D-Amino Acid Oxidase

III. STUDIES OF FLAVIN ADENINE DINUCLEOTIDE BINDING*

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SUMMARY

A recently described spectrophotometric assay for D-amino acid oxidase was used to study the inhibition of the enzyme by a series of adenine and flavin derivatives. AMP, ADP, and ADP-ribose inhibited the enzyme competitively with respect to FAD. No instantaneous inhibition was observed with flavin derivatives. These inhibition studies indicated that the adenosine moiety and the pyrophosphate group of the FAD molecule contributed to the effective binding of FAD to D-amino acid oxidase. The isoalloxazine moiety of FAD appears to be less tightly bound than the adenosine moiety. FMN and riboflavin were found to inactivate the D-amino acid oxidase apoenzyme by a time-dependent photochemical process. This photoinactivation of the enzyme in the presence of flavin derivatives is enhanced by the adenine compounds.

The various forms of D-amino acid oxidase were studied by gel titration. This technique indicated that the apoenzyme form and holoenzyme form each have a molecular weight of approximately 50,000. This value corresponds to the minimum molecular weight calculated per FAD-binding site. The benzoate complex at low protein concentrations was found to contain a 50,000 molecular weight species and a 100,000 molecular weight species. At higher concentrations, the benzoate complex was found to exist entirely as the dimer.

D-Amino acid oxidase (D-amino acid: O₂ oxidoreductase (deaminating), EC 1.4.3.3) provides a convenient system for studying flavin-protein interactions, since FAD can be readily removed from the holoenzyme to yield apoenzyme. The resulting active apoenzyme can be used to study the recombination of FAD and apoenzyme to form holoenzyme. It was of interest to study the effect of compounds structurally analogous to portions of the FAD molecule on this recombination process. Previous studies (1-4) showed that both adenine derivatives and isoalloxazine derivatives inhibit D-amino acid oxidase. However, it is not clear how these derivatives function as inhibitors. Some investigators reported that the compounds studied were FAD competitive inhibitors (1-3), and some found that at least adenosine and riboflavin were noncompetitive inhibitors with respect to FAD (4). DeLuca, Weber, and Kaplan (5) observed no inhibition of the enzyme by flavin mononucleotide. Because of this controversy, a number of adenine and isoalloxazine derivatives were studied as inhibitors of D-amino acid oxidase.

Massey and Curti (6) demonstrated that there are two phases in the re-formation of the D-amino acid oxidase holoenzyme from FAD and apoenzyme. It was suggested that there was a rapid binding of FAD to the enzyme, followed by slower, secondary changes, which were interpreted as conformational changes of the protein. It was of interest to determine whether the binding of adenine derivatives or isoalloxazine derivatives to the apoenzyme brought about such conformational changes of the protein.

Dixon and Kleppe (7) demonstrated that there is a spontaneous dissociation of the D-amino acid oxidase holoenzyme into FAD and apoenzyme on dilution. Since most recombination and kinetic studies are performed with dilute enzyme solutions, the effect of the binding of FAD on the subunit structure of D-amino acid oxidase is of importance. D-Amino acid oxidase is generally thought to have a molecular weight of approximately 100,000, and to consist of two protein subunits, each binding 1 FAD molecule. Recently, Yagi et al. (8) demonstrated that the molecular weight of the apoenzyme form of the enzyme was 55,000, while the molecular weight of the holoenzyme form was approximately 100,000. These molecular weight studies were performed with relatively high concentrations of protein. It was of interest to determine the molecular weight of the enzyme at low protein concentrations. The various forms of D-amino acid oxidase were studied by Sephadex chromatography in an attempt to learn more about the relationship between the binding of FAD to the apoenzyme and the molecular weight and size of the protein.

EXPERIMENTAL PROCEDURE

D(-)-α-Phenylglycine, lumichrome, and lumazine were purchased from Aldrich. The D-phenylglycine was recrystallized...
from water. Adenosine, AMP, ADP, ADP-ribose, riboflavin, FMN, FAD (Grade III), and crystallized and lyophilized bovine serum albumin were obtained from Sigma. Three times crystallized ovalbumin was a product of Nutritional Biochemicals. Twice crystallized and once lyophilized yeast alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) was purchased from Worthington. Sephadex G-200 was a product of Pharmacia. DEAE-cellulose was purchased from Bio-Rad Laboratories.

Electrophoretically purified D-amino acid oxidase was obtained from Worthington, and analytical grade D-amino acid oxidase was purchased from Boehringer-Mannheim as the crystalline benzoate complex suspended in 1.8 m ammonium sulfate solution. Unless otherwise specified, the D-amino acid oxidase obtained from Worthington was used in all experiments. The apoenzyme form of D-amino acid oxidase was prepared according to Massey and Curti (6), and stock solutions were stored at 4° in 0.1 M sodium pyrophosphate, pH 8.5. Prior to use, the stock solutions were diluted with 0.05 M sodium pyrophosphate, pH 8.5. To the required concentration. Stock solutions of the holoenzyme were stored at 4° in 0.05 M sodium pyrophosphate, pH 8.5, containing 1 × 10^{-5} M FAD. Stock solutions of D-phenylglycine, FAD, and inhibitors were all prepared in 0.05 M sodium pyrophosphate and adjusted to pH 8.5.

The D-amino acid oxidase-catalyzed reactions were studied by the spectrophotometric assay, in which the optical density changes accompanying the production of benzoylformic acid from D-phenylglycine were followed (9). The reactions were studied at 25° in 3-ml reaction mixtures containing 0.05 m sodium pyrophosphate buffer, pH 8.5, and 0.015 M D-phenylglycine. The concentrations of the other components of the reaction mixtures—FAD, inhibitors, and enzyme—will be included in specific descriptions of individual experiments. Reactions were initiated by the addition of enzyme. Reaction rates were measured by following the increase in optical density at 243 μm, and 20-sec readings were obtained for 8 min. Upon initiation of the reaction by the addition of enzyme, the reaction proceeds through a lag phase and reaches a constant velocity within 6 min at 25°. This constant velocity is taken as the velocity measurement.

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° with a Radiometer pH meter, type PHM 4c, with a G-200-B glass electrode.

The photoinactivation of D-amino acid oxidase was carried out by illuminating 1.0-ml samples containing enzyme and flavin in 0.05 M sodium pyrophosphate, pH 8.5. The reaction mixtures were both prepared and studied in a darkened room. The reaction mixtures were incubated in a 3-ml cuvette at 25° in a temperature-controlled cell compartment of an Amino-Bowman spectrophotofluorometer and were illuminated from the side with a 150-watt xenon lamp. The desired wave length was obtained with the monochromator of the Amino-Bowman spectrophotofluorometer. The energy of the light used was measured with a YSI model 65 Radiometer thermopile. The light intensity was varied by the insertion of aluminum aerogel in the light path. The time course of the photoinactivation of D-amino acid oxidase was determined by removing 0.03-ml samples from the reaction mixture after successive periods of illumination and assaying for enzyme activity.

Fluorescence measurements were performed in a temperature-controlled cell compartment of an Amino-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 FMN and FAD used in the fluorescence studies were purified by chromatography on DEAE-cellulose according to Massey and Swoboda (10). In other experiments, the commercial FMN and FAD were used.

Sephadex G-200 was suspended in 0.05 M sodium pyrophosphate, pH 8.5, and was allowed to swell for 7 hours at 90°. The fine particles were removed, and the swollen gel was washed several times with buffer. The Sephadex G-200 column was equilibrated at 4° with 0.05 M sodium pyrophosphate, pH 8.5.

RESULTS

Determination of Inhibitor Dissociation Constants.—The inhibition of D-amino acid oxidase by various analogues of portions of the FAD molecule was studied. Inhibitor dissociation constants were determined for each of these compounds in two different experiments. The inhibitors were first studied as a function of varying FAD concentration and three constant inhibitor concentrations. The data obtained in this manner were plotted according to Lineweaver and Burk (11). Fig. 1 shows the double reciprocal plot obtained when AMP was used as the inhibitor. The inhibition observed was competitive with respect to FAD, and the average inhibitor dissociation constant was calculated to be 1.04 × 10^{-4} M. In a second set of experiments, the inhibitors were studied as a function of varying inhibitor concentration with two concentrations of FAD. The data obtained in these studies were plotted according to Dixon (12). Fig. 2 shows the inhibition of the enzyme when varying concentrations of AMP were used. The K_i value calculated from the intersection of the lines was 1.11 × 10^{-4} M.

Two independent methods were used to determine the inhibitor dissociation constants, since the two methods permitted
a check on the $K_I$ values obtained under different conditions. The Lineweaver-Burk plots indicate the type of inhibition, such as competitive or noncompetitive. The Dixon plots permit one to look for other aspects of inhibition, such as "partial" inhibition, which cannot be recognized with Lineweaver-Burk plots. Webb (13) has pointed out the advisability of using at least two different plotting methods to determine the type of inhibition and the $K_I$ values.

AMP, ADP, and ADP-ribose all inhibited D-amino acid oxidase competitively with respect to FAD. The $K_I$ values calculated for these compounds by the two plotting methods are presented in Table I. Adenosine was also studied as an inhibitor, but did not inhibit the enzyme up to a concentration of $1 \times 10^{-4}$ M (Table I). At higher concentrations, the absorption of adenosine at 243 m\(\mu\) interfered with the spectrophotometric assay of the enzyme. The binding of the adenine compounds becomes better in going from adenosine to AMP to ADP. ADP and ADP-ribose have approximately the same $K_I$ values.

The flavin analogues lumazine, lumichrome, riboflavin, and FMN did not instantaneously inhibit the enzyme up to concentrations of $6 \times 10^{-5}$ M (Table I). This concentration is about one-half the $K_I$ value obtained for AMP, and somewhat greater than the $K_I$ value for FAD.

**Photoinactivation Experiments**—Although the isalloxazine derivatives did not competitively inhibit D-amino acid oxidase at the concentrations tested, lumichrome, riboflavin, and FMN caused a time-dependent inhibition of the enzyme. This time-dependent inactivation was observed only when the reaction mixtures were exposed to light; therefore, this reaction was studied as a photochemical process. The logarithm of the activity of the apoenzyme in the presence of a flavin was plotted against time. Such a plot for the inactivation of the apoenzyme by lumichrome, riboflavin, and FMN is greatly accelerated by the addition of an adenine derivative. The logarithm of enzyme activity with respect to time is shown in Fig. 3 for the inactivation of the apoenzyme by riboflavin and FMN and 450-m\(\mu\) light is shown in Fig. 3. This photoinactivation of D-amino acid oxidase followed first order kinetics in all cases. The half-time values and first order rate constants for the inactivation of the apoenzyme by lumichrome, riboflavin, and FMN are presented in Table II. The first order rate constants were calculated from the equation

$$k_{obs} = \frac{0.693}{t}$$

Lumazine did not give a time-dependent inactivation of the apoenzyme under the conditions used. The inactivation observed in the presence of riboflavin or FMN was more rapid than that with lumichrome.

The photoinactivation of D-amino acid oxidase in the presence of riboflavin or FMN is greatly accelerated by the addition of an adenine derivative. The logarithm of enzyme activity with respect to time is shown in Fig. 3 for the inactivation of the apoenzyme by a combination of riboflavin and AMP (Line 3) and by FMN and AMP (Line 4). All combinations of adenine and flavin derivatives resulted in a first order inactivation of the enzyme. The half-time values and first order rate constants are listed in Table II. Each of the adenine derivatives—adenosine, AMP, ADP, ADP-ribose—increased the rate of inactivation of the apoenzyme by riboflavin or FMN. The adenine compounds, when incubated alone with the apoenzyme, did not cause a time-dependent inhibition of D-amino acid oxidase. The inhibition observed with these adenine derivatives is fully reversible upon dilution. The enhancement of the flavin inactivation of the enzyme by the adenine derivatives is linear with respect to the concentration of the adenine compound; that is, with an increase in the adenine concentration, there is a proportional increase in the rate of photoinactivation at a given flavin concentration.

The presence of FAD in the incubation mixture containing FMN and apoenzyme resulted in some protection of the apoenzyme against photoinactivation. FAD at a concentration of $1 \times 10^{-6}$ M reduced the first order rate constant of inactivation in the presence of $5 \times 10^{-4}$ M FMN by 19%. The first order rate constant in the presence of $3 \times 10^{-4}$ M FAD was 34% of that obtained with $5 \times 10^{-5}$ M FMN alone.

### Table I

Inhibitor dissociation constants for compounds structurally related to FAD

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_I$ (M) Lineweaver-Burk plots</th>
<th>$K_I$ (M) Dixon plots</th>
</tr>
</thead>
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<tr>
<td>Adenosine</td>
<td>$1.04 \times 10^{-4}$</td>
<td>$1.11 \times 10^{-4}$</td>
</tr>
<tr>
<td>AMP</td>
<td>$5.2 \times 10^{-5}$</td>
<td>$5.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>ADP</td>
<td>$5.9 \times 10^{-5}$</td>
<td>$5.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>$1.11 \times 10^{-4}$</td>
<td>$1.11 \times 10^{-4}$</td>
</tr>
<tr>
<td>Lumazine</td>
<td>$5.4 \times 10^{-5}$</td>
<td>$5.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>Lumichrome</td>
<td>$5.7 \times 10^{-4}$</td>
<td>$5.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>$5.8 \times 10^{-4}$</td>
<td>$5.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>FMN</td>
<td>$5.9 \times 10^{-4}$</td>
<td>$5.9 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

* No inhibition up to $1 \times 10^{-4}$ M.

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**Fig. 2.** Inhibition of D-amino acid oxidase as a function of the concentration of AMP. Reaction mixtures contained 0.05 M sodium pyrophosphate (pH 8.5), 0.015 M D-phenylglycine, 15 \(\mu\)g of D-amino acid oxidase apoenzyme, FAD, and inhibitor, in a total volume of 3 ml. The AMP concentration was varied from zero to $1.4 \times 10^{-4}$ M. Line 1, $2 \times 10^{-7}$ M FAD; Line 2, $4 \times 10^{-7}$ M FAD.
The effect of varying concentrations of FMN on the inactivation of D-amino acid oxidase apoenzyme while being illuminated at two different light intensities is presented in Fig. 4. At both light intensities, the rates of inactivation leveled off at a maximum value at the higher concentrations of FMN. The concentration of FMN at half the maximum inactivation rate was approximately 4 x 10^-4 M when the light intensity used was 1.65 x 10^4 ergs cm^-2 sec^-1, and 2 x 10^-4 M when the light intensity was 7.4 x 10^3 ergs cm^-2 sec^-1. Since the rates of inactivation leveled off at lower FMN concentrations when the system was illuminated with the lower intensity of light, it appeared that this effect was due to absorption of the incident light energy by the higher concentrations of FMN.

The effect of various wave lengths of light on the inactivation of D-amino acid oxidase in the presence of 1 x 10^-4 M FMN is shown in Fig. 5. The light intensity at the various wave lengths used is represented by the open circles. The light intensity was relatively constant from 310 to 500 nm. The action spectrum of the photoinactivation of the enzyme is represented by the solid circles, and it appears that 450-nm light is most efficient in the photoinactivation process. The action spectrum in the visible region is very similar to the spectrum of FMN, which is represented by the dashed line. The intensity of light used to illuminate the enzyme fell off below 310 nm. For this reason, 270-nm light of an intensity of 5.1 ergs cm^-2 sec^-1 was used to illuminate the apoenzyme and 1 x 10^-4 M FMN, and a first order rate of inactivation of the enzyme were 0.0100, 0.0257, and 0.0373 min^-1, respectively. The photoinactivation of D-amino acid oxidase was a linear function of light intensity in the region of light intensities used. If the riboflavin or FMN was first incubated in the presence of light for 30 min, after which the apoenzyme was added and the incubation mixture was placed in the dark, there was no loss of enzymatic activity after 75 min.

The half-time values and first order rate constants were obtained from studies carried out under conditions described in Fig. 3.

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The importance of flavins alone and in the presence of AMP. The reaction mixtures were illuminated with 450-nm light at an intensity of 7.4 x 10^3 ergs cm^-2 set^-1. Since the rates of inactivation leveled off at lower FMN concentrations when the light intensity used was 7.4 x 10^3 ergs cm^-2 set^-1, the first order rates of inactivation of the enzyme were 0.0100, 0.0257, and 0.0373 min^-1, respectively. The photoinactivation of D-amino acid oxidase was a linear function of light intensity in the region of light intensities used. If the riboflavin or FMN was first incubated in the presence of light for 30 min, after which the apoenzyme was added and the incubation mixture was placed in the dark, there was no loss of enzymatic activity after 75 min.

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The effect of various wave lengths of light on the inactivation of D-amino acid oxidase in the presence of 1 x 10^-4 M FMN is shown in Fig. 5. The light intensity at the various wave lengths used is represented by the open circles. The light intensity was relatively constant from 310 to 500 nm. The action spectrum of the photoinactivation of the enzyme is represented by the solid circles, and it appears that 450-nm light is most efficient in the photoinactivation process. The action spectrum in the visible region is very similar to the spectrum of FMN, which is represented by the dashed line. The intensity of light used to illuminate the enzyme fell off below 310 nm. For this reason, 270-nm light of an intensity of 5.1 ergs cm^-2 sec^-1 was used to illuminate the apoenzyme and 1 x 10^-4 M FMN, and a first order rate of inactivation of the enzyme were 0.0100, 0.0257, and 0.0373 min^-1, respectively. The photoinactivation of D-amino acid oxidase was a linear function of light intensity in the region of light intensities used. If the riboflavin or FMN was first incubated in the presence of light for 30 min, after which the apoenzyme was added and the incubation mixture was placed in the dark, there was no loss of enzymatic activity after 75 min.

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order rate constant of 0.008 min$^{-1}$ was obtained. This rate constant is still much lower than that observed when the system was illuminated with 450-nm light.

Since adenine derivatives increased the rate of photoinactivation of L-amino acid oxidase by riboflavin or by FMN, it was thought that the adenine and flavin derivatives may have formed a complex that was more reactive than the flavin alone. Therefore, it was of interest to determine the dissociation constants of the adenine-flavin complexes. The dissociation constants were determined graphically according to the method described by Weber (14) and are listed in Table III. The lowest dissociation constant obtained was that for the riboflavin adenine complex. This complex forms more readily than does the FMN-adenosine complex, which in turn forms more readily than the FAD-adenosine complex. Flavin-AMP, -ADP, and -ADP-ribose complexes all have higher dissociation constants than the corresponding flavin-adenosine complexes. The dissociation constants obtained in this study agree well with previously reported values (1, 14, 15), which are also listed in Table III.

The time-dependent inactivation of L-amino acid oxidase apoenzyme by riboflavin and FMN was studied as a function of temperature. Four inactivation reactions were studied: heat inactivation of the apoenzyme, inactivation by riboflavin, and inactivation by FMN with two different intensities of illumination. First order rate constants obtained at different temperatures for the inactivation reactions are shown plotted on a log arithmetic scale against the reciprocal of the absolute temperature in Fig. 6. The solid triangles represent the inactivation of the apoenzyme by FMN or riboflavin, and the data were obtained with mixtures incubated in a Forma-Temp Jr., constant temperature, circulating water bath (Forma Scientific Inc., Marietta, Ohio) in a laboratory with normal lighting. The solid circles represent the inactivation of the apoenzyme by FMN, when the incubation mixtures were illuminated directly in the water bath by a 150-watt bulb placed 10 inches from the incubation mixtures.

![Figure 5](http://www.jbc.org/) Comparison of absorption spectrum of FMN with the action spectrum for the photoinactivation of L-amino acid oxidase by FMN. --- ---, absorption spectrum for $3 \times 10^{-4}$ M FMN; $\circ$ --- $\circ$, the energy of light used in the action spectrum; --- ---, first order rate constants obtained for the inactivation of the apoenzyme by FMN with illumination by a xenon lamp at various wave lengths. The reaction mixtures contained 0.05 M sodium pyrophosphate (pH 8.5), 0.1 mg of L-amino acid oxidase apoenzyme, and $1 \times 10^{-4}$ M FMN, in a total volume of 1 ml. After intervals of illumination, 0.03-ml aliquots were assayed for enzymatic activity by the spectrophotometric assay.

### Table III

Dissociation constants of complexes of flavin and adenine derivatives

<table>
<thead>
<tr>
<th>Complex</th>
<th>Concentration of adenine compound</th>
<th>$K$ observed</th>
<th>$K$ reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin-adenosine</td>
<td>$0.537 \times 10^{-3}$</td>
<td>0.0092</td>
<td>0.0074 at 5$^\circ$C</td>
</tr>
<tr>
<td>Riboflavin-AMP</td>
<td>$0.537 \times 10^{-3}$</td>
<td>0.013</td>
<td>0.0147$^{acc}$</td>
</tr>
<tr>
<td>FMN-adenosine</td>
<td>$0.534 \times 10^{-3}$</td>
<td>0.020</td>
<td>0.020$^{acc}$</td>
</tr>
<tr>
<td>FMN-AMP</td>
<td>$0.342 \times 10^{-3}$</td>
<td>0.021</td>
<td>0.021$^{acc}$</td>
</tr>
<tr>
<td>FMN-ADP</td>
<td>$1.02 \times 10^{-4}$</td>
<td>0.045</td>
<td>0.040$^{acc}$</td>
</tr>
<tr>
<td>FMN-ADP-ribose</td>
<td>$2.10 \times 10^{-4}$</td>
<td>0.045</td>
<td>0.037$^{acc}$</td>
</tr>
<tr>
<td>FAD-adenosine</td>
<td>$1.02 \times 10^{-3}$</td>
<td>0.021</td>
<td>0.021$^{acc}$</td>
</tr>
<tr>
<td>FAD-AMP</td>
<td>$1.52 \times 10^{-4}$</td>
<td>0.045</td>
<td>0.040$^{acc}$</td>
</tr>
<tr>
<td>FAD-ADP</td>
<td>$1.02 \times 10^{-4}$</td>
<td>0.045</td>
<td>0.037$^{acc}$</td>
</tr>
<tr>
<td>FAD-ADP-ribose</td>
<td>$2.10 \times 10^{-4}$</td>
<td>0.045</td>
<td>0.037$^{acc}$</td>
</tr>
</tbody>
</table>

* From Weber (14).

* From Tsibris, McCormick, and Wright (15).

* From Burton (1).
The concentration of riboflavin was $1 \times 10^{-6}$ M for the inactivation of the apoenzyme by riboflavin.

The greater light intensity used in these experiments resulted in faster rates of inactivation. The heat inactivation of the apoenzyme (open circles) has a much greater slope than the inactivation of the enzyme by flavins. In Table IV are listed the values obtained for the activation energy ($E_a$), the free energy of activation ($\Delta F^\ddagger$), the enthalpy of activation ($\Delta H^\ddagger$), and the entropy of activation ($\Delta S^\ddagger$) for the various inactivation reactions. The heat inactivation of the enzyme (open circles) has a much greater slope than the inactivation by flavins. The activation energy ($E_a$) for the activation by flavins was 15.9 kcal/mol, and fluorescence emission was measured at 340 nm. Upon mixing $6 \times 10^{-4}$ M apoenzyme with $5 \times 10^{-5}$ M FMN, riboflavin, adenosine, AMP, ADP, or ADP-ribose, there was an instantaneous quenching of protein fluorescence, owing to the absorption of these compounds; however, there was no further decrease in the protein fluorescence with time. A combination of AMP and FMN also resulted in no time-dependent quenching of protein fluorescence.

**Determination of Molecular Weight of l-Amino Acid Oxidase by Gel Filtration**—The effect of FAD binding on the molecular weight of l-amino acid oxidase was studied by gel filtration. The enzyme obtained from Boehringer was studied by chromatography on Sephadex G-200, and the results are shown in Fig. 7. The elution volumes for the various samples run on Sephadex G-200 are listed in Table V. When the l-amino acid oxidase holoenzyme was studied, the column had been equilibrated with $0.05$ M sodium pyrophosphate, pH 8.5, and $1 \times 10^{-4}$ M FAD. Chromatography of the benzoate complex was conducted after the column had been equilibrated with $0.05$ M sodium pyrophosphate (pH 8.5), $1 \times 10^{-4}$ M FAD, and $1 \times 10^{-4}$ M benzoic acid. FAD and benzoic acid were used in these chromatography procedures to prevent the holoenzyme from dissociating into apoenzyme and free FAD, and the benzoic acid from dissociating into benzoic acid, FAD, and the apoenzyme. All other samples were applied to the column after equilibration with $0.05$ M sodium pyrophosphate.

The l-amino acid oxidase purchased from Boehringer is in the form of the benzoate complex. When the benzoate complex was studied by gel filtration, the Boehringer enzyme was used directly. The apoenzyme used was prepared from the benzoate complex according to Massey and Curti (6). The holoenzyme was prepared by incubating the apoenzyme with $1 \times 10^{-4}$ M FAD.

**Table IV**

<table>
<thead>
<tr>
<th>Method of inactivation</th>
<th>$E_a$ (kcal/mol)</th>
<th>$\Delta F^\ddagger$ (kcal/mol)</th>
<th>$\Delta H^\ddagger$ (kcal/mol)</th>
<th>$\Delta S^\ddagger$ (cal deg$^{-1}$ mol$^{-1}$)</th>
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<tbody>
<tr>
<td>Heat</td>
<td>57.20</td>
<td>23.90</td>
<td>56.60</td>
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<tr>
<td>Riboflavin</td>
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<td>23.21</td>
<td>12.88</td>
<td>-34.1</td>
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<tr>
<td>FMN</td>
<td>13.43</td>
<td>23.23</td>
<td>12.63</td>
<td>-34.2</td>
</tr>
<tr>
<td>FMN with illumination</td>
<td>11.51</td>
<td>23.04</td>
<td>11.60</td>
<td>-39.7</td>
</tr>
</tbody>
</table>

**Table V**

<table>
<thead>
<tr>
<th>Material</th>
<th>Elution volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue dextran</td>
<td>13.4</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>27.5</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>26.0</td>
</tr>
<tr>
<td>Yeast alcohol dehydrogenase</td>
<td>21.0</td>
</tr>
<tr>
<td>l-Amino acid oxidase apoenzyme</td>
<td></td>
</tr>
<tr>
<td>0.23 mg per ml</td>
<td>27.3</td>
</tr>
<tr>
<td>1.0 mg per ml</td>
<td>27.4</td>
</tr>
<tr>
<td>3.86 mg per ml</td>
<td>27.5</td>
</tr>
<tr>
<td>l-Amino acid oxidase holoenzyme</td>
<td></td>
</tr>
<tr>
<td>0.32 mg per ml</td>
<td>27.0</td>
</tr>
<tr>
<td>1.0 mg per ml</td>
<td>27.0</td>
</tr>
<tr>
<td>3.38 mg per ml</td>
<td>27.0</td>
</tr>
<tr>
<td>l-Amino acid oxidase-benzoate complex</td>
<td></td>
</tr>
<tr>
<td>0.5 mg per ml</td>
<td>23.5, 27.0</td>
</tr>
<tr>
<td>1.0 mg per ml</td>
<td>20.7</td>
</tr>
<tr>
<td>5.0 mg per ml</td>
<td>23.5</td>
</tr>
</tbody>
</table>
oxidase have elution volumes slightly less than ovalbumin, which has a molecular weight of 45,000. The elution volumes of the apoenzyme and holoenzyme are greater than that obtained with bovine serum albumin, which has a molecular weight of approximately 65,000. The benzoate complex, applied to the column as 0.5-mL samples containing 1 or 5 mg of protein per mL, has an elution volume which is significantly different from those obtained with the apoenzyme or holoenzyme forms of D-amino acid oxidase. When 0.5 mL of the benzoate complex at a concentration of 0.3 mg per mL was applied to the column, two peaks were obtained. One peak had an elution volume which corresponded to those obtained with the apoenzyme and holoenzyme, and the other peak had an elution volume similar to that obtained when the benzoate complex was used in the more concentrated form.

The Stokes radii for the three forms of D-amino acid oxidase were obtained from the gel filtration data according to Siegel and Monty (16). By this method, the Stokes radius is determined from a plot of $K_d$ with respect to the Stokes radius of the proteins, where $K_d$ is the distribution coefficient. The Stokes radii used for the standard proteins were those reported by Ackers (17). The molecular weights of each form of D-amino acid oxidase were calculated by using the Stokes radii and either the frictional ratios and the partial specific volumes or the sedimentation coefficients reported by Yagi et al. (8). The apoenzyme was calculated to have a Stokes radius of 31 Å and a molecular weight of 48,000. The holoenzyme was calculated to have a Stokes radius of 31.5 Å and a molecular weight of 50,000. No aggregation of the apoenzyme or holoenzyme was observed for the concentrations studied. The benzoate complex at a concentration of 1 or 5 mg per mL was found to have a Stokes radius of 30.5 Å and a molecular weight of 65,300. The benzoate complex at a concentration of 0.3 mg per mL was resolved into two fractions, which corresponded to molecular weights of 48,200 and 95,300.

**Discussion**

The adenine derivatives AMP, ADP, and ADP-ribose were found to inhibit the D-amino acid oxidase-catalyzed reaction competitively with respect to FAD. The observation that adenosine does not inhibit the enzyme at concentrations equivalent to the $K_d$ value obtained for AMP (Table I) suggests that AMP is bound more effectively to the enzyme than is adenosine. This observation, and the fact that ADP and ADP-ribose are bound better than is AMP, suggest that the phosphate groups of AMP, ADP, and ADP-ribose contribute to the binding of these compounds to the enzyme. It has been shown previously (4) that AMP is a competitive inhibitor of D-amino acid oxidase, and that adenosine is a very poor noncompetitive inhibitor of the enzyme. Walaas and Walaas (4), on the basis of the inhibition of the enzyme by phosphate and other anions, suggested that the phosphate groups of FAD contribute to its binding to the enzyme.

Previously, the inhibition of dehydrogenases by adenine derivatives had been investigated in an attempt to learn the importance of the phosphate groups of NAD in the binding of NAD to these dehydrogenases. In the case of rabbit muscle $\alpha$-glycerophosphate dehydrogenase (6 - glycerol - 3 - phosphate : NAD oxidoreductase, EC 1.1.1.8), it was observed that there was a stepwise increase in binding in going from adenosine to adenosine monophosphate to adenosine diphosphate (18). Such an increase in binding with the phosphorylation of the adenine derivatives was similar to that observed with D-amino acid oxidase, and it was suggested that there was a region of the NAD-binding sites of $\alpha$-glycerophosphate dehydrogenase that was capable of interacting with a pyrophosphate grouping. On the other hand, such a stepwise interaction was not observed in the binding of the adenine derivatives to yeast alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1) (19), and it was concluded that the phosphate groups of NAD did not contribute to the binding of NAD to yeast alcohol dehydrogenase.

Since ADP-ribose was no more effectively bound than was ADP (Table I), it appears that the terminal ribose of ADP-ribose does not contribute to the binding of this compound to D-amino acid oxidase. This observation does not exclude the possibility that the ribityl moiety of FAD is involved in the binding of the prosthetic group to the enzyme.

The isoalloxazine derivatives luxazine, lumichrome, riboflavin, and FMN do not inhibit D-amino acid oxidase substantially up to concentrations of $6 \times 10^{-5}$ M (Table I). AMP, ADP, and ADP-ribose all give marked inhibition of the enzyme at this concentration. In extending these observations to FAD, it appears that the adenosine moiety and the pyrophosphate group of FAD must contribute to the binding of the prosthetic group to the enzyme. The isoalloxazine moiety of FAD appears to be less tightly bound than the adenosine moiety. This less rigid binding of the functional portion of the coenzyme may provide more flexibility for this moiety in the catalytic process.

In contrast to these results, previous studies had indicated that isoalloxazine derivatives were effective inhibitors of the enzyme. When D-amino acid oxidase activity was measured manometrically, it was found that FMN (4) and flavin monosulfate (2) were FAD-competitive inhibitors of the enzyme. However, DeLuca et al. (5) observed no inhibition by FMN when enzyme activity was assayed by coupling the D-amino acid oxidase-catalyzed reaction with the lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) system. Since no inhibition by FMN was observed with the spectrophotometric assay (9) in the present study, it appeared that there was some interaction taking place between FMN and the enzyme in the manometric assay, but not in the other two methods of assay.

Lumichrome, riboflavin, and FMN were found to inactivate D-amino acid oxidase in a time-dependent process (Table II), whereas the inhibition by the adenine derivatives was not time-dependent. The time-dependent inactivation of the enzyme by flavins was shown to be a light-sensitive reaction and was affected by the intensity of the light used. The photoinactivation of D-amino acid oxidase by FMN is due to the photosensitization of FMN by light, since the action spectrum of the photoinactivation process is very similar to the absorption spectrum of FMN in the visible region. These observations explain the results obtained when the inhibition of D-amino acid oxidase by FMN was studied with the manometric assay, in which the assay mixtures are exposed to light during the entire period of pre-equilibration and measurement of rates. Therefore, FMN would appear to have inhibited the enzyme. It was found in the present study that FAD protects against inactivation by FMN; therefore, FMN could have been an FAD-competitive inhibitor in the manometric assays.

The photoinduced inactivation of various enzymes in the presence of dyes, including riboflavin and FMN, has been demonstrated in several studies (20-22). The photoinactivation of
proteins by these dyes is due to photochemical oxidation of various amino acids of the protein. The photochemical oxidation of amino acids and proteins is considered to be a complex process. There have been several theories proposed concerning the mechanism of photochemical oxidation of some systems. In one proposal, the dye is photoactivated with light and then interacts with the protein; another suggestion is that the dye combines with the protein first, and this complex is then photoactivated.

It was found that adenine derivatives accelerated the photoinactivation of D-amino acid oxidase apoenzyme in the presence of riboflavin or FMN (Table II). The rates of photoinactivation depended on the concentration of the adenine compound present. There are several possible explanations for this enhancement by the compounds. The adenine derivatives, upon binding to the adenine region of the FAD-binding site, may provide a more favorable environment for the binding of flavins. However, since adenosine enhances the rate of photoinactivation at a concentration at which it does not cause instantaneous inhibition of the enzyme, it does not appear that adenosine functions by prior binding to the enzyme. A second possibility is the formation of adenine-flavin complexes that interact with the enzyme more effectively than either the flavin or adenine derivative alone. The dissociation constants for the adenine-flavin complexes range from 9.2 × 10⁻⁴ M for the adenosine-riboflavin complex to 2.1 × 10⁻³ M for the FMN-ADP-ribose complex (Table III). Although very small amounts of these complexes would form at the concentrations used in the photoinactivation experiments, the better binding of these complexes to the enzyme could enhance the photochemical inactivation. Since adenosine forms complexes with the flavin derivatives studied, the ability of the adenine and flavin derivatives to interact with one another does appear to be of importance in the facilitation of the photoinactivation process. Whether such interactions occur prior to binding to the enzyme or after each component has been independently bound to the FAD-binding site cannot be established on the basis of the data available.

In the photoinactivation process, it is not clear whether there is prior binding of the flavin to D-amino acid oxidase and excitation of this flavin-enzyme complex with light, or whether the flavin in an excited state binds to or interacts with the enzyme. The observation that the addition to the enzyme of riboflavin or FMN that had been irradiated produced no effect on the enzymatic activity in the dark indicates that the flavin must be in an excited state before binding to or in the presence of the enzyme. If the flavin were bound to the enzyme first, and the enzyme then became irreversibly photoxidized, one would expect to obtain an enzyme saturation curve with increasing amounts of FMN. A saturation curve was obtained; however, the concentration of FMN at which the inactivation rates level off depends on the intensity of light used for illumination (Fig. 4). This leveling off of inactivation rates may be due to the fact that the higher concentrations of FMN absorb more light, thus decreasing the light available for photoactivation of enzyme-flavin complex.

The effect of temperature on the inactivation of D-amino acid oxidase by riboflavin and FMN was studied in an attempt to learn more about the mechanism of the photochemical reaction. In most cases, the denaturation of proteins is very sensitive to temperature and is highly endothermic. Furthermore, a large entropy of activation is generally associated with denaturation of proteins (23-25). In the present study, the heat inactivation of D-amino acid oxidase apoenzyme proceeds with an enthalpy of activation of 56.6 kcal per mole and an entropy of activation of 105.2 cal deg⁻¹ mole⁻¹. The high positive value of ΔS₁ in protein denaturation is generally attributed to an increased disorganization of the molecular structure, or to large changes in the conformation of the protein. The inactivation of the apoenzyme by riboflavin or FMN proceeded with a ΔH₂ of 12.8 kcal per mole, a value much lower than that obtained in the heat inactivation of the enzyme. This low ΔH₂ and the negative ΔS₁ obtained for the inactivation by riboflavin or by FMN suggest that the enzyme does not undergo the large conformational changes observed with heat denaturation.

It has been suggested that the binding of FAD to D-amino acid oxidase causes a conformational change in the protein (6). By studying changes in flavin absorbance and in flavin and protein fluorescence, and by measuring catalytic activity, Massey and Curti (6) found that the formation of holoenzyme from apoenzyme and FAD proceeded through two stages. The first stage was thought to be a rapid binding of FAD, followed by slow, secondary changes, which were related to the appearance of catalytic activity and were interpreted as conformational changes of the protein. Upon mixing of FAD with apoenzyme, there is a small, rapid quenching of protein fluorescence, followed by a large, slow change. Adenine derivatives do not cause a change in the fluorescence of the D-amino acid oxidase apoenzyme. Isoalloxazine derivatives also do not cause a time-dependent quenching of the fluorescence of the apoenzyme. Furthermore, a combination of FMN and AMP mixed with the apoenzyme did not cause any decrease in the fluorescence of the protein. Therefore, it appears that the intact FAD molecule is required for the induction of the changes in protein structure which accompany the measurable fluorescence changes. Neither adenine derivatives nor flavin derivatives, nor a combination of the two, can convert the apoenzyme to a catalytically active form.

There has been some lack of agreement in the literature concerning the molecular weight of D-amino acid oxidase. It was generally believed that all three forms of the enzyme, apoenzyme, holoenzyme, and benzoate complex, have a molecular weight of approximately 100,000 (26-28). However, some investigators have reported a higher molecular weight for the holoenzyme (29). This discrepancy was thought to be due to a tendency for the enzyme to polymerize with increasing protein concentration (26, 29). Recently, Yagi et al. (8) calculated a molecular weight of 55,000 for the apoenzyme from sedimentation and diffusion studies.

In the present study, it was found by gel filtration that the apoenzyme form and holoenzyme form of D-amino acid oxidase each have a molecular weight of approximately 50,000. There was no effect of protein concentration on the molecular weights of the apoenzyme and holoenzyme in the concentration range studied. At high concentrations (1 to 5 mg per ml), the benzoate complex has a molecular weight of 95,500; however, at a lower concentration (0.3 mg per ml), the benzoate complex consists of two fractions having molecular weights of 48,200 and 95,500. It appears that the apoenzyme and holoenzyme forms of D-amino acid oxidase are present as monomers having one FAD-binding site per molecule of protein, and that these forms of the enzyme do not tend to aggregate. On the other hand, it appears that the benzoate complex has a molecular weight of 50,000 only at
low protein concentration. At higher concentrations, the benzoate complex forms dimers. The binding of benzoic acid to the holoenzyme may bring about a conformational change in the protein, which makes the protein more susceptible to aggregation. It has been suggested previously that there are conformational changes in the protein upon the binding of benzoic acid to the holoenzyme (8, 30).

In previous studies, in which D-amino acid oxidase was purified as the stabilized benzoate complex, what was believed to be the holoenzyme may still have been the benzoate complex. Therefore, when the molecular weight was determined for concentrations of 1 mg per ml or greater, a molecular weight of 100,000 was obtained. Generally, molecular weight determinations have been made with these higher protein concentrations. The sensitivity of the spectrophotometric assay described by Fonda and Anderson (9) enables one to measure enzyme activity at low concentrations of D-amino acid oxidase. Therefore, the enzyme could be studied by gel filtration at concentrations similar to those used in the inactivation experiments.

REFERENCES
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Margaret L. Fonda and Bruce M. Anderson


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