Conformation of Porcine D-Amino Acid Oxidase as Studied by Protein Fluorescence and Optical Rotatory Dispersion†

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ABSTRACT: An inner-filter effect arising from an uneven absorption of tryptophan or protein fluorescence by flavine is described, and revised mathematical corrections for such an effect are detailed. The corrected protein fluorescence maximum for D-amino acid oxidase is progressively blue shifted from that of the apoprotein by the presence of increasing levels of flavine adenine dinucleotide (FAD). A maximal blue shift of 11 nm is observed at saturating levels of FAD. This indicates that some tryptophanyl residues in the active holoenzyme are in a more nonpolar environment than those in the apoenzyme. The conformations of holoenzyme and the holoenzyme–benzoate complex are significantly different from the apoenzyme, but all three forms appear to have the same helix content of 15 ± 1%. Both fluorescence and optical rotatory dispersion measurements show that the oxidase starts to unfold at 1 M guanidine hydrochloride and becomes nearly random at 6 M of this denaturant. Guanidine hydrochloride at 1 M probably perturbs the enzyme conformation to a limited but significant level, which may depress the FAD-binding affinity and enhance the dissociation of enzyme dimers to monomers. Effects of pH and salt concentrations on the binding of FAD to enzyme have been studied by fluorescence polarization measurements. Involvements of the N-3 proton of FAD and ionic interactions between FAD and the oxidase in the binding of the coenzyme are implicated. The average fluorescence quantum yields of tyrosyl and tryptophanyl residues in the apoenzyme are estimated to be 0.02 and 0.11, respectively. The weak fluorescence activity of tyrosyl residues is demonstrated to be, in part, a result of energy transfer to tryptophanyl residues. The average efficiency for such energy transfer is approximately 0.35. To account for this, some tyrosyl residues must be proximal to tryptophanyl residues.

The complexing propensities of the isoalloxazine ring portion of flavines with indoles and phenols (Tollin, 1968) in general have been noted. The intermolecular associations of flavine with tyrosine (Harbury and Foley, 1958) and tryptophan (Harbury and Foley, 1958; Wilson, 1966) have been studied in aqueous solution. Although some charge-transfer character may be present in the intermolecular cases, hydrophobic forces are undoubtedly involved in the intramolecular associations in flavinyl tryptophan and flavinyl tyrosine peptides (Föry *et al.*, 1968, 1970; MacKenzie *et al.*, 1969).

Furthermore, the formation of both inter- (Draper and Ingraham, 1970) and intramolecular (Wu *et al.*, 1970a) complexes of flavine and aromatic amino acids is favored whether the flavine is in an oxidized, semiquinoid, or fully reduced state.

Probable interactions of flavine coenzymes with certain tyrosyl and tryptophanyl residues of flavoproteins may contribute significantly to the overall flavine-binding affinity. The loss of flavine-binding ability of apoproteins as a result of modification of specific tyrosyl residues has been demonstrated (Strittmatter, 1961; Hinkson, 1968). Chemical modification of flavodoxin (McCormick, 1970) suggests that a tryptophanyl residue is required in flavine mononucleotide (FMN) binding. Recent X-ray analysis has established that both tyrosyl and tryptophanyl residues interact in parallel stacks with the FMN bound to different flavodoxins (Watenpaugh *et al.*, 1972; Anderson *et al.*, 1972). Tyrosyl residues have even been found vicinyl to the covalent 8α-flavine adenine dinucleotide (FAD) within mammalian monoamine oxidase (Walker *et al.*, 1971), the *Chromatium* cytochrome c_{552} flavoprotein (Kenney *et al.*, 1973), and a bacterial D-6-hydroxynicotine oxidase (Brühmüller and Decker, 1973).

Porcine D-amino acid oxidase [D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3] is a FAD-dependent enzyme. The possible involvements of tyrosyl (Yagi *et al.*, 1959) and tryptophanyl (Wu *et al.*, 1970b) residues of this enzyme in the binding of FAD have been suggested. Recent photochemical studies (Tu and McCormick, 1973) further show that a tyrosyl residue is at or near the active site of the oxidase. Such a residue could function simply as a site for complexing FAD or even as a nucleophilic phenolate moiety in the bond making and breaking steps. However, little is known about the exact structure of the FAD-binding site and the nature of the flavine–protein interactions in this enzyme.

An improved method for complete purification of D-amino acid oxidase has been developed (Tu *et al.*, 1973). After DEAE-Sephadex column chromatography, the enzyme obtained was shown to be pure by disc and sodium dodecyl sulfate gel electrophoreses and by sedimentation equilibrium. In the present work, protein fluorescence and, in some cases, optical rotatory dispersion have been used to investigate the conformation of the pure oxidase. The present work was carried out to examine the nature and consequences of FAD binding and also to delineate the chemical environment of tyrosyl and tryptophanyl residues in the oxidase. Such knowledge is of fundamental importance for better understanding flavine–protein interactions in general.

Experimental Procedures

Materials. The method of Massey *et al.* (1961) for the purification of D-amino acid oxidase from hog kidney has been

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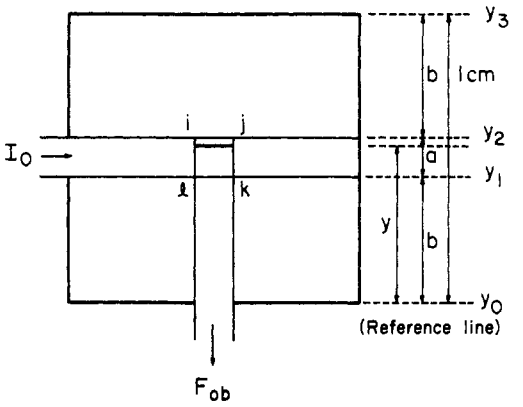

FIGURE 1: Diagrammatic top view of a quartz fluorescence cuvet. The meanings for symbols are given in the text.

recently modified (Tu *et al.*, 1973) to include DEAE-Sephadex column chromatography for the final step in purification. The apoenzyme so obtained was used for the entire work. Holoenzyme was reconstituted from apoprotein and FAD. Guanidine hydrochloride (Ultra Pure) and FAD (monosodium salt) were obtained from Mann Research Laboratories and Eastman Organic Chemicals, respectively. Unless otherwise stated, enzyme samples were prepared in 0.1 M sodium pyrophosphate buffer (pH 8.3).

Fluorescence Measurements. Both excitation and emission spectra were taken at 23° and pH 8.3 with a Perkin-Elmer fluorescence spectrophotometer, Model MPF-3. A scanning speed of 60 nm/min was used for all spectral measurements. Excitation and emission spectra were not corrected for variations in photon output and phototube response, respectively. Fluorescence polarization was measured at 23° using an Aminco-Bowman spectrophotofluorometer equipped with a Glan prism accessory. The method of Azumi and McGlynn (1962) was used to calculate the values of fluorescence polarization.

Optical Rotatory Dispersion Measurements. Measurements were obtained at 23° and pH 8.3 with a Jasco Model ORD/UV-5. The reduced mean residue rotation at 233 nm, $[m']_{233}$, was calculated and taken as representative of the helical content (Simmons *et al.*, 1961).

Corrections of Protein Fluorescence Spectra in the Presence of Flavine. In a protein (or tryptophan)–flavine, two-component system, the peak position of the fluorescence spectrum of bound or free tryptophan will be artificially blue shifted. This is an inner-filter effect caused by the flavine, which has an absorption maximum near 373 nm and preferentially absorbs the longer wavelength portion of the tryptophan (bound or free) fluorescence in the 300–390-nm region. For the gross correction of such an artifact, a method of mathematical approximation was first used (Wu *et al.*, 1970b). Following the same principle, equations have now been derived, as follows, to correct more exactly the inner-filter effect.

The top view of a 4.5-ml quartz fluorescence cuvet (1 × 1 cm cross section) situated in the cell holder of a fluorescence spectrophotometer is shown in Figure 1. The excitation light slit, with a width (in centimeters) of a, and the fluorescence light slit are shown as openings in the cuvet walls. The distance (in centimeters) between the near side of the excitation beam and that of the cuvet (the reference line) with respect to the fluorescence detector is designated as b. The excitation light (280 nm), with an intensity of I_0, comes in from the left side of the cuvet, and the fluorescence emitted by those tryptophan (or protein) molecules located within the area $ijkl$ is detected in the direction perpendicular to the excitation beam, with observed fluorescence intensity F_{obsd}. Let F_i be the initial fluorescence intensity at a given wavelength emitted by a layer of tryptophan or protein molecules, dy (shown as a darkened band in Figure 1), and F be the intensity of the detectable portion of F_i at y_0. According to the Beer–Lambert law

$$F = F_i e^{-\mu c y} \tag{1}$$

where μ is the absorption constant (2.3 × molar extinction coefficient) of flavine at the wavelength of interest and c is the molar concentration of flavine. The total fluorescence intensity, F_t, and the observed intensity, F_{obsd}, at a given wavelength emitted from those tryptophan or protein molecules within the area of $ijkl$ can be expressed as follows

$$F_t = \int_{y_1}^{y_2} F_i dy = F_i(y_2 - y_1) \tag{2}$$

$$F_{obsd} = \int_{y_1}^{y_2} F dy \tag{3}$$

Substituting expression 1 for F in eq 3 and integrating, one can solve for F_i[1]

$$F_i = F_{obsd}\left(\frac{\mu c}{e^{-\mu c y_1} - e^{-\mu c y_2}}\right) \tag{4}$$

Substitution of expression 4 for F_i in eq 2, of b for y_1, and of $a + b$ for y_2 yields

$$F_t = F_{obsd}\left[\frac{\mu c a}{e^{-\mu c b} - e^{-\mu c(a+b)}}\right] \tag{5}$$

Using eq 5, the total fluorescence intensity, F_t, at a given wavelength can be calculated from the observed fluorescence intensity, F_{obsd}.

Usually, a can be selected so that μca will be $\ll 1$. Then, using an approximation, $e^{\mu ca} = 1 - \mu ca$, F_t can be calculated from[2]

$$F_t = F_{obsd} e^{\mu c b} \tag{6}$$

Results

The corrected protein fluorescence spectra of D-amino acid oxidase apo- and holoenzyme are shown in Figure 2B. The presence of FAD not only markedly quenches the protein fluorescence intensity but also causes a blue shift of the emission spectrum. The observed emission maxima (results not shown) of the oxidase in the presence of 10^{-5}, 5×10^{-5}, and 10^{-4} M FAD are blue shifted by 3, 8, and 17 nm, respectively, from that of apoenzyme at 332 nm. After being corrected for the inner-filter effect, the corresponding blue shifts were found to be 2, 5, and 11 nm, respectively. FAD concentrations higher than 10^{-4} M do not shift the fluorescence maximum any further. The addition of 10^{-4} M benzoate (a competitive inhibitor with respect to the substrate) to enzyme samples containing FAD causes a little additional quenching in protein fluorescence but does not shift the emission maxima. As experimental controls, the fluorescence spectra of tryptophan were also investigated in the presence and absence of FAD (Figure 2A). At 5×10^{-5} and 10^{-4} M FAD, the emission maxima of tryptophan solutions were observed to be blue shifted by 6 and 11 nm, respec-

[1] Note that one ignores the z (vertical) direction, since F_i is independent of both y and z.

[2] Percentage errors introduced by this approximation are 1, 2.4, and 4.8 when μca is 0.02, 0.05, and 0.1, respectively.

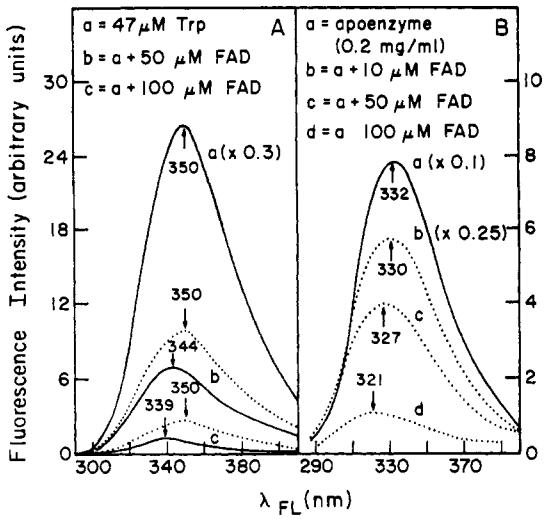

FIGURE 2: Fluorescence spectra of tryptophan (A) and D-amino acid oxidase apoprotein (B) in the presence and absence of FAD. Observed spectra (–) and those corrected for the inner-filter effect (· · ·) using eq 5 and, for *b* in B, eq 6. Slit widths of 1.29 and 0.39 mm were used for excitation light (10-nm bandwidth centered at 280 nm) and for fluorescence emission (3-nm bandwidth), respectively. Emission maxima are indicated in nanometers for each spectrum.

tively, from that of the tryptophan alone. Importantly, after being corrected for the inner-filter effect, all the tryptophan samples have the same emission maximum at 350 nm.

The unfolding of D-amino acid oxidase apoenzyme (E), holoenzyme (EF), and the holoenzyme–benzoate complex (EFB) by guanidine hydrochloride was investigated by analyzing the corrected protein fluorescence maxima and by measuring the optical rotatory dispersion. Results in Figure 3 show that most of the unfolding of apoenzyme happens at guanidine hydrochloride concentrations of 1–5 M, as indicated by the red shifts in emission maxima. At 6 M guanidine hydrochloride, the apoenzyme has a fluorescence maximum at 347 nm, which is quite close to 350 nm for the free tryptophan in aqueous solution. As described before, the presence of 10^{-5} M FAD with or without additional 10^{-4} M benzoate shifts the emission maximum of D-amino acid oxidase from 332 to 330 nm in the absence of denaturant. Nevertheless, the unfolding patterns of those samples are indistinguishable from that of apoenzyme at guanidine hydrochloride concentrations higher than 1 M. Using reduced mean residue rotation at 233 nm as an indication of helix content of protein, and allowing 5–10% for experimental variations, the results in Figure 4 show that the helix contents of D-amino acid oxidase apoenzyme at 0–8 M guanidine hydrochloride are generally the same as that of holoenzyme and the holoenzyme–benzoate complex. Assuming the enzyme is completely unfolded in 8 M guanidine hydrochloride, the helix content of the oxidase in the absence of denaturant is approximately 15 ± 1%, as estimated by the method of Simmons *et al.* (1961). Again, most of the unfolding of the oxidase happens at guanidine hydrochloride concentrations of 1–5 M.

The fluorescence characteristics of the apoenzyme are quite different in both emission intensity and maximum as compared with holoenzyme and the holoenzyme–benzoate complex. However, no significant differences in helix content can be detected. It is interesting to examine further some other physicochemical properties of the oxidase in the three different forms. The rates of thermal denaturation of D-amino acid oxidase are shown in Figure 5. The presence of FAD greatly

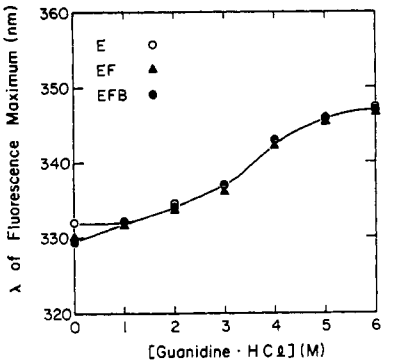

FIGURE 3: Effect of varying concentrations of guanidine hydrochloride on the protein fluorescence maximum of D-amino acid oxidase. Fluorescence spectra were corrected for the inner-filter effect as described in Figure 2. Data are for 0.2 mg of apoenzyme/ml alone (○), in the presence of 10^{-5} M FAD (●), and in the presence of 10^{-5} M FAD plus 10^{-4} M benzoate (▲).

stabilizes the oxidase against thermal denaturation, and the copresence of benzoate provides some additional protection.

The fluorescence polarization of a chromophore should increase when bound to a macromolecule, such as protein. In the present case, effects of pH and salt concentrations on the binding of FAD to D-amino acid oxidase apoenzyme were studied by measuring the fluorescence polarization of FAD in the presence and absence of the apoenzyme (Figure 6). For the polarization measurements, an instrumental variation of approximately 10% has been found. Qualitatively, the FAD-binding affinity of D-amino acid oxidase reaches an apparent optimum at pH 8, decreases gradually toward pH 9, and drops sharply from pH 9 to 10 and from pH 8 to 7. At pH 8.3, the apoenzyme has higher FAD-binding affinities at KCl concentrations less than 0.06 M. At high KCl concentrations, 0.6–1 M, the fluorescence polarization of FAD is only slightly raised by the presence of apoenzyme. This indicates a poor binding of FAD by the enzyme under such conditions.

Monomeric D-amino acid oxidase contains 9 tryptophanyl and 13 tyrosyl residues (Tu *et al.*, 1973). Information about the relative fluorescence activities of tryptophanyl and tyrosyl residues in the oxidase would help one understand better the chemical environment of those residues in the enzyme. To avoid complications caused by the presence of FAD, which absorbs strongly in the ultraviolet range, only apoenzyme was used for such studies. The absolute and difference fluorescence spectra of apoenzyme with excitation light at 275, 285, and

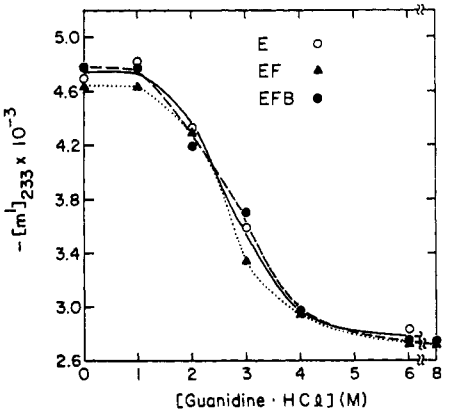

FIGURE 4: Effect of varying concentrations of guanidine hydrochloride on the reduced mean residue rotation at 233 nm of D-amino acid oxidase. Samples were the same as described in Figure 3, except that the concentration of protein was 0.064 mg/ml.

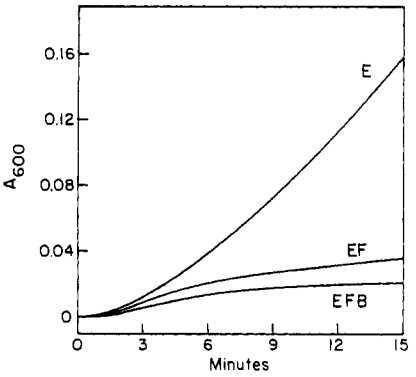

FIGURE 5: Thermal denaturation of D-amino acid oxidase apoenzyme (E), holoenzyme (EF), and the holoenzyme–benzoate complex (EFB). Samples were the same as described in Figure 4. Each sample (1 ml) was incubated at 50° and turbidity measured by following the increases in absorbance at 600 nm.

293 nm are shown in Figure 7. As shall be seen (Figure 9), tryptophanyl residues are the only chromophore absorbing at 293 nm. Therefore, using 293-nm excitation light, the observed protein fluorescence spectrum (Figure 7) is constructed entirely from the tryptophan emission. At shorter wavelengths, *e.g.*, 285 and 275 nm, tyrosyl residues absorb some of the excitation light, and the shape of the protein fluorescence spectra must be different from the previous case if tyrosyl residues also fluoresce. Results in Figure 7 show that the difference spectra obtained, using 285- and 275-nm *vs.* 293-nm excitation, have the shape characteristic of tyrosine emission. It is clear that most of the fluorescence of the apooxidase is from tryptophanyl residues and only a small portion of emission, which can be detected using shorter wavelengths of excitation light, is from tyrosyl residues. The relative emission activities of tryptophanyl and tyrosyl residues were also examined by analyzing the excitation spectra of the apoenzyme

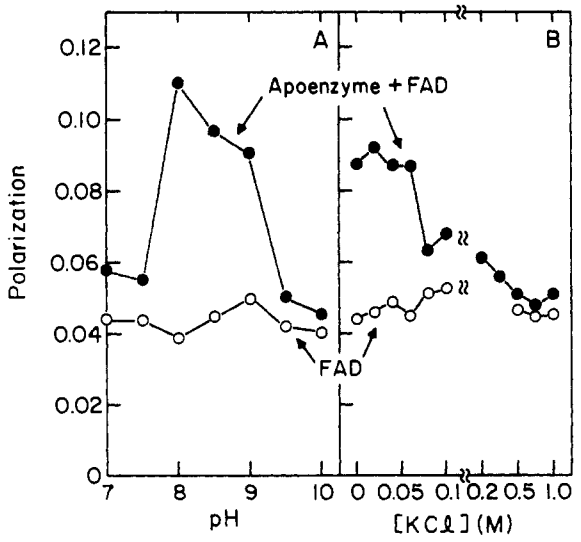

FIGURE 6: Fluorescence polarization of FAD in the presence (●) and absence (○) of D-amino acid oxidase apoenzyme, as functions of pH (A) and KCl concentrations (B). Excitation light was set at 450 nm and fluorescence monitored at 530 nm. Solutions contained 2×10^{-6} M FAD (A) or 2.4×10^{-6} M FAD (B) in the presence or absence of 0.475 mg of apoenzyme per ml. Samples were maintained at pH 7–8 with 0.1 M sodium phosphate and 8.5–10 with 0.1 M sodium pyrophosphate for the studies of pH effects. Sodium pyrophosphate buffer (2 mM and pH 8.3), containing different concentrations of KCl, was used to study the effects of ionic strength. Each point is an average of two measurements.

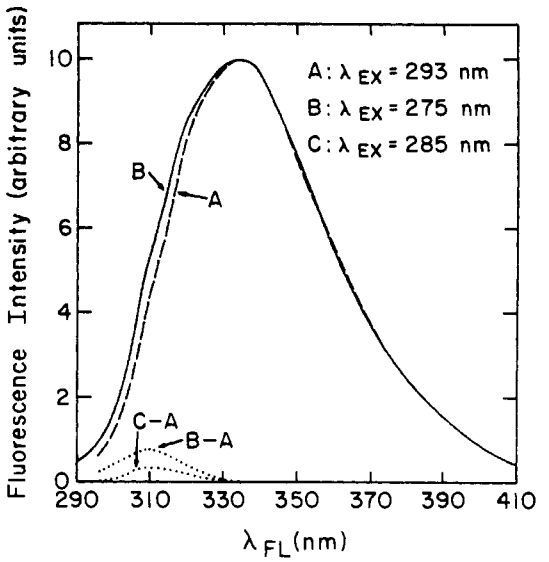

FIGURE 7: Fluorescence spectra of D-amino acid oxidase apoenzyme (0.2 mg/ml) with varying excitation light. Slit widths for excitation and fluorescence emission were selected to give spectral resolutions of 3 and 5 nm, respectively.

with fluorescence monitored at different wavelengths. Solutions containing tryptophan alone and a mixture of tryptophan plus tyrosine in a molar ratio of 9:13, simulating their contents in D-amino acid oxidase (Tu *et al.*, 1973), were also investigated for comparison. Two peaks, one at 284 nm and the other at 292 nm, appear in the excitation spectra of both tryptophan (Figure 8A) and apoenzyme (Figure 8C) when the fluorescence is monitored at different wavelengths as indicated. However, in the case of a mixture of tryptophan and tyrosine (Figure 8B) with both chromophores capable of absorbing and emitting light, the 284-nm peak in the excitation spectra becomes more intense, and the 292-nm peak is greatly depressed when the fluorescence is monitored at wavelengths closer to the tyrosine fluoresence peak (310 nm). Interestingly, the absorption spectrum of tyrosine in aqueous solution shows a minor peak near 283 nm. A closer comparison of the shape of excitation spectra between apoenzyme and tryptophan reveals that the intensity of the 284-nm peak relative to the 292-nm peak for the former sample becomes slightly greater when the fluorescence is monitored at 320 nm and slightly less with the emission followed at 360 or 370 nm. This, again, leads to the conclusion that tyrosyl residues emit a small fraction of the fluorescence when the apoenzyme is excited at shorter wavelengths.

The efficiency of intramolecular energy transfer from tyrosyl to tryptophanyl residues has been shown to exist in many proteins, and the efficiency for such a process can be estimated by the method of Eisinger (1969). Firstly, the fractions of ultraviolet light absorbed by tryptophanyl, tyrosyl, or phenylalanyl residues in a protein can be calculated from the amino acid composition and the molar extinction coefficients of those aromatic amino acids (Eisinger, 1969). The results of such calculations for D-amino acid oxidase are presented in Figure 9. Secondly, when the protein fluorescence is measured at a wavelength where a tryptophanyl residue is the only emitting chromophore, then the quantum yield of the protein, $\Phi_{pp}(\lambda)$, at an excitation wavelength of λ can be expressed by: $\Phi_{pp}(\lambda) = \Phi_{Trp}[f_{Trp}(\lambda) + e_{Tyr}f_{Tyr}(\lambda) + e_{Phe}f_{Phe}(\lambda)]$, where $f_{Trp}(\lambda)$, $f_{Tyr}(\lambda)$, and $f_{Phe}(\lambda)$ are the fractions of light at the specified wavelength absorbed by tryptophanyl, tyrosyl, and phenylalanyl residues, respectively; Φ_{Trp} is the wavelength-independent quantum

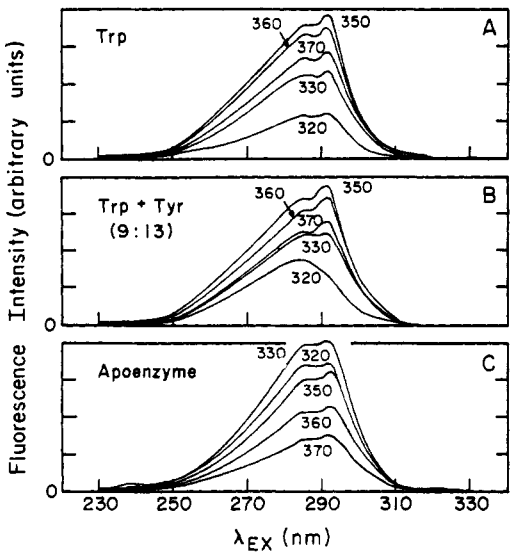

FIGURE 8: Fluorescence excitation spectra of tryptophan (4.5×10^{-5} M), a mixture of tryptophan (4.5×10^{-5} M) and tyrosine (6.5×10^{-5} M), and apooxidase (0.2 mg/ml). The fluorescence emission was monitored at various wavelengths from 320 to 370 nm as indicated for each spectrum. All samples were prepared in 0.1 M sodium pyrophosphate (pH 8.3). Spectral resolutions of 3 and 5 nm were selected for excitation light and fluorescence emission, respectively.

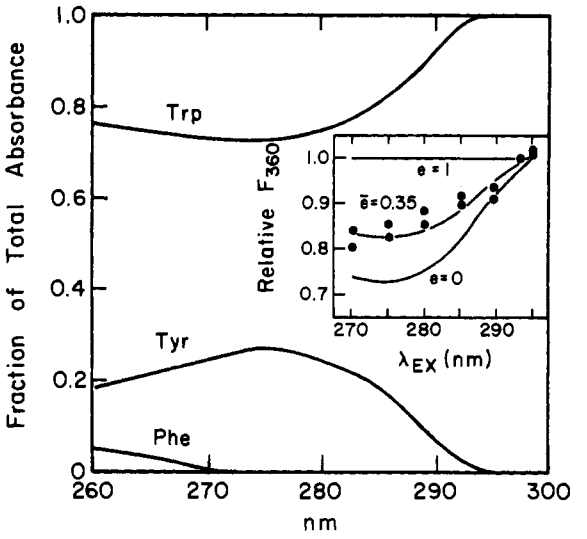

FIGURE 9: Fractional absorbances, in the ultraviolet range, by tryptophanyl, tyrosyl, and phenylalanyl residues in D-amino acid oxidase apoenzyme. In the insert, the normalized excitation spectrum of the apoenzyme, with fluorescence monitored at 360 nm, is compared with theoretical curves calculated for different efficiencies of energy transfer from tyrosine to tryptophan. Both the experimental values (●) and the theoretical curves are normalized at λ 293 nm.

yield of a tryptophanyl residue in the absence of energy transfer; and e_{Tyr} and e_{Phe} are the wavelength-independent efficiencies of energy transfer from tyrosyl and phenylalanyl to a tryptophanyl residue, respectively. The term $e_{Phe}f_{Phe}(\lambda)$ can be omitted when λ exceeds 270 nm, because phenylalanine no longer absorbs any excitation light. Therefore, the profile of Φ_{pp} as a function of λ (Figure 9, insert) in the range of 270–295 nm should be exactly the same as the fractional absorbance profile of tryptophanyl residues when e_{Tyr} is zero, and should be a horizontal line when e_{Tyr} is 1. Accordingly, different curves can be constructed for different values of e. The excitation spectrum of D-amino acid oxidase with fluorescence measured at 360 nm (Figure 8C) was normalized to reflect the fluorescence quantum yield and was compared with theoretical curves calculated for different values of e. As shown in the insert of Figure 9, the experimental results indicate that the tyrosine to tryptophan energy transfer does exist in the apoenzyme, with the average efficiency estimated to be approximately 0.35. The average quantum yield of tryptophanyl residues, Φ_{Trp}, in the apooxidase has been determined to be 0.11 using 295-nm light for excitation. The average quantum yield of tyrosyl residues, Φ_{Tyr}, in the enzyme was then calculated to be 0.02 using the following equation (Eisinger, 1969)

$$\frac{A_{Tyr}}{A_{Trp}} = \frac{f_{Tyr,275}(1 - \bar{e}_{Tyr})\Phi_{Tyr}}{[f_{Trp,275} + \bar{e}_{Tyr}f_{Tyr,275}]\Phi_{Trp}}$$

where A_{Tyr} and A_{Trp} are the areas under the tyrosyl and tryptophanyl fluorescence spectra obtained by excitation at 275 nm (Figure 7).

Discussion

D-Amino acid oxidase apoenzyme has an emission maximum at 332 nm, which is blue shifted by 18 nm from that of free tryptophan in aqueous solution. This is commonly seen with simple proteins and considered to indicate a more hydrophobic environment for the tryptophanyl residues. With partially purified D-amino acid oxidase, we observed (Wu *et al.*, 1970b) that addition of different concentrations of FAD to the apoenzyme results in further blue shifts in the protein emission. After further correction for the inner-filter effect, such progressive blue shifts of protein emission spectra (*cf.* Figure 2B) have now been demonstrated with the pure D-amino acid oxidase apoenzyme upon adding FAD. These findings indicate that at least some of the tryptophanyl residues in the apoenzyme are shifted to a relatively nonpolar environment upon binding FAD and are thus less exposed to the aqueous medium. In the present studies, no correction was made for the partial absorption of excitation light by FAD present in some of the protein samples. Such correction will increase the protein fluorescence intensity, but will not change the shape of the emission spectrum. By following the quenching of flavine and protein fluorescence and the appearance of catalytic activity after mixing apoenzyme with FAD, Massey and Curti (1966) concluded that a protein conformational change accompanies re-formation of the active holoenzyme. Our present observations also suggest such a conformation change initiated by binding FAD. However, this could also be a consequence of enzyme dimerization, known to be favored by high concentrations of FAD (Neims and Hellerman, 1970).

Several interesting features have been revealed by the studies of guanidine hydrochloride denaturation and thermal denaturation of D-amino acid oxidase. The conformations of holoenzyme and the holoenzyme–benzoate complex are significantly different from the apoenzyme. This is indicated by the fluorescence quenching, blue shifts in protein fluorescence, and the increased thermal stabilities (*cf.* Figure 5) of the former enzyme forms. Nevertheless, the helix contents of the three forms of enzyme in the native state are approximately the same (*cf.* Figure 4). Their resistance to the unfolding by guanidine hydrochloride also appears to be similar (*cf.* Figures 3 and 4). No significant differences in helix contents are observed at 1 M guanidine hydrochloride between apoenzyme and that of holoenzyme and the holoenzyme–benzoate complex. Nevertheless, the differences in protein

fluorescence maxima between apoenzyme and the other two enzyme forms are no longer present in 1 M guanidine hydrochloride (*cf.* Figure 3). These observations suggest that 1 M guanidine hydrochloride probably perturbs the conformation of holoenzyme and the holoenzyme–benzoate complex to a limited but significant level. This structural change may result in either decreased FAD-binding affinities or enhanced dissociations of enzyme dimers to monomers. In this connection, it has been demonstrated that D-amino acid oxidase holoenzyme and the benzoate complex exist as monomers in 2 M urea (Yagi *et al.*, 1973), and the interaction between FAD and the oxidase decreases upon addition of urea (Sugiura *et al.*, 1973).

Optimal FAD binding has been found to be near pH 8. It is known that alkylation at the N-3 position of FAD results in a total loss of coenzyme activity and a considerable loss of ability to bind with D-amino acid oxidase (Chassy and McCormick, 1965). The pK_a for the ionization of the N-3 proton is also known to be near pH 10 (Hemmerich *et al.*, 1965). Therefore, the decrease in FAD-binding affinity from pH 9 to 10, as shown by the decrease in fluorescence polarization (Figure 6A), indicates that the N-3 hydrogen is probably required for FAD binding. Using different D-amino acids with similar ionization characteristics as substrate, optimal catalytic activities of D-amino acid oxidase were found to be at different pH values, which could be as high as 10–11 (Dixon and Kleppe, 1965). The present fluorescence polarization measurements show that the binding of FAD in the absence of substrate is disfavored at pH values higher than 9. It is quite possible that the simultaneous binding of D-amino acids and FAD may modify the FAD-binding affinity of the enzyme.

The importance of ionic interactions in the binding of flavine coenzymes by flavoproteins has long been noted. Theorell and Nygaard (1954) showed that binding of FMN to the old yellow enzyme was considerably weakened at high concentrations of monovalent anions. Recently, Meighen and MacKenzie (1973) demonstrated that high concentrations of anions enhance the binding of neutral flavines to bacterial luciferase, but phosphate competes with FMN for the binding. Previously, the binding of FAD to D-amino acid oxidase has been shown to be depressed at high salt concentrations (Walaas and Walaas, 1956), and apoenzyme can be obtained by dialyzing the holoenzyme against 1 M KBr (Massey and Curti, 1966). Now, similar salt effects on FAD binding have been observed with the pure D-amino acid oxidase. These observations confirm that ionic interactions between FAD and the oxidase must be involved in the binding.

It is interesting to note thay tyrosyl residues in D-amino acid oxidase emit little fluorescence, with an average quantum yield estimated to be 0.02. On the other hand, tryptophanyl residues in the apoenzyme fluoresce strongly with an average quantum yield of 0.11, which is only slightly less than the quantum yield of tryptophan (0.14) in aqueous solution (Eisinger, 1969). At least two possible quenching mechanisms can be considered to account for the low quantum yield of tyrosyl residues: (1) the tertiary structure of the enzyme provides an environment that either allows an effective, radiationless relaxation of the singlet tyrosyl residues, or reduces the photoexcitability of ground-state tyrosyl residues; (2) the energy of the light-excited tyrosyl residues is transferred to tryptophanyl residues, which subsequently emit fluorescence. The first mechanism is probable but difficult to quantitate. The existence of the second mechanism, *viz.* Förster energy transfer, has now been demonstrated (*cf.* Figure 9, insert). Based on the estimated quantum yield of tyrosyl residues in the apoenzyme, the Förster distance (R_0) for energy transfer with 50% efficiency (Förster, 1948, 1966) is calculated to be 9.8 Å using the following equation

$$R_0^6 = 8.8 \times 10^{-25} k^2 \Phi_D n^{-4} J_{AD}$$

where k^2 is a transition moment orientation factor, Φ_D is the fluorescence quantum yield of the donor, and n is the refraction index of the medium. The term J_{AD} is an overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor and can be expressed as

$$J_{AD} = \int_0^\infty F_D(\nu)\epsilon_A(\nu)\nu^{-4}d\nu$$

where $F_D(\nu)$ is the normalized emission frequency distribution of the donor, $\epsilon_A(\nu)$ is the molar decadic absorption coefficient of the acceptor, and ν is the frequency in reciprocal centimeters. Furthermore, based on the average energy transfer efficiency (e) of 0.35 from tyrosyl to tryptophanyl residues in the apoenzyme, the distance (r) between the donor and acceptor is calculated to be 10.9 Å using the following equation: $r = (e^{-1} - 1)^{1/6}R_0$. The distance actually represents a weight-average result, which is a function of both distance and orientation of tyrosine–tryptophan pairs in the enzyme. Nevertheless, comparing with a molecular Stokes radius of 25.1 Å for monomeric D-amino acid oxidase (Henn and Ackers, 1969), an average distance of 10.9 Å between tyrosyl and tryptophanyl residues suggests that at least some tyrosyl residues are within fairly close vicinity to tryptophanyl residues. Possibly both tyrosyl (Yagi *et al.*, 1959) and tryptophanyl (Wu *et al.*, 1970b) residues of the oxidase are involved in the binding of FAD. The presence of a tyrosyl residue at the active center of the enzyme has also been demonstrated by recent photochemical studies (Tu and McCormick, 1973). It is possible that there is a particular tyrosine–tryptophan pair present at the active center of D-amino acid oxidase, as in the case of one of the flavodoxins. When these two residues are within close proximity, they may, together, contribute more effectively to the FAD-binding affinity through coplanar interactions with the isoalloxazine ring of FAD at the enzyme-active center.

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Activity of Liver Alcohol Dehydrogenase with Various Substituents on the Amino Groups†

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ABSTRACT: Modification of amino groups at the active sites of horse liver alcohol dehydrogenase increased the activity of the enzyme up to tenfold, as assayed at pH 7 with high concentrations of substrates. Reductive alkylation and amidination by a variety of imido esters (new compounds are described) activated the most. Carbamylation changed the activity little, and succinylation inactivated. As the sizes of positively charged substituents on the amino groups were increased, the Michaelis and inhibition constants for all four substrates and the turnover numbers for the reactions catalyzed by the modified enzymes generally increased. Substituents that changed the net charge of the amino groups decreased the rates of binding of NAD^+ or NADH to modified enzymes; most substituents increased the rates of dissociation of the enzyme–coenzyme complexes. Modification of amino groups also differentially affected the binding of ethanol and acetaldehyde.

Most attempts to accelerate ethanol metabolism have given small or inconsistent effects (Lundquist, 1971). Since liver alcohol dehydrogenase catalyzes a rate-limiting step in the metabolism (Hawkins and Kalant, 1972), activation of the enzyme could accelerate ethanol metabolism.

Picolinimidylation of one ϵ-amino group at each active site of horse liver alcohol dehydrogenase increases the turnover number for the reaction of NAD^+ and ethanol about tenfold. The modified enzyme has an ordered bi–bi mechanism, in which the rate of dissociation of NADH (the rate-limiting step for the native enzyme) is so fast that transfer of hydrogen in the ternary complex becomes at least partially rate limiting (Plapp, 1970; Plapp *et al.*, 1973). The ordinary imido esters probably cannot be used to activate the enzyme *in vivo* because they nonspecifically modify most ϵ-amino groups of proteins (Hunter and Ludwig, 1972).

As part of a program to study the role of amino groups in enzymic activity and to design specific active-site directed activators of liver alcohol dehydrogenase, we have modified the amino groups with substituents of varied size, shape, and charge and studied the effects on the activity and kinetics of the enzyme.

Experimental Section

Chemistry. Many of the imido esters used in this study have been prepared previously (Hunter and Ludwig, 1962; Mc-

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