 with *O*-(2,4-dinitrophenyl)-hydroxylamine in the absence (A) and presence (B) of benzoate. The peptide fraction eluting at 28.5 min was isolated from tryptic digests of protein modified to a residual activity of 6% and from enzyme modified in the presence of benzoate (see Figure 1) and rechromatographed on a C_{18} reverse-phase column equilibrated at pH 7.4 into three major peaks with retention times of 25.3, 31.7, and 32.9 min. The peak at 25.3 min rechromatographed as a single peak at low pH, with a retention time of 36 min, and was found by phenylthiocarbamyl amino acid analysis to have a composition consistent with T22 (see Table I). Sequence analysis of tryptic peptide (A) isolated from enzyme modified in the absence of benzoate gave the sequence of T22. All cycles including cycle 6 yielded an identifiable phenylthiohydantoin derivative consistent with unmodified T22 peptide. Under the above conditions phenylthiohydantoin-histidine eluted at 6.6 min while the phenylthiohydantoin derivative of im-aminohistidine eluted at 10.6 min. As a final confirmation of the absence of modification of His-217, tryptic peptide T22 isolated from enzyme modified in both the absence (A) and presence (B) of benzoate was subjected under identical conditions to cleavage by *Staphylococcus aureus* protease (4% w/w) at pH 7.8, 50 mM phosphate buffer. In addition to unhydrolyzed starting material, two peptides of retention times 11.7 and 29.8 min were obtained after separation by C_{18} reverse-phase HPLC at low pH. The amino acid compositions of the two fragments were consistent with specific cleavage of T22 at Asp-215 adjacent to the histidine residue (Table I). Carboxypeptidase Y cleavage of the 29.8-min heptapeptide fragment (212–218) of T22 and subsequent analysis of the amino acids released by manual *o*-phthalaldehyde amino acid analysis confirmed the absence of any modification of His-217; see Table II. As the known chemistry of RR'N-NH_2 compounds (Epsztajn et

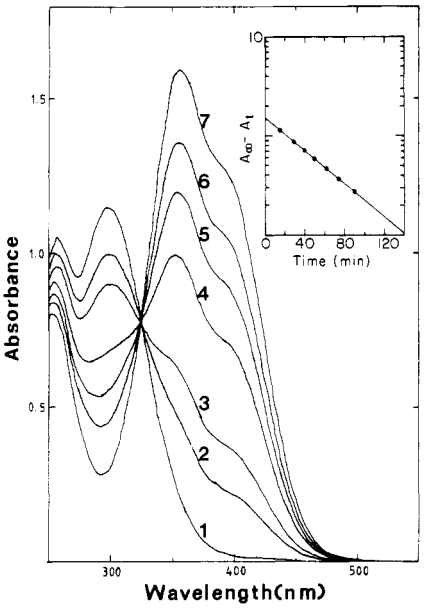
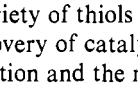


FIGURE 4: Reaction of *N*-acetyl-L-methionine with *O*-(2,4-dinitrophenyl)hydroxylamine (DNPHA) in solution at 25 °C. (1) Spectrum of 0.1 mM DNPHA in 50 mM sodium phosphate, pH 7.5. (2–7) Spectra of 0.1 mM DNPHA after addition of 2 mM *N*-acetyl-L-methionine: (2) 7 min; (3) 15 min; (4) 40 min; (5) 62 min; (6) 90 min; (7) 150 min. The final spectrum (7) is that of 2,4-dinitrophenol. Inset: Semilog plot of ΔA_{390} vs. time.

Table III: Second-Order Rate Constant (k_2) for Reaction of Amino Acids with DNPHA in Solution at 25 °C

amino acid	pH	k_2 (M^{-1} min^{-1})	product
N-acetyl-L-cysteine	6.0	3.6	R–S–S–R[a]
	7.5	68.5	R–S–NH–SR[b]
			RSO_2NH_2[b]
N-acetyl-L-methionine	6.5	9.6	
	7.5	9.6	
	9.0	9.6	
N-acetyl-L-histidine	7.5	2.3×10^{-3} [d]	N–NH_2[a–c]
N^{α}-acetyl-L-lysine	7.5	1.3×10^{-3} [d]	R–NH–NH_2[a–c]
N-acetyl-L-tryptophan	7.5	1.2×10^{-3} [d]	
N-acetyl-L-tyrosine	7.5	7.2×10^{-5} [d]	
oxidized glutathione	7.5	0.145[d]	
sodium phosphate buffer, 50 mM	7.5	$t_{50\%}$ = 904 h	

[a] Oae and Yamamoto (1973). [b] Sheradsky et al. (1972). [c] Tamura et al. (1973). [d] $k_{2\,app}$ values (k_{obsd}/[amino acid]).

al., 1970) indicates that they are stable to the mild reaction conditions used in carboxypeptidase Y and manual *o*-phthalaldehyde analysis, we may assume that this result, in conjunction with the sequence data, excludes His-217 as the target of this modification.

Reactivity of O-(2,4-Dinitrophenyl)hydroxylamine with Amino Acids in Solution. The absence of modification of His-217 suggested that the group modified was either one of the residues previously identified at the active site or a completely new residue. The latter possibility suggested that a knowledge of the reactivity of the reagent with functional groups present on amino acids, under conditions used in the modification of D-amino acid oxidase, could afford an indication as to the nature of the amino acid residue modified. The reaction of various amino acids was monitored by following the release of 2,4-dinitrophenol at 390 nm as shown in Figure 4 for *N*-acetyl-L-methionine at pH 7.5. The results with various amino acids are shown in Table III. In all cases the reaction was found to conform to pseudo-first-order behavior for >3 half-lives. In the case of *N*-acetyl-L-cysteine, 1 mM EDTA was included to correct erratic behavior and a nonzero intercept. The inactivation rate of D-amino acid oxidase by *O*-(2,4-dinitrophenyl)hydroxylamine at pH 7.5 is more than 300000 times greater than with the nonenzymic rate of known residues at or near the active site, i.e., His, Tyr, or Lys. The relative unreactivity of His, Tyr, and Lys would make them unlikely candidates for modification. Of the remaining residues, both disulfides and thiols can be eliminated on the basis of previous modifications (Swenson et al., 1982, 1983, 1984a) and the known sequence of the enzyme (Ronchi et al., 1982), leaving only the thioether side chain of a methionine residue as a likely candidate.

Table IV: Evaluation of Aminated DAAO with Thiols at 5 °C[a]

thiol	concn (M)	pH	% activity [time (h)]
L-cysteine	0.01	8.5	7.3 (22)
3-mercaptopropionic acid	0.01	8.5	9.0 (68).
dithiothreitol	0.01	8.5	12.0 (48)
dithiothreitol	0.2	9.0	100.0 (11)
dithiothreitol[b]	0.2	9.0	95.0 (11)
mercaptoethanol	0.2	9.0	100.0 (11)
mercaptoethanol[b]	0.2	9.0	95.0 (11)

[a] Conditions: aminated DAAO (11 μM), residual activity 6%, in 20 mM buffer, pH 8.5 or 9.0, containing added FAD (100 μM), was incubated under nitrogen with varying concentrations of thiols adjusted to pH 8.5 or 9.0. [b] No added FAD.

Evaluation of the Stability of Modification to Thiolysis. If the loss in activity of DAAO on reaction with DNPHA is due to formation of an *S*-aminomethionine sulfonium salt as inferred from kinetic studies, then as the corresponding *S*-methylmethionine sulfonium salts slowly regenerate methionine on incubation with a vast excess of thiols (Christie et al., 1979), some degree of reactivation may be expected for aminated enzyme under similar conditions. The maximum catalytic reactivation obtained on incubation of modified DAAO at 5 °C with a variety of thiols is shown in Table IV. As can be seen, the recovery of catalytic activity is dependent on both the concentration and the nature of the thiol used. The condition found for the complete reactivation of catalytic activity was 0.2 M mercaptoethanol or DL-dithiothreitol, pH 9.0, with 100 μM FAD. At lower concentrations of thiol (10 mM) a negligible amount of catalytic activity was recovered. The spectrum of aminated enzyme after complete reactivation with dithiothreitol, in the presence of 100 μM FAD, and removal of reagents by Sephadex G-25 chromatography, was found to be identical with that of native enzyme. The reversibility of the modification, as ascertained by the recovery of both catalytic and spectral properties relative to native enzyme, is consistent with the chemistry of sulfonium salts and with assignment of modification of DAAO by DNPHA to formation of an *S*-aminomethionine sulfonium salt.

Elimination of Thiol Residues as a Target of DNPHA. The complete reversibility of the modification on thiolysis could suggest the formation of a disulfide as a product of DNPHA modification of DAAO. However, from a total of two determinations with 5,5′-dithiobis(2-nitrobenzoic acid) at pH 7.4, a mean value of 5.1 thiol residues was calculated per mole of enzyme-bound FAD monomer (the same as with native enzyme), thus excluding thiols as a target of DNPHA modification (Ronchi et al., 1982).

Discussion

O-(2,4-Dinitrophenyl)hydroxylamine and its derivatives form a unique class of electrophilic compounds capable of incorporating a small amine group into nucleophilic residues. In this work we have shown that of the three derivatives evaluated only DNPHA was capable of abolishing the catalytic

activity of D-amino acid oxidase. The overall inactivation process has been shown to obey second-order kinetics and proceeds very quickly in comparison to the corresponding inactivation reaction observed with the comparable reagent methyl *p*-nitrobenzenesulfonate. The apparent second-order rate constant k_2 (k_{obsd}/[DNPHA]) for the inactivation of D-amino acid oxidase by *O*-(2,4-dinitrophenyl)hydroxylamine at pH 7.6 was 700 M^{-1} min^{-1} (Figure 3).

The reaction of DNPHA with D-amino acid oxidase is more specific than that with MNBS. A total of 1–1.5 mol of amine residues per D-amino acid oxidase monomer was calculated to be incorporated by DNPHA while reaction with MNBS resulted in the incorporation of 3.2 methyl residues per molecule of monomer in the absence of benzoate (Swenson et al., 1984a).

The lack of modification of His-217 indicates that the group of pK_a = 6.3 identified from the pH vs. $k_{2\,app}$ profile to be involved in inactivation is not the apparent pK_a of the group being modified but one associated with the binding of the reagent or one controlling its accessibility to the residue being modified. The pK_a values of 6.3 and 8.8 observed in the inactivation profile are consistent with values previously identified for groups in the active site associated with benzoate binding of 6.3 and 9.3 (Quay & Massey, 1977), indicating that this reagent probably binds at the same site. The high degree of protection against inactivation afforded by benzoate is clearly consistent with this explanation. The difference in pK_a of 0.5 unit betwwen the values of the more basic of these two residues may reflect base weakening due to the possible interaction of this residue with a nitro group located on the ligand. In addition, we may also conclude from these results that, apart from the two specific pK_a values associated with binding or accessibility, a pH-independent rate must exist for the residue being modified to account for a nonlimiting rate of 150–200 M^{-1} min^{-1} observed in the pH profile (Figure 2). The second-order dependence of the rate of inactivation vs. [DNPHA] can be explained if the rate of inactivation is fast compared to the rate of dissociation of the proposed EI complex. Precedence exists for binding, as nitroaromatic compounds are good ligands of DAAO: i.e., the K_i of 2,4-dinitrophenol with DAAO is 2.3×10^{-4} M (Nishino et al., 1980b). The pH-independent rate of inactivation of DAAO by DNPHA is consistent with assignment of the residue being modified to the thioether side chain of a methionine group as no apparent pH dependence is observed in the reaction of this amino acid with DNPHA in solution (Table III), as could be expected on the basis of its chemical properties. Apart from methionine, only the disulfide bond and the indole ring of tryptophan may be expected to exhibit pH-independent kinetics of modification.

The visible absorption spectrum of aminated D-amino acid oxidase is similar to that of methylated enzyme with the 455-nm flavin absorption in both cases blue-shifted by 7–9 nm relative to native enzyme. Evaluation of aminated DAAO with respect to benzoate binding shows that the enzyme is capable of binding this competitive inhibitor but binding is substantially weakened by ca. 300-fold at pH 8.0. This is consistent with the hypothesis of Scheme I, in which the positively charged group responsible for initial binding of DNPHA is freed when dinitrophenol is released. The lowered affinity of benzoate binding could be associated with steric hindrance of the amino group introduced in the modification.

The reaction of DNPHA with neutral nucleophiles is a typical S_N2 reaction on a sp^3 nitrogen (Oae & Yamamoto, 1973). The observed order of nucleophilicity at the sp^3 nitrogen center of this reagent is S^- > $S-CH_3$ > -S-S- > imidazole-His > NH_3^+-Lys > indole-Trp > OH-Tyr (Table III). The reaction with oxidized glutathione was assumed to be primarily due to reaction of the -S-S- bond as both the other candidates, the NH_3^+ and COO^- functional groups, are present on *N*-acetyllysine and show negligible reactivity in comparison.

The observed order of nucleophilicity is consistent with studies on an analogous reagent hydroxylamine-*O*-sulfonate (Krueger et al., 1979) and can be explained in terms of a marked preference of this reagent for soft rather than hard nucleophiles where polarizability and ease of oxidation play a more significant role in reactivity than does proton basicity. The sp^3 nitrogen center of this reagent responds to nucleophiles in a manner parallel to that of peroxide oxygen (very soft center) and this is not surprising in view of the isoelectronic similarity of the two functional groups.

The complete catalytic and spectral reactivation of aminated enzyme on thiolysis indicates that *S*-aminomethionine sulfonium salts undergo preferential cleavage of the S–N bond in favor of either of the S–C bonds and so in their chemistry behave like sulfoxides where S–O bond cleavage predominates (Allinger et al., 1972).

It is quite evident from the kinetic data that the specificity of modification observed in the use of this reagent with D-amino acid oxidase is in part due to the soft character of the electrophilic center as well as the fortuitous absence of several soft nucleophiles at the active site of this enzyme. Assignment of modification of D-amino acid oxidase to the thioether side chain of a methionine residue is supported by pH dependence data, solution kinetic studies, the known structure of the active site of the enzyme, and studies undertaken on the stability of the modification to thiols. Modification of the thioether side chain of a methionine group to an *S*-aminosulfonium salt changes a neutral hydrophobic residue into a positively charged hydrophilic group. The formation of such a group at or near the active site would account for the loss in activity of DAAO as the electrostatic field created by such a group may be expected to alter the acid/base properties of residues located in the near vicinity or change the redox properties of the bound flavin. Alternatively, the loss in activity may be a direct consequence of the involvement of this group in catalysis or a combination of the above two effects. On the basis of this tentative assignment we can calculate a rate enhancement for the reaction of DNPHA with a methionine residue at the active site of this enzyme relative to a methionine residue in solution at pH 7.5 to be 73-fold. We may assume from this value that adsorption of the reagent by the enzyme has to occur prior to modification, as inferred from the pH dependence data, and that the relative orientation of the reagent to the residue modified must be favorable for reaction to occur. The presence of a methionine group located near the active site of a flavoprotein is not without precedence. For example, Met-56 in flavodoxin is in van der Waals contact with the bound flavin ring (Smith et al., 1977), while in *p*-hydroxybenzoate hydroxylase, Met-347 forms the wall of the substrate binding site (Hofsteenge, 1981).

Further work defining the catalytic properties of the modified enzyme in the two half-reactions is in progress. We hope that these studies may suggest a role for this residue and allow its incorporation into a proposed model of the active site (Williams et al., 1984).

ACKNOWLEDGMENTS

We thank Sheila Norton, under the direction of Dr. G. Tarr, Protein Sequencing Facility, University of Michigan, for

performing the automated sequence analyses and L. David Arscott for many helpful discussions.

Registry No. DNPHA, 17508-17-7; D-amino acid oxidase, 9000-88-8; methionine, 63-68-3; benzoate, 65-85-0; *N*-acetyl-L-methionine, 65-82-7; *N*-acetyl-L-cysteine, 616-91-1; *N*-acetyl-L-histidine, 2497-02-1; N^{α}-acetyl-L-lysine, 1946-82-3; *N*-acetyl-L-tryptophan, 1218-34-4; *N*-acetyl-L-tyrosine, 537-55-3; oxidized glutathione, 27025-41-8.

REFERENCES

Allinger, N. L., Cava, M. P., De Jongh, D. C., Johnson, C. R., Lebel, N. A., & Stevens, C. L. (1972) in *Organic Chemistry*, pp 685–686, Worth, New York.

Beutler, E., Duron, O., & Kelly, B. M. (1963) *J. Lab. Clin. Med. 61*, 882–888.

Bright, H. J., & Porter, D. J. T. (1975) *Enzymes (3rd Ed.) 12B*, 421–505.

Christie, D. L., Hancock, W. S., & Barling, P. M. (1979) *Endocrinology (Baltimore) 105*, 1020–1030.

Curti, B., Ronchi, S., Branzoli, U., Ferri, G., & Williams, C. H., Jr. (1973) *Biochim. Biophys. Acta 327*, 266–273.

Epsztajn, J., Lunt, E., & Katritzky, A. R. (1970) *Tetrahedron 26*, 1665–1673.

Ferti, C., Curti, B., Simonetta, M. P., Ronchi, S., Galliano, M., & Minchiotti, L. (1981) *Eur. J. Biochem. 119*, 553–557.

Fitzpatrick, P. F., & Massey, V. (1983) *J. Biol. Chem. 258*, 9700–9705.

Fonda, M. L., & Anderson, B. M. (1967) *J. Biol. Chem. 242*, 3957–3962.

Hill, D. W., Walters, F. H., Wilson, T. D., & Stuart, J. D. (1979) *Anal. Chem. 51*, 1338–1341.

Hofsteenge, J. (1981) Ph.D. Dissertation, University of Groningen, The Netherlands.

Krueger, J. H., Sudbury, B. A., & Blanchet, P. F. (1974) *J. Am. Chem. Soc. 96*, 5733–5736.

Marcotte, P. & Walsh, C. (1978) *Biochemistry 17*, 2864–2868.

Nakagawa, Y., & Bender, M. L. (1970) *Biochemistry 9*, 259–267.

Nishino, T., Massey, V., & Williams, C. H., Jr. (1980a) *J. Biol. Chem. 255*, 3610–3616.

Nishino, T., Nishino, T., & Massey, V. (1980b) *Biochem. Biophys. Res. Commun. 95*, 312–319.

Oae, S., & Yamamoto, F. (1973) *Tetrahedron Lett. 51*, 5143–5146.

Okamoto, T., Hirobe, M., & Yamazaki, T. (1966) *Chem. Pharm. Bull. 14*, 512–517.

Porter, D. J. T., & Bright, H. J. (1972) *Biochem. Biophys. Res. Commun. 46*, 571–577.

Porter, D. J. T., Voet, J. G., & Bright, H. J. (1973) *J. Biol. Chem. 248*, 4400–4416.

Porter, D. J. T., Voet, J. G., & Bright, H. J. (1977) *J. Biol. Chem. 252*, 4464–4473.

Quay, S., & Massey, V. (1977) *Biochemistry 16*, 3348–3354.

Ronchi, S., Galliano, M., Minchiotti, L., Curti, B., Rudie, N. G., Porter, D. J. T., & Bright, H. J. (1980) *J. Biol. Chem. 255*, 6044–6046.

Ronchi, S., Minchiotti, L., Galliano, M., Curti, B., Swenson, R. P., Williams, C. H., Jr., & Massey, V. (1982) *J. Biol. Chem. 257*, 8817–8823.

Sheradsky, T., Salemnick, G., & Nir, Z. (1972) *Tetrahedron 28*, 3833–3843.

Smith, W. W., Burnett, R. M., Darling, G. D., & Ludwig, M. L. (1977) *J. Mol. Biol. 117*, 195–225.

Swenson, R. P., Williams, C. H., Jr., & Massey, V. (1982) *J. Biol. Chem. 257*, 1937–1944.

Swenson, R. P., Williams, C. H., Jr., & Massey, V. (1983) *J. Biol. Chem. 258*, 497–502.

Swenson, R. P., Williams, C. H., Jr., & Massey, V. (1984a) *J. Biol. Chem. 259*, 5585–5590.

Swenson, R. P., Williams, C. H., Jr., & Massey, V. (1984b) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C., & Mayhew, S. G., Eds.) pp 301–304, Walter de Gruyter, Berlin.

Tamura, Y., Minamikawa, J., Sumoto, K., Fujii, S., & Ikeda, M. (1973) *J. Org. Chem. 38*, 1239–1241.

Tarr, G. E. (1986) in *Microcharacterization of Polypeptides: A Practical Manual* (Shively, J. E., Ed.) pp 155–194, Humana, Clifton, NJ.

Walsh, C. T., Schonbrunn, A., & Abeles, R. H. (1971) *J. Biol. Chem. 246*, 6855–6866.

Walsh, C. T., Krodel, E., Massey, V., & Abeles, R. H. (1973) *J. Biol. Chem. 248*, 1946–1955.

Weygand, C., & Hilgetag, G. (1972) in *Preparative Organic Chemistry* (Hilgetag, G., & Martini, A., Eds.) pp 585–587, Wiley, Berlin.

Williams, C. H., Jr., Arscott, L. D., & Swenson, R. P. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C., & Mayhew, S. G., Eds.) pp 95–109, Walter de Gruyter, Berlin.

Yagi, K., & Ozawa, T. (1962) *Biochim. Biophys. Acta 56*, 413–419.