D-Amino Acid Oxidase

IV. INACTIVATION BY MALEIMIDES*

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SUMMARY

A series of N-alkylmaleimides was shown to effectively inhibit D-amino acid oxidase at pH 7.0 and 7.5. Apparent second order rate constants for maleimide inactivation of the enzyme increased with increasing chain length of the alkyl substituents of the maleimide derivatives. It is suggested that this chain length effect is due to an enhanced binding of the longer chain compounds through nonpolar interactions with the enzyme. N-Phenylmaleimide and N-(4-dimethylamino-3,5-dinitrophenyl)maleimide were used to label D-amino acid oxidase. Product studies indicated that the maleimides were reacting only with cysteine residues of the enzyme. Peptide mapping was used to show that D-amino acid oxidase is composed of identical subunits having a molecular weight of 50,000. Product studies and spectrophotometric studies indicated that 3 moles of maleimide were bound per 50,000 g of protein.

D-Amino acid oxidase was shown to be saturated with high concentrations of the N-alkylmaleimides, thus indicating the binding of the maleimides to the enzyme before the rate-limiting inactivation step. FAD and adenine derivatives were very effective in protecting the enzyme against inactivation by N-ethylmaleimide. Prior inactivation of the enzyme by N-ethylmaleimide prevented the binding of FAD to the enzyme. These results indicated that there is at least one reactive sulfhydryl group made less accessible by the binding of adenine derivatives at the FAD-binding site of D-amino acid oxidase.

EXPERIMENTAL PROCEDURE

D(-)-Phenylglycine, purchased from Aldrich, was recrystallized from water. Adenosine, AMP, ADP, ADP-ribose, and FAD (Grade III) were obtained from Sigma. Lyophilized and twice crystallized trypsin was purchased from Worthington.

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N-Butylymaleimide was purchased from Eastman. N-Butylmaleimide and N-phenylmaleimide were products of Nutritional Biochemicals. N-Pentyl-, N-hexyl-, N-heptyl-, and N-octylmaleimides were prepared according to the method described by Heitz, Anderson, and Anderson (21). DDPM\(^1\) was obtained from Columbia Organic Chemicals. DEAE-cellulose, Dowex AG-I-X8 (formate), and Dowex AG-50WX (hydrogen) were purchased from Bio-Rad; Sephadex G-25 was a product of Pharmacia.

S-Succinylcysteine, prepared by the acid hydrolysis of the product obtained from the reaction of L-cysteine with N-ethylmaleimide, was purified further by chromatography on Dowex-I-formate. The S-succinylcysteine had the properties the same as those reported for the compound prepared by different methods (22-25). The ninhydrin color yield for the synthetic S-succinylcysteine, expressed as aspartic acid equivalents, was 1.07, which is in good agreement with the color yield of 1.09 reported by Smyth, Blumenfeld, and Konigsberg (24) for this compound.

Electrophoretically purified N-amino acid oxidase was obtained from Worthington, and analytical grade N-amino acid oxidase was purchased from Boehringer Mannheim as the crystalline benzoxa complex. Unless otherwise specified, the N-amino acid oxidase obtained from Worthington was used in all experiments.

The apoenzyme form of N-amino acid oxidase was prepared according to the method of Massey and Curti (26), and stock solutions were stored at 4°C in 0.1 M sodium pyrophosphate, pH 8.5.

The time-dependent inactivation of N-amino acid oxidase by the maleimides was studied in incubation mixtures maintained at 25°C. The reaction mixtures consisted of 100 μg of apoenzyme in 3 ml of 0.05 M sodium pyrophosphate buffer containing 1% ethanol and varying concentrations of the maleimides. At various times after the addition of apoenzyme to the incubation mixture, 0.3-ml aliquots of the incubation mixture were added to 2.7 ml of the standard assay mixture. The assay mixture contained 0.05 M sodium pyrophosphate (pH 8.5), 8.22 × 10\(^{-4}\) M FAD, and 0.017 M ω-phenylglycine. The N-amino acid oxidase-catalyzed reactions were studied by the spectrophotometric assay in which the optical density changes accompanying the production of benzyolformic acid from ω-phenyglycine were followed (27). The change in absorbance at 243 μm was used as a measure of enzymatic activity.

Spectrophotometric measurements were carried out in a temperature-controlled cell compartment of a Zeiss PMQ II spectrophotometer or a Gilford model 2000 recording spectrophotometer, with 1-cm light path cuvettes used in all studies. Measurements of pH were made at 25°C with a Radiometer pH meter, type PHM 4e, with a G-200-B glass electrode.

Fluorescence measurements were performed in a temperature-controlled cell compartment of an Aminco-Bowman spectrophotofluorometer with a xenon lamp. The instrument was equipped with a Moseley Autograph model 135A X-Y recorder and a Pacific photometric recording photometer, model 15, fitted with an EMI 9502 photocell. The FAD used in the fluorescence studies was purified by chromatography on DEAE cellulose according to the procedure of Massey and Swoboda (28).

The amino acid analyses of N-amino acid oxidase were performed with the enzyme obtained from Boehringer. Samples containing 1 to 2 mg of untreated apoenzyme or apoenzyme which had been inactivated by N-phenylmaleimide were lyophilized and hydrolyzed in 1 ml of glass-distilled constant boiling HCl at 110°C for 48, and 72 hours in evacuated sealed glass tubes. The hydrolysates were lyophilized, and the residues were taken up in 0.2 M sodium citrate, pH 2.2. Quantitative analyses of the amino acids were performed on aliquots according to the method of Speckman, Stein, and Moore (90) with a Beckman/Spinco model 116 amino acid analyzer.\(^2\) The aniline formed during hydrolysis of the N-phenylmaleimide-labeled protein was determined by the Bratton-Marshall method (30, 31). In order to detect small amounts of aniline (0.2 to 2.0 μg), smaller amounts of the reagents were used. The aniline was diazotized and coupled with N-(1-naphthyl)ethylenediamine to form a purple dye.

The N-amino acid oxidase obtained from Boehringer was used for peptide mapping. The proteolytic digestion was carried out by a procedure similar to that described by Helinski and Yanofsky (32). The apoenzyme form of N-amino acid oxidase was treated with 6 M urea in 0.3 M ammonium bicarbonate, pH 8.3, for 15 min. After the solution was diluted 3-fold with water, trypsin, in an amount equal to 2% of the N-amino acid oxidase by weight, was added. The solution was stirred for 4 hr at 23°C. The digest was adjusted to pH 3 and applied to a Dowex 50-X2 column (50 to 100 mesh, H\(^+\) form, 1 × 10 cm) which had previously been washed with water. The urea was eluted from the column with water, and the peptides were eluted with 4 M NH\(_4\)OH.

Peptide mapping was performed according to a modification of the method described by Katz, Dreyer, and Anfinsen (33). Freshly prepared 1-butanol-acetic acid-water (4:1:5) was used as the solvent for the descending paper chromatography. The high voltage electrophoresis in the second direction was performed with a buffer composed of acetic acid-formic acid-water (3:1:30), pH 1.9. Refrigerated high voltage electrophoresis was run at 1800 volts, 70 ma, and −8°C for 1 hr in a Horuma Minipherograph equipped with platinum block electrodes.

RESULTS

1. Inactivation of N-Amino Acid Oxidase by N-Alkylmaleimides—When N-amino acid oxidase apoenzyme is incubated with N-ethylmaleimide, the enzymatic activity decreases with increasing time of incubation. Fig. 1 shows the linear relationship obtained when the logarithm of the enzymatic activity was plotted against time of incubation. Pseudo first order rate constants of inactivation were calculated from such relationships by the equation, \(k_1 = 0.693/t\). These first order rate constants were then plotted versus the concentration of N-ethylmaleimide as shown in Fig. 2. A linear dependency on N-ethylmaleimide concentration was observed in this concentration range, although at higher concentrations of N-ethylmaleimide, a limiting rate of inactivation was achieved. Thus, the inactivation process cannot be described in terms of simple second order kinetics but rather should take into account an enzyme saturation effect observed by the prior binding of maleimide derivatives. These properties of the inactivation process will be discussed later. The effect of maleimide concentration on the inactivation process can be described by the following equation.

\[^1\] The abbreviation used is: DDPM, N(4-dimethylamino-3,5-dinitrophenyl)maleimide.

\[^2\] We would like to thank Mrs. T. M. Tucker for the protein amino acid analyses.
Fig. 1. Time-dependent inactivation of D-amino acid oxidase apoenzyme by N-ethylmaleimide at 25°C. The reaction mixtures contained 0.05M sodium pyrophosphate (pH 7.0), 1% ethanol, 100 μg of apoenzyme, and inhibitor as indicated, in a total volume of 3 ml. Line 1, no inhibitor; Line 2, 6.40 × 10⁻⁴ M N-ethylmaleimide; Line 3, 1.04 × 10⁻³ M N-ethylmaleimide; Line 4, 1.58 × 10⁻³ M N-ethylmaleimide. The assays were performed as described under "Experimental Procedure."

Fig. 2. The effect of N-ethylmaleimide concentration on the first order rate constants of D-amino acid oxidase inactivation at pH 7.0 and 25°C. The reaction mixtures contained 0.05M sodium pyrophosphate (pH 7.0), 1% ethanol, 100 μg of D-amino acid oxidase apoenzyme, and varying concentrations of N-ethylmaleimide, in a total volume of 3 ml.

\[-d[E]/dt = k_1/K_T [EI]\]

where \(k_1\) equals the pseudo first order rate constant of inactivation, \(K_T\) equals the dissociation constant for the enzyme-maleimide complex, and \(EI\) represents an enzyme-maleimide complex formed prior to the inactivation reaction. For the sake of comparison of the different maleimide derivatives, we can let \(k_1/K_T\) equal \(k'\) and define \(k'\) as an inactivation constant.

The inactivation of D-amino acid oxidase apoenzyme by N-ethyl- and N-butyl- through N-octylmaleimides was studied at pH 7.0 and pH 7.5. In all cases, linear relationships were obtained when the logarithm of the enzymatic activity was plotted against time. Inactivation constants were obtained from the plot of the first order rate constants versus the maleimide concentration. Such constants obtained for all of the alkylmaleimides at the two pH values are listed in Table I. The logarithms of the inactivation constants, obtained at pH 7.0 and pH 7.5 and plotted against the number of carbons in the respective alkyl chains, is shown in Fig. 3. A straight line relationship was obtained at both pH values. It can be seen that the rate of inactivation of the enzyme increases with increasing chain length of the maleimide derivative. Inactivation constants (\(k'\)) were calculated from inactivation studies carried out under conditions described in Fig. 2. At least six concentrations of the maleimides were used in each case. The inactivation constant (\(k'\)) equals \(k_1/K_T\) as described in the text.

Table I

<table>
<thead>
<tr>
<th>N-Substituted maleimide</th>
<th>(k') (liter mole⁻¹ minute⁻¹)</th>
<th>(k') (liter mole⁻¹ minute⁻¹)</th>
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<tr>
<td>Ethyl-</td>
<td>27.2</td>
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<tr>
<td>Butyl-</td>
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<td>186</td>
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<tr>
<td>Pentyl-</td>
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<td>Hexyl-</td>
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<td>Heptyl-</td>
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<td>Octyl-</td>
<td>403</td>
<td>1020</td>
</tr>
<tr>
<td>Phenyl-</td>
<td>320</td>
<td>—</td>
</tr>
<tr>
<td>4-Dimethylamino-3,5-dinitrophenyl-</td>
<td>710</td>
<td></td>
</tr>
</tbody>
</table>

* Not measured.

Fig. 3. The relationship of the logarithm of the inactivation constants to the chain length of the alkyl substituents of the maleimides. •—•, pH 7.5; ○—○, pH 7.0.
The effects of high concentrations of N-alkylmaleimides on rates of inactivation of D-amino acid oxidase. The 3-ml incubation mixtures contained 0.05 M sodium pyrophosphate (pH 6.5), 1% ethanol, 100 µg of D-amino acid oxidase, and varying concentrations of the alkylmaleimides.

Constants obtained for each of the alkylmaleimides are 2½ to 3 times greater at pH 7.5 than at pH 7.0. Ethanol (1%) was used in the incubation mixtures in order to solubilize longer chain alkylmaleimides and was also included in the incubation mixtures used as "controls." The presence of the ethanol had no effect on the stability of the enzyme alone nor on the first order rate constants of inactivation by N-ethylmaleimide.

As already indicated, a maximum rate of inactivation of the enzyme could be reached by increasing the concentration of the maleimide. The first order rate constants of inactivation of the apoenzyme were determined over a greater range of concentrations of N-ethyl, N-butyl, and N-pentylmaleimides at pH 6.5. The lower pH was used in this study since higher concentrations of the maleimides at pH 7.0 resulted in rates of inactivation too rapid to measure accurately. The relationship of the first order rate constants versus the concentrations of the alkylmaleimides is presented in Fig. 4. A maximum rate of inactivation is approached at the higher concentrations of the maleimides. A replot of the data as a double reciprocal plot indicated that the same maximum rate of inactivation is approached with each of the maleimides.

Several compounds known to interact at the FAD-binding site were used in an attempt to protect D-amino acid oxidase against inactivation by N-ethylmaleimide at pH 7.0. The results of these protection experiments are listed in Table II. AMP, ADP, ADP-ribose, and FAD were studied at concentrations equivalent to 1, 2, and 3 times their dissociation constants (K₁), which had been reported previously (1). All of these compounds give very efficient protection against inactivation by N-ethylmaleimide. These compounds had no effect on the absorption of N-ethylmaleimide at 305 nm, indicating no interaction with N-ethylmaleimide in the absence of the enzyme.

The K₁ values for AMP, ADP, and ADP-ribose had been previously measured (1) at pH 8.5, whereas the protection experiments were conducted at pH 7.0. A K₁ value was determined for AMP at pH 7.0. In this kinetic study, the reactions were carried out in 0.061 M sodium pyrophosphate, pH 7.0, and the concentration of D-phenylglycine was 0.015 M. The FAD concentration was varied from 2.61 × 10⁻² M to 1.31 × 10⁻¹ M, and five concentrations of AMP varying from 0 to 1.45 × 10⁻¹ M were used. The K₁ value (8 × 10⁻⁴ M) obtained from a double reciprocal plot of the data is listed in Table III and is very similar to that of 1.04 × 10⁻⁴ M reported for AMP at pH 8.5 (1).

The dissociation constants for FAD were determined graphically at pH 8.5 and pH 7.0 from experiments in which enzyme velocities were measured as a function of varying FAD concentrations at several constant concentrations of the substrate, D-phenylglycine. The data obtained were plotted as the reciprocal

\[
\frac{1}{v} = \frac{1}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \frac{1}{K_M}
\]

The values obtained were very similar to those reported previously (1). The Michaelis constants for D-phenylglycine at pH 8.5 and pH 7.0 were 3.2 × 10⁻² M and 4.43 × 10⁻² M, respectively. The dissociation constant for FAD-apoenzyme complex at pH 8.5 and pH 7.0 was 1.05 × 10⁻⁴ M and 1.26 × 10⁻⁴ M, respectively.

### Table II

<table>
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<tr>
<th>Protecting compound and concentration</th>
<th>K₁</th>
<th>Protection</th>
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<tr>
<td>AMP</td>
<td>0.0336</td>
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<td>1.07 × 10⁻⁴</td>
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<td>2.15 × 10⁻⁴</td>
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<tr>
<td>3.22 × 10⁻⁴</td>
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<tr>
<td>ADP</td>
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<td>5.42 × 10⁻⁴</td>
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<td>59.3</td>
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<tr>
<td>1.08 × 10⁻⁴</td>
<td>0.0147</td>
<td>71.5</td>
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<tr>
<td>2.16 × 10⁻⁴</td>
<td>0.0103</td>
<td>75.7</td>
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<tr>
<td>ADP-ribose</td>
<td>0.0327</td>
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<td>5.65 × 10⁻⁴</td>
<td>0.0187</td>
<td>57.8</td>
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<tr>
<td>1.13 × 10⁻⁴</td>
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<tr>
<td>2.36 × 10⁻⁴</td>
<td>0.0060</td>
<td>69.7</td>
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<tr>
<td>FAD</td>
<td>0.0270</td>
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<td>1.02 × 10⁻⁴</td>
<td>0.0109</td>
<td>64.5</td>
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<tr>
<td>2.03 × 10⁻⁴</td>
<td>0.0090</td>
<td>61.4</td>
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<tr>
<td>3.05 × 10⁻⁴</td>
<td>0.0065</td>
<td>76.7</td>
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### Table III

<table>
<thead>
<tr>
<th>Constants</th>
<th>pH 7.0</th>
<th>pH 8.5</th>
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<tbody>
<tr>
<td>Michaelis constant for D-phenylglycine</td>
<td>3.2 × 10⁻²</td>
<td>4.43 × 10⁻²</td>
</tr>
<tr>
<td>Michaelis constant for FAD</td>
<td>1.65 × 10⁻⁴</td>
<td>2.94 × 10⁻⁷</td>
</tr>
<tr>
<td>Dissociation constant for FAD-apoenzyme complex</td>
<td>1.05 × 10⁻⁴</td>
<td>1.26 × 10⁻⁴</td>
</tr>
<tr>
<td>K₁ for AMP</td>
<td>8.0 × 10⁻⁴</td>
<td>1.04 × 10⁻⁴</td>
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Fig. 5. Double reciprocal plot with FAD as variable substrate at fixed concentrations of D-phenylglycine. The reactions were studied at 25° in 3-mL reaction mixtures containing 0.05 M sodium pyrophosphate, pH 8.5, and 15 μg of D-amino acid oxidase. The concentration of FAD was varied from 1.45 × 10⁻⁵ M to 8.71 × 10⁻⁵ M. Line 1, 2.5 × 10⁻³ M D-phenylglycine; Line 2, 3 × 10⁻³ M D-phenylglycine; Line 3, 4 × 10⁻³ M D-phenylglycine; Line 4, 6 × 10⁻³ M D-phenylglycine; Line 5, 1.2 × 10⁻² M D-phenylglycine.

Fig. 6. Replot of the data obtained from Fig. 5. A, slopes versus the reciprocal concentration of D-phenylglycine; B, intercepts versus the reciprocal concentration of D-phenylglycine.

Fig. 7. Changes in FAD fluorescence on binding to D-amino acid oxidase at 15.5°. ——, 1.71 × 10⁻⁵ M chromatographically pure FAD mixed with 1.83 × 10⁻⁵ M D-amino acid oxidase apoenzyme; ——, 1.71 × 10⁻⁵ M chromatographically pure FAD mixed with 1.83 × 10⁻⁵ M apoenzyme which had been inactivated by N-ethylmaleimide. The maleimide-treated enzyme had been incubated with 2 × 10⁻⁴ M N-ethylmaleimide in 0.05 M sodium pyrophosphate, pH 7.0, for 30 min and was approximately 90% inactivated. The reaction mixtures used for measuring the fluorescence intensity contained 0.1 M sodium pyrophosphate, pH 8.5, and 2 × 10⁻⁵ M N-ethylmaleimide, in a total volume of 1.5 mL. Flavin fluorescence was measured at 530 nm with excitation at 470 nm. The molarity of D-amino acid oxidase was based on a FAD-combining weight of 50,000.

of the velocity versus the reciprocal of the FAD concentration (34, 35). Fig. 5 shows the data obtained for D-amino acid oxidase at pH 8.5. Fig. 6 shows the data obtained for D-amino acid oxidase at pH 8.5 plotted in this manner. The slopes and intercepts of the lines of the double reciprocal plot of Fig. 5 were then replotted versus the reciprocal of the D-phenylglycine concentrations. This method has been described for bireactant sequential mechanisms by Florini and Vestling (35) and also by Cleland (36). The linear relationships obtained from these treatments of the data at pH 8.5 are presented in Fig. 6. The Michaelis constants for FAD and D-phenylglycine and the dissociation constants for FAD calculated from the slopes and intercepts of these replots are presented in Table III. The same type of experiment was conducted at pH 7.0 since this was the pH at which the N-ethylmaleimide protection experiments were carried out. Since the dissociation constants for FAD and for AMP did not vary with the pH change used, it was assumed that dissociation constants obtained for ADP and ADP-ribose at pH 8.5 would be the same at pH 7.0.

Massey and Curti (26) have demonstrated changes in flavin and protein fluorescence that occur upon mixing the D-amino acid oxidase apoenzyme with FAD. A rapid decrease followed by a second much slower decrease in FAD fluorescence was observed. The initial rapid decrease in fluorescence was suggested to be due to a rapid binding of FAD to the apoenzyme. It was of interest to study the effect of N-ethylmaleimide on the interaction between the apoenzyme and FAD. Fig. 7 shows the decrease in fluorescence intensity of FAD upon mixing with the intact apoenzyme (closed circles). Rate constants for
the rapid and slow quenching processes obtained in the present study agreed well with those reported by Massey and Curti (26). There was only a very slight decrease in the flavin fluorescence when FAD was mixed with apoenzyme that had been inactivated with N-ethylmaleimide. The final concentration of N-ethylmaleimide in the reaction mixture used for measuring the FAD fluorescence was \(2 \times 10^{-8} \text{ M}\). The presence of N-ethylmaleimide at this concentration had no effect on the initial rapid decrease in the FAD fluorescence upon the binding of FAD to the intact apoenzyme.

At pH 7.0 or lower and at low concentrations of maleimide, the reaction of maleimides with most proteins can be quite specific for sulphydryl groups. However, maleimides can react with amino acid derivative, which had an elution pattern identical to that of the synthetic N-succinylcysteine, was obtained with the reaction of maleimides with most proteins. Maleimides can be quite specific which had been inactivated by N-phenylmaleimide. A new amino acid derivative, which had an elution pattern identical to that of the synthetic S-succinylcysteine, was obtained with maleimides reacting with the maleimide-treated protein. An average value of 5.82 moles of S-succinylcysteine was calculated per 100,000 g of protein. The maleimide-treated enzyme also contained 5.6 moles of aniline per 100,000 g of protein determined according to the method of Bratton and Marshall (30). Other than the appearance of the S-succinylcysteine, there is no significant change in the quantity of any of the amino acids measured after treatment of the protein with N-phenylmaleimide.

The number of sulfhydryl groups of d-amino acid oxidase reacting with the maleimides was also determined spectrophotometrically. The loss of absorption of N-ethylmaleimide at 305 nm was measured for the S-succinylcysteine and aniline. An amino acid analysis of d-amino acid oxidase apoenzyme was listed in Table IV. Also shown in Table IV is the analysis obtained for the apoenzyme which had been inactivated by N-phenylmaleimide. A new amino acid derivative, which had an elution pattern identical to that of the synthetic S-succinylcysteine, was obtained with the maleimide-treated enzyme. An average value of 5.82 moles of S-succinylcysteine was calculated per 100,000 g of protein. The maleimide-treated enzyme also contained 5.6 moles of aniline per 100,000 g of protein determined according to the method of Bratton and Marshall (30). Other than the appearance of the S-succinylcysteine, there is no significant change in the quantity of any of the amino acids measured after treatment of the protein with N-phenylmaleimide.

### Table IV

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Untreated apoenzyme</th>
<th>Apoenzyme inactivated by N-phenylmaleimide</th>
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<tbody>
<tr>
<td></td>
<td>24-hr hydrolysis</td>
<td>72-hr hydrolysis</td>
</tr>
<tr>
<td></td>
<td>moles amino acid/10^8 g protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>moles amino acid/10^8 g protein</td>
</tr>
<tr>
<td>Lysine</td>
<td>27.9</td>
<td>28.4</td>
</tr>
<tr>
<td>Histidine</td>
<td>18.0</td>
<td>19.0</td>
</tr>
<tr>
<td>Ammonia</td>
<td>74.8b</td>
<td>94.1</td>
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<tr>
<td>Arginine</td>
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<td>Aspartic acid</td>
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<td>Threonine</td>
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<td>Serine</td>
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<td>Glutamic acid</td>
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<td>Proline</td>
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<td>Glycine</td>
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<td>Alanine</td>
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<td>Valine</td>
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<td>Methionine</td>
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<td>Isoleucine</td>
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<td>Leucine</td>
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<tr>
<td>S-Succinylcysteine</td>
<td>5.0b</td>
<td>5.0b</td>
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a Calculated according to Tristram and Smith (39).

b Extrapolated value to zero time.

c Value at 72 hrs.

d Determined according to the diazo coupling method of Bratton and Marshall (30).
The stoichiometry of the reaction of DDPM with D-amino acid oxidase was also studied by taking advantage of the absorption of DDPM in the visible region. As in the case of the other maleimide derivatives studied, the inactivation of the apoenzyme by DDPM followed pseudo first order kinetics. The inactivation constant calculated for the DDPM reaction is listed in Table I. D-Amino acid oxidase at a concentration of $1 \times 10^{-5}$ M was incubated with $3.4 \times 10^{-6}$ M DDPM in 0.05 M sodium pyrophosphate, pH 7.0, containing 1% ethanol. After 30 min, when only 10% of the enzymatic activity remained, excess DDPM was removed by one of two methods. The protein in one portion of the reaction mixture was precipitated with ammonium sulfate (90% saturation). The protein pellet obtained after centrifugation was washed with saturated ammonium sulfate solution. The protein was dissolved in 0.05 M sodium pyrophosphate, pH 7.0, and the spectrum of the labeled protein was measured. The amount of DDPM bound was determined by using the molar extinction coefficient of 3,000 reported for DDPM-N-acetylcysteine (40), and the amount of protein present was determined using the 278-nm reading after correction for DDPM. There were 4.8 moles of DDPM bound per 100,000 g of D-amino acid oxidase. A second portion of the DDPM-labeled protein was applied to Sephadex G-25 (1 x 30 cm) and eluted with water. The amount of DDPM bound to the protein was determined to be 4.96 moles per 100,000 g of D-amino acid oxidase.

Since a number of methods indicated that five to six sulfhydryl groups per 100,000 g of D-amino acid oxidase reacted with maleimides, it was of interest to determine the number of reactive sulfhydryl groups per active unit of the enzyme. Previous studies have indicated that D-amino acid oxidase is composed of two subunits per 100,000 g. However, it has not been made clear whether these subunits are identical. In order to learn more about the subunit structure of D-amino acid oxidase, peptide mapping of trypsin digests of the apoenzyme was performed. In theory, the number of peptides obtained should be approximately 76 if D-amino acid oxidase is composed of two different subunits. If the protein is composed of two identical subunits, one would expect to obtain approximately 38 peptides. The digestion process was performed six times, with the most reproducible results obtained when the protein was digested with trypsin for 4 to 5 hours. The pattern of the peptide spots which were consistently observed is shown in Fig. 8. The observation of 35 peptides would indicate that D-amino acid oxidase is composed of two identical subunits.

Since D-amino acid oxidase is composed of two identical subunits, it would be expected that the maleimides react with three sulfhydryl groups per subunit. In peptide patterns of trypsin digests of D-amino acid oxidase labeled with DDPM, only two DDPM-labeled peptides were observed. These labeled peptides are currently being separated and purified for further study.

DISCUSSION

A series of N-alkylmaleimides have been used to investigate the environment of reactive sulfhydryl groups of D-amino acid oxidase. In the inactivation of the apoenzyme by these compounds, chain length effects were observed (Fig. 3). For example, inactivation by N-octylmaleimide is 14- to 15-fold faster than that obtained with N-ethylmaleimide. Since the alkyl chains of the N-alkylmaleimides are located on the ring nitrogen, they would not be expected to affect the reactivity of the double bond of the maleimide ring. Heitz et al. (21) observed no chain length effect in the reactions of N-ethylemaleimide and N-heptylmaleimide with cysteine or glutathione.

It has been shown previously that maleimides can react with functional groups of proteins other than sulfhydryl groups (15, 16, 37) and for this reason, the inactivation of D-amino acid oxidase was studied under conditions where reactions with other functional groups were minimized. Acid hydrolysis of the enzyme inactivated with N-phenylmaleimide yielded equal amounts of S-succinylcysteine and aniline. These product studies indicate that the maleimides are reacting with only sulfhydryl groups of D-amino acid oxidase when the inactivation reactions are performed at pH 7.0 and low concentrations of maleimides. Previously, product studies have indicated that maleimides react selectively with sulfhydryl groups of a number of enzymes (40-42). Since sulfhydryl groups are involved in the inactivation of D-amino acid oxidase by maleimides, the chain length effects observed in the inactivation by N-alkylmaleimides...
suggest that the essential sulfhydryl groups of the enzyme are located in a nonpolar region of the enzyme.

Maleimides have been shown to be hydrolyzed at basic pH (11); however, under the conditions used in the present study, there was no significant loss of maleimide due to a hydrolytic side reaction. It has been shown that the hydrolysis of N-ethylmaleimide is specific base-catalyzed and that at pH 7.0 has a t1/2 of 2160 min (21). The alkylation of sulfhydryl groups by maleimides likewise occurs most rapidly under basic conditions. The rates of inactivation of N-amino acid oxidase by alkylmaleimides were 2- to 3-fold faster at pH 7.5 than at pH 7.0. If this difference is pH was affecting only the ionization of sulfhydryl groups, one would expect approximately a 2-fold increase in the rate of reaction in going from pH 7.0 to pH 7.5. Since the same difference in the rates of inactivation at the two pH values was observed with all the maleimides, it appears that there is no effect of pH on the interaction of the alkyl groups with the enzyme.

There are several mechanisms by which enzymes can be inactivated by maleimides. The data obtained with N-amino acid oxidase are most consistent with a two-step process. The first step could be a rapid noncovalent binding of the maleimide in the vicinity of the sulfhydryl groups. The second step would be a slower alkylation of sulfhydryl groups which results in the inactivation of the enzyme. The second step would be rate-limiting and should not be influenced by the length of the alkyl chain since there is no chain length effect on the rate of reaction of maleimides with sulfhydryl compounds, such as cysteine and glutathione (21). The first step, or binding process, is suggested to involve the interaction of the maleimide with a nonpolar region of the enzyme. The longer the alkyl group of the maleimide derivative, the more effective this interaction would be. Thus, the enzyme-maleimide complex would be stabilized by hydrophobic interactions, and a higher concentration of the complex would be formed with the longer chain derivatives. Such a mechanism, involving the binding of the maleimide prior to reaction with a sulfhydryl group and inactivation of the enzyme, is consistent with the observation that the enzyme can be saturated with higher concentrations of N-ethylmaleimide, N-butylmaleimide, and N-pentylmaleimide at pH 6.5 (Fig. 4), and the rates of inactivation with these compounds are the same at saturation. The effect of increasing concentrations of N-alkylmaleimides on the first order rates of inactivation, therefore, is related to the binding of maleimides to the apoenzyme and required the linear response observed at lower concentrations of maleimides (Fig. 2) to be considered in terms of inactivation constants (Table I).

Another mechanism which cannot be ruled out is a process which consists of binding of the maleimide followed by a conformational change which leads to an inactive enzyme. Sulfhydryl groups released by the conformational change now react rapidly with the maleimide in a step unrelated to the inactivation process. One should observe a chain length effect in the initial binding of the maleimide. An enzyme saturation curve would also be expected with this mechanism.

N-Alkylmaleimides have been used previously in attempts to investigate the nature of the environment surrounding active sulfhydryl groups of dehydrogenases. Heitz et al. (21) observed a chain length effect on the rates of inactivation of yeast alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1) by alkylmaleimides. It was concluded in this study that the alkylmaleimides interact with a nonpolar region of the enzyme which is located near sulfhydryl groups that are of importance in the catalytic activity of the enzyme. In contrast to this, the second order rate constants obtained for the inactivation of yeast glucose-6-phosphate dehydrogenase (N-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49) by N-ethylmaleimide and N-heptylmaleimide were approximately the same, and it was concluded that there was no nonpolar region near the functional sulfhydryl groups of this enzyme (21). Therefore, chain length effects in the inactivation of enzymes by N-alkylmaleimides appear to be related to the presence of a nonpolar environment around the reacting functional groups of the protein.

The ability of adenine derivatives and FAD to protect N-amino acid oxidase apoenzyme against inactivation by N-ethylmaleimide was studied (Table II). All of these compounds offered very effective protection against inactivation by N-ethylmaleimide. At concentrations equivalent to their K1 values measured at pH 8.5, AMP, ADP, and ADP-ribose each gave greater than 40% protection. With the use of kinetic analyses to determine the dissociation constants of the FAD-apoenzyme complex at pH 8.5 and pH 7.0, it was found that the dissociation constant for FAD at these two pH values was essentially the same (Table III). The good agreement between inhibitor dissociation constants for AMP measured at pH 7.0 and pH 8.5 (Table III) suggests that the binding of adenine derivatives likewise does not vary too greatly with pH in this region. Since almost 50% protection is observed with FAD at a concentration equivalent to its dissociation constant, the sulfhydryl group or groups essential to the functioning of the enzyme are not available in the holoenzyme for reaction with N-ethylmaleimide. Furthermore, the smaller adenine derivatives offer as much protection as FAD. Prior binding of N-ethylmaleimide to N-amino acid oxidase prevents the binding of FAD to the enzyme as determined by changes in FAD fluorescence. These observations may indicate that the maleimides are interacting with a sulfhydryl group at the FAD binding site or more specifically, at the adenosine region of the FAD-binding site; however, one cannot rule out the possibility that conformational changes of the protein are responsible for these protection effects. Yagi and Ozawa (6) observed that p-chloromercuribenzoate inhibited N-amino acid oxidase and that FAD protected against this inhibition. These investigators, on the basis of multiple inhibition kinetics, concluded that p-chloromercuribenzoate combined with the apoenzyme in competition with the adenine portion of FAD.

In the present study, 3 moles of maleimide were found to be bound per 50,000 g of N-amino acid oxidase. It is impossible to conclude from the data presented whether all three sulfhydryl groups are necessary for activity or whether there is one essential sulfhydryl group. It is possible that upon reaction of the essential sulfhydryl group there is a conformational change which causes the exposure of other sulfhydryl groups. Hellerman, Coffey, and Neims (2) observed that 6 to 8 moles of several sulfhydryl reagents per 100,000 g of protein were required to completely inhibit N-amino acid oxidase. These workers suggested that conformational changes may occur after the binding of a sulfhydryl reagent to a first fast reacting sulfhydryl group.

There has been some discrepancy in the literature concerning the molecular weight and subunit structure of N-amino acid oxidase. Until recently it was reported that the enzyme had a
molecular weight of 100,000 (43, 44). However, Yagi et al. (45)
calculated a molecular weight of 55,000 for the apoenzyme form
of d-amino acid oxidase, and Fonda and Anderson (1) dem-
onstrated that both the apoenzyme and holoenzyme forms had
molecular weights of approximately 50,000, while the benzozate
complex existed as a 100,000 molecular weight species at higher
protein concentrations. The enzyme contains 1 mole of FAD
per 50,000 g. It is not known if the enzyme normally functions
as a dimer and is broken down into monomers only in dilute
solution. Furthermore, it had never been clearly demonstrated
that the monomeric units of the enzyme were identical.

Kotaki, Harada, and Yagi (46) had suggested that the enzyme was made
up of two identical subunits since only one amino acid was found
as the N-terminal residue. The peptide mapping studies pre-
' sented here add additional evidence to this suggestion that the
subunits of d-amino acid oxidase are identical.

The amino acid composition for d-amino acid oxidase had been
determined previously by Hellerman and Coffey (44) and by
Kotaki et al. (46). However, there was a large discrepancy in
the values reported by the two laboratories for the basic amino
acids. The values obtained in the present study for the neutral
and acidic amino acids are in reasonable agreement with those
reported previously. The values obtained for the basic amino
acids fall between the two sets of values reported previously.

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