CHARACTERISTICS OF THE FLUORESCENCE SPECTRA OF APOENZYME AND FLAVIN PORTIONS OF D-AMINO ACID OXIDASE[1]

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The tryptophan fluorescence maxima for semiholo- and holoenzymes of D-amino acid oxidase are blue-shifted from that of the apoenzyme by 10 and 20 nm, respectively. These findings indicate that tryptophan residues of semiholo and holo forms are in a more nonpolar environment and less accessible to water than those in the apoenzyme. Overall, results suggest that some tryptophan residues may be near or at the flavin active site in D-amino acid oxidase.

D-Amino acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) provides a convenient system for studying flavin-protein interactions, since the apoenzyme can be readily obtained by recently developed methods (1,2). Massey and Curti (1) studied the effect of mixing apoenzyme with FAD (flavin-adenine dinucleotide) by following the changes in flavin and protein fluorescence and concluded that a protein conformational change accompanies reformation of the active holoenzyme. However, as far as we are aware, no comparison between the position of the emission maxima of the apoenzyme and that of the reconstituted holoenzyme has been made. Based on our previous fluorescence studies of the synthetic flavinyl peptides (3), these comparisons may reveal the nature of the environment surrounding the aromatic amino acids in the native enzyme. This study, then, was undertaken to examine the positions of both protein and flavin fluorescence maxima of a reconstituted enzyme as

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compared to that of the apoenzyme or FAD portion alone. From these results and other data, such as from photochemical studies (4,5), one may be able to detect the presence and proximity of a specific aromatic amino acid near or at a flavin coenzyme in D-amino acid oxidase.

MATERIALS AND METHODS

Electrophoretically purified D-amino acid oxidase was obtained from Worthington Biochemical Corp. The apoenzyme was prepared by treatment with KBr and dialysis (1). The semiholoenzyme is that obtained by reconstitution with FAD at 50% maximal activity. The holoenzyme is that with sufficient FAD for just maximal catalytic activity.

Measurements of enzyme activity were made both spectrophotometrically (6) and oxygraphically. For the former method, the absorbance changes at 252 nm accompanying the production of benzoylformic acid from D-phenylglycine were followed in a Gilford model 2000 recording spectrophotometer. For the latter, a Gilson oxygraph was used to measure oxygen uptake following addition of DL-alanine as substrate.

Fluorescence measurements were performed in a temperature-controlled cell compartment of an Aminco-Bowman spectrophotofluorometer with a 150 W xenon lamp. All measurements were carried out in a cuvette with 1-cm lightpath at 25° in a darkened room. A tryptophan solution (2.5×10^{-5} M) having the same fluorescence intensity at emission maximum (350 nm) as that of apoenzyme with the same instrument settings was mixed with the FAD solutions equivalent to semiholo- and holoenzyme. These solutions were used as controls.

The tryptophan fluorescence spectra of semiholo- and holoenzyme, and the above controls were corrected for the inner-filter effect due to the partial absorption of emitted fluorescence from bound or free tryptophan in the 310-350 nm region by FAD with one maximum near 375 nm. The following equation was derived and used: $I_c = I_o/[1-(^{2.3}/_2)Aa]e^{-2.3Ab}$

where $\underline{I}_c$ and $\underline{I}_o$ are the corrected and observed fluorescence intensities, respectively; $\underline{A}$ is the absorbances of FAD by itself in solution at emission

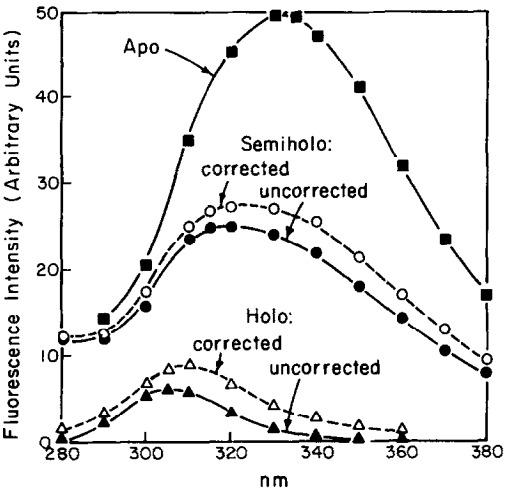
wavelengths of apoenzyme or tryptophan; a is the excitation slit width (0.2 cm); b is the distance (0.4 cm) between the near side of the excitation beam and that of the cuvette with respect to the fluorescence detector. No correction has been made for the inner-filter effect due to the absorption of the exciting light by FAD in the cases of semiholo- and holoenzyme. This latter correction will increase the fluorescence intensity, but will not shift the emission maximum of the fluorescence spectrum.

RESULTS

The results of the measurements of enzyme activity by both methods are approximately the same. The molar ratios of FAD to apoenzyme (50,000 mol wt) in semiholo and holo forms are averaged to be 1.75 and 20 to 1, respectively.

When apoenzyme was excited at 275, 280, or 292 nm, the emission spectra with maxima at 330 nm had the same shape which suggests no significant contribution from tyrosine (7,8). The emission maximum of tryptophan and FAD combined was blue-shifted about 5 nm from that of tryptophan alone. After applying the equation given above for correction of inner-filter effect, the emission maxima of the two becomes the same. This experimentally indicates the equation used is correct.

The fluorescence spectra of the protein portions in semiholo- and holoenzyme as compared to that of apoenzyme are shown in Fig. 1. The corrected emission maxima of semiholo and holo forms are blue-shifted by 10 and 20 nm, respectively, from that of apoenzyme. The extent of fluorescence quenching of protein is greater for holo- than for semiholo- with respect to the apoenzyme fluorescence. Figure 2 shows the fluorescence spectra of the flavin in semiholoenzyme, holoenzyme, and equivalent FAD solutions. In these four cases, the emission maxima stay the same at 520 nm, but holo- and semiholoenzyme have less fluorescence than the equivalent concentrations of free FAD. The extent of fluorescence quenching is greater for the latter than the former.



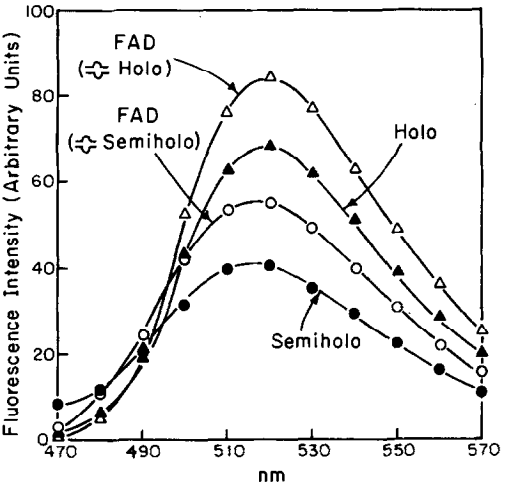


Fig. 1. The protein fluorescence spectra of apo-, semiholo-, and holoenzyme excited at 280 nm.
Fig. 2. The flavin fluorescence spectra of FAD, semiholo-, and holoenzyme excited at 450 nm. Concentrations for the above are 1×10^{-5}, 1.75×10^{-5}, and 2×10^{-4} M for apoenzyme, FAD equivalent to semiholo- and holoenzyme, respectively, in 0.05 M sodium pyrophosphate buffer, pH 8.5.

DISCUSSION

The emission maximum of the tryptophan in the apoenzyme is blue-shifted by 20 nm as compared with that of free tryptophan. This is in agreement with that reported for simple protein (9) and for flavinyl peptides (3) wherein emission maxima are blue-shifted as the solvent or environment changes from polar to less polar. Moreover, the protein emission maxima of the semiholo- and holoenzyme are even more blue-shifted from that of apoenzyme. These observations suggest that the environment for at least some of the tryptophan residues in the catalytically functional oxidase is relatively nonpolar or less exposed to the aqueous medium. Hence, some progressive "tightening" of protein structure occurs upon binding of coenzyme. This may be the result of protein conformational changes accompanying reconstitution of the active enzyme or dimerization known to be favored by higher levels of FAD (10). That certain of the tryptophan residues may be near or at the FAD-binding site in D-amino acid oxidase is compatible with the additional observation of quenching of the flavin fluorescence.

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