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INTERACTION OF STEROIDS WITH D-AMINO ACID OXIDASE

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Summary

1. Progesterone inhibited D-amino acid oxidase (D-amino acid : O_2 oxidoreductase (deaminating), EC 1.4.3.3) in competition with its substrate, D-alanine. Binding of progesterone brought about the increase in both fluorescence intensity and fluorescence polarization of FAD, which indicates that the environment surrounding FAD chromophore is modified due to a conformational change in the apoenzyme.

2. Ethinyl estradiol, testosterone, testosterone propionate, corticosterone and aldosterone also inhibited the enzyme slightly in the same manner. Their binding also produced a slight increase in FAD fluorescence without decreasing the fluorescence polarization.

3. Cholesterol did not inhibit the enzyme, though it increased the fluorescence polarization of FAD. This indicates the binding of cholesterol with the enzyme at a site other than the substrate binding site.

Introduction

Recent studies indicate that the use of steroid hormones by women for the purpose of contraception provokes biochemical changes akin to riboflavin deficiency [1—3]. However, subsequent experiments to elucidate the biochemical basis of this effect show that the administration of contraceptive steroids to adult female rats leads to the increase in the activity of some flavin enzymes, one of them being D-amino acid oxidase (D-amino acid : O_2 oxidoreductase (deaminating), EC 1.4.3.3) [4]. The change in the activity of an enzyme in vivo may arise from alteration in the concentration of the enzyme or through the modification of the activity of the existing enzyme. The present report deals with the latter alternative, using purified D-amino acid oxidase as a model system.

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Materials and Methods

Hog kidney D-amino acid oxidase was purified by the method of Yagi et al. [5,6]. Progesterone, ethinyl estradiol, testosterone, testosterone propionate, cholesterol, D-alanine and FAD were obtained from Nakarai Chemicals Co., Kyoto, corticosterone from Tokyo Kasei Kogyo Co., Tokyo, and aldosterone from Merck, Darmstadt. Solutions of steroids were prepared with ethanol (concentration, 26—87 mM).

Enzyme activity was assayed by measuring oxygen consumption in a Beckman Oxygen Sensor equipped with a recorder at 25° C. To observe the competition between steroid and substrate, the holoenzyme was added to the reaction mixture containing FAD and graduated amounts of D-alanine in the presence and absence of steroid in 0.1 M pyrophosphate buffer (pH 8.3).

Absorption spectra were recorded on a Union Giken spectrophotometer SM-401. Fluorescence spectra were recorded on a Shimadzu recording spectrofluorometer RF-502. The instrument was fitted with a Polacoat ultraviolet polarizing filters for the measurement of polarization at 530 nm. Excitation was done at 450 nm. Polarization degree, P, was calculated from the following equation:

$$P = \frac{I_{\parallel} - CI_{\perp}}{I_{\parallel} + CI_{\perp}}$$

where C denotes an instrumental correction factor determined as described elsewhere [7]. $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular to the plane of polarization of the exciting beam. Both values were obtained by correcting for the light scattering [8] which was observed on the addition of ethanol solution of steroids to the buffer, as follows:

$$I_{\parallel} = I_{\parallel}^{s} - I_{\parallel}^{c}$$

$$I_{\perp} = I_{\perp}^{s} - I_{\perp}^{c}$$

where the supercripts, s and c, mean a sample solution containing both the enzyme and a steroid, and a control solution containing only a steroid, respectively.

The concentrations described are final ones.

Results

Kinetic analysis

Double reciprocal plots of the velocity of the enzymatic reaction versus the concentration of the substrate, D-alanine, in the presence of progesterone and an excess amount of FAD revealed that progesterone inhibited D-amino acid oxidase in competition with the susbtrate, D-alanine (Fig. 1). Ethinyl estradiol, testosterone, testosterone propionate, corticosterone and aldosterone also inhibited the enzyme slightly in the same manner. The dissociation constant of the enzyme · inhibitor complex, K_i, obtained for each steroid is as follows: progesterone, 18 μM; ethinyl estradiol, 150 μM; testosterone, 180 μM; testosterone propionate, 140 μM; corticosterone, 320 μM; aldosterone, 120 μM. Cholesterol

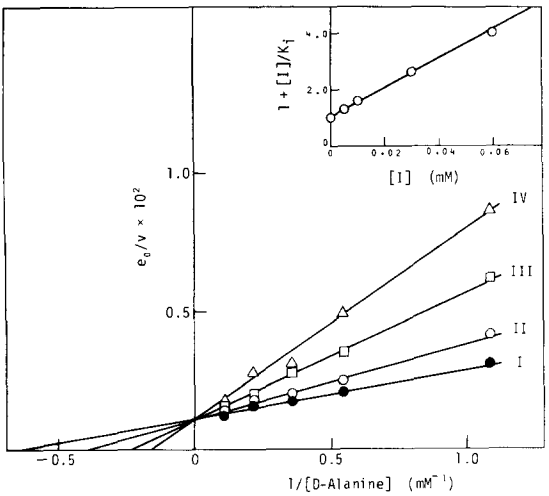

Fig. 1. Double reciprocal plot of the catalytic oxidation of D-alanine by D-amino acid oxidase in the presence of progesterone. Oxygen consumption was measured at 25°C with reaction mixture containing the enzyme (0.06 μM), FAD (27 μM) and various amounts of progesterone in 0.1 M pyrophosphate buffer (pH 8.3). Curve I: in the absence of progesterone; Curve II: I + 10 μM progesterone; Curve III: I + 30 μM progesterone; Curve IV: I + 60 μM progesterone. The insert shows a plot of $1 + [I]/K_i$ vs. [I]. [I] is the concentration of progesterone and K_i the dissociation constant for the enzyme · inhibitor complex.

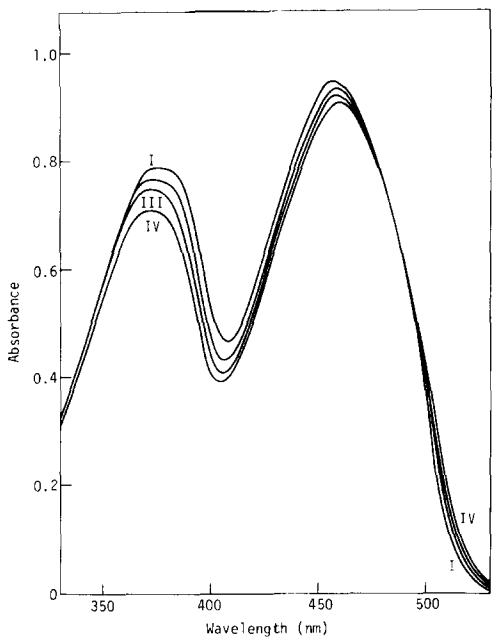

Fig. 2. Effect of progesterone on the visible absorption spectrum of D-amino acid oxidase. Curve I: the enzyme (84 μM) dissolved in 0.017 M pyrophosphate buffer (pH 8.3); Curve II: I + 91 μM progesterone; Curve III: I + 180 μM progesterone; Curve IV: I + 270 μM progesterone.

had no effect on the enzyme activity. None of the steroids when added along with progesterone could modify the progesterone effect. Although the steroids were dissolved into ethanol, the effects of steroids on D-amino acid oxidase cannot be ascribed to the presence of ethanol, since ethanol at the concentration used does not affect this enzyme [9].

Absorption and fluorescence measurements

Progesterone brought about a slight decrease in the intensity of visible absorption bands, accompanied by a small red shift of the 455 nm peak (Fig. 2). Corticosterone also brought about a similar but less pronounced effect, but other steroids showed no effect.

The increase in the fluorescence intensity of D-amino acid oxidase was observed with all the steroids tested, the most significant increase being in the case of progesterone (Fig. 3). In the case of progesterone, fluorescence polarization also increased markedly. Considerable increase in fluorescence polarization was also found in the cases of corticosterone, cholesterol and testosterone propionate, but the increase was slight with ethinyl estradiol. It should be emphasized that none of the steroids tested diminished the fluorescence polarization. In addition, it was observed that progesterone brought about a slight but definite blue shift in the fluorescence emission band (Fig. 4). Any appreciable change in the spectroscopic characteristics did not occur at the concentration of progesterone lower than 24 μM.

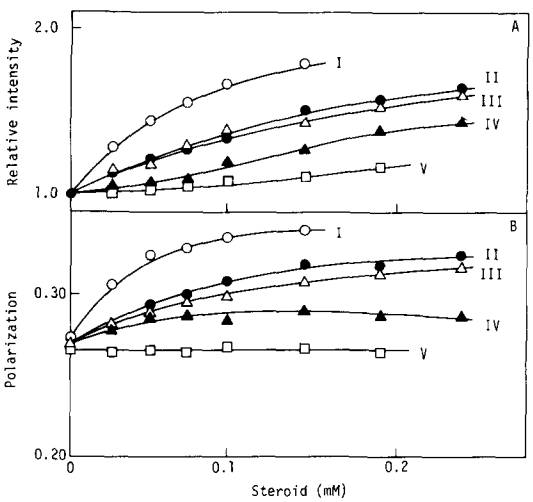

Fig. 3. Effect of steroids on the fluorescence intensity and polarization degree of D-amino acid oxidase. The fluorescence intensity and polarization degree were measured at various concentrations of steroids. These values were corrected for the light scattering, which was observed on the addition of ethanol solution of steroid to the buffer, as described in the text. The enzyme (12 μM) was dissolved in 0.017 M pyrophosphate buffer (pH 8.3). A: relative fluorescence intensity; B: polarization degree. Emission: 530 nm; excitation: 450 nm. Curve I: progesterone; Curve II: corticosterone; Curve III: cholesterol; Curve IV: testosterone propionate; Curve V: ethinyl estradiol.

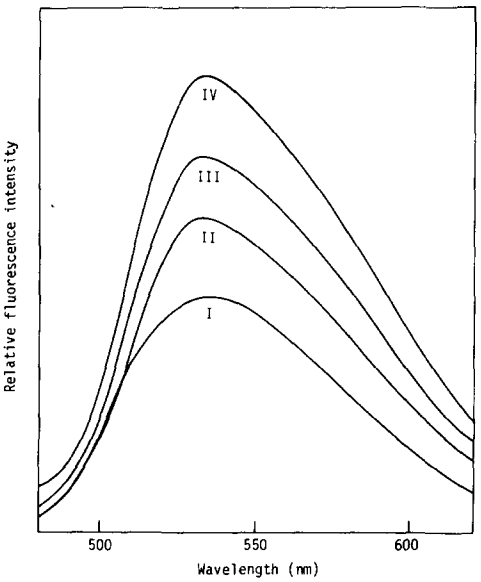

Fig. 4. Effect of progesterone on the fluorescence emission spectrum of D-amino acid oxidase. The enzyme (12 μM) was dissolved in 0.017 M pyrophosphate buffer (pH 8.3). The fluorescence spectra were corrected for a slight light scattering. Excitation: 450 nm. Curve I: in the absence of progesterone; Curve II: I + 24 μM progesterone; Curve III: I + 49 μM progesterone; Curve IV: I + 72 μM progesterone.

Discussion

One of the most striking features of this study is the inhibition of D-amino acid oxidase by progesterone in competition with the substrate. Since progesterone bears no structural resemblance to the substrate, the inhibition must be due to a steric block of the substrate binding site. This is conceivable since the oxidase has a wide substrate specificity with flexibility in its conformation, and the locus surrounding the substrate binding site is hydrophobic in nature [10,11]. However, inhibition by such a bulky molecule having no ionic groups is exceptional, since other competitive inhibitors such as carboxylic acids [12,13] and 1-anilinonaphthalene-8-sulfonate [10,11] contain ionic groups. The inhibition by the other steroids may be ascribed to the mechanism similar to that of progesterone, but the affinity of these inhibitors for the enzyme molecule is weaker than that of progesterone.

Data on fluorescence and absorption characteristics support a close interaction between the enzyme and the inhibitor molecules. The increase in both fluorescence intensity and polarization rules out the possibility of FAD dissociation, and indicates an increase in hydrophobicity of the environment surrounding the flavin chromophore as a result of the interaction.

The interaction between these steroid hormones and the enzyme is considered to occur at the substrate binding site of the enzyme. However, cholesterol combines with the enzyme to induce a similar fluorescence change without the competition with the substrate, viz., cholesterol combines with the enzyme at a site other than the substrate binding site. This led us to suppose that in the cases of the hormones the possibility of their binding to the enzyme

at a site other than the substrate binding site cannot be excluded. This might be a reason why the K_i values obtained by kinetic analysis do not agree with dissociation constants expected from the results by the spectroscopic methods. Furthermore, it should be noted that the concentrations of the enzyme in the enzymatic assay and in the spectroscopic analysis are different. At different concentrations of the enzyme, the concentrations of the monomeric enzyme become different [14,15].

In relation to the present finding, the report of Mason et al. [16] should be mentioned. They observed that the addition of estrogens in vitro inhibits some pyridoxal phosphate enzymes by interfering with the binding of the coenzyme. This has been suggested as a possible molecular basis of the effects of steroids on pyridoxal phosphate enzymes in vivo. This is not the case for D-amino acid oxidase.

The present data show that the increase in the enzyme activity in vivo observed by Ahmed and Bamji [4] by administering steroidal contraceptives to rats may not be explained by the result obtained from the in vitro experiment with the purified enzyme. However, a possibility of the stabilization of the existing enzyme through binding with steroid molecules merits consideration.

To the best of our knowledge, this is the first instance of the binding of steroids with a flavin enzyme. It is conceivable that similar interactions may be seen even with other flavoproteins. This is yet another example of binding of a steroid with a protein.

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