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COMPLEX FORMATION OF D-AMINO ACID OXIDASE WITH BARBITURATES AND URACIL DERIVATIVES

YOSHIMASA KYOGOKU[a*], MORIMITSU NISHIKIMI[b], BYUNG-SUL YU[a**] AND KUNIO YAGI[b]

[a] *Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo (Japan) and* [b] *Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya (Japan)*

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SUMMARY

1. Phenobarbital and barbital modified the absorption and circular dichroism (CD) spectra of D-amino acid oxidase [D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3] and inhibited the enzyme in competition with the substrate, but barbituric acid did not. 1-Methyl-5-bromouracil, 1-methyluracil, 1-methyl-4-thiouracil and 9-ethyladenine also produced changes in the absorption and CD spectra of the enzyme, and acted as inhibitor. Among these, 1-methyl-4-thiouracil was unique in that it produced diffuse absorption extending to 700 nm and positive CD in the corresponding wavelength region. However, uridine, 5-bromouridine and adenosine did not affect the absorption and CD spectra of the enzyme, indicating that bulky, hydrophilic groups attached to bases disturbed the binding.

2. The K_i of phenobarbital was not lowered with decreasing pH from 8 to 6. Since the pK_a of phenobarbital is 7.5, this result indicates that the non-ionized form of the compound effectively combines with the enzyme. No correlation was found between the K_i values (at pH 8.3) and the pK_a values of barbiturates and uracil derivatives. Thus, these compounds are considered to bind to the enzyme without essential participation of an anionic group, in contrast to the other known inhibitors such as benzoate.

INTRODUCTION

Barbiturates reduce oxygen consumption in various mammalian tissues and inhibit respiration in cell free preparations of liver and brain mitochondria[1,2]. The inhibition takes place in the flavoprotein site of the mitochondrial electron transport chain[3–5]. Moreover, it has been shown that phenobarbital inhibits a number of purified flavoenzymes and produces modifications of the absorption and fluorescence spectra[6].

* Present address: Institute for Protein Research, Osaka University, Suita, Osaka, Japan.
** Present address: College of Pharmacy, Seoul National University, Seoul, Korea.

The latter suggests that phenobarbital might interact with the enzyme-bound flavins. In the present paper, we report the interrelation between the chemical structure of uracil derivatives which have a structure common to barbiturates and their complex formation with D-amino acid oxidase [D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3], a flavoenzyme.

MATERIALS AND METHODS

D-Amino acid oxidase was prepared according to the method of Yagi *et al.*[7]. Its concentration was expressed in terms of the bound FAD.

The following chemicals of reagent grade were purchased: phenobarbital from Sigma, St. Louis; barbital from Merck, Darmstadt; barbituric acid from Nakarai Chemicals, Kyoto; 1-methyl-5-bromouracil, 1-methyluracil, 1-methyl-4-thiouracil, uridine, 5-bromouridine, adenosine and 9-ethyladenine from Cyclo Chemical, Los Angeles.

Absorption spectra were measured in a Beckman DK-2A spectrophotometer. CD spectra were measured in a Jasco spectropolarimeter, model J-20. The CD intensity was calibrated with an aqueous solution of 10-(+)-camphorsulfonic acid assuming $\Delta\varepsilon = 2.2$ at 291 nm. The results were expressed in terms of molar ellipticity, in degrees·cm^2·dmole^{-1}. Fluorescence measurements were carried out with a Shimadzu RF-502 spectrofluorometer.

Oxygen consumption due to the catalytic oxidation of D-alanine by D-amino acid oxidase was measured using a Beckman Oxygen Sensor as described previously[7]. The reaction was initiated by adding the enzyme to solutions containing 10 μM FAD and D-alanine of various concentrations (0.83–4.0 mM). Inhibition constants (K_i) were calculated from Lineweaver–Burk plots obtained in the absence and presence of inhibitors (concentrations close to the values of K_i for inhibitors).

All the measurements were performed in 17 mM pyrophosphate buffer (pH 8.3) at 20 °C, unless otherwise stated.

RESULTS AND DISCUSSION

When an excess of phenobarbital was added to a solution of D-amino acid oxidase, the absorption band at 450 nm of the enzyme shifted towards longer wavelength, accompanied with the appearance of the three-banded structure. At the same time, slight hypochromicity was observed at both the absorption peaks in the visible wavelength region (Curve II in Fig. 1A). These changes are identical with those reported by Giuditta and Casola[6]. Essentially identical changes were observed with barbital, but barbituric acid gave no change. The spectral changes of the enzyme observed upon addition of phenobarbital or barbital were similar to those observed upon addition of benzoate[8] or straight-chain fatty acids such as *n*-valerate[9].

1-Methyl-5-bromouracil provoked a less remarkable change; slight red shifts of both the peaks with slight hypochromicity (Curve III in Fig. 1A). The spectral change caused by 1-methyluracil was similar to that of 1-methyl-5-bromouracil. 9-Ethyladenine produced blue shifts of both the peaks (Curve II in Fig. 1B). By contrast, 1-methyl-4-thiouracil provoked a different type of change. As shown by Curve III in Fig. 1B, a diffuse absorption extending to 700 nm was observed. Similar

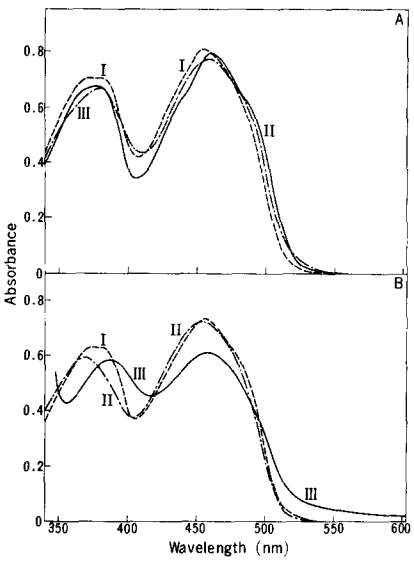

Fig. 1. Visible absorption spectra of the mixtures of D-amino acid oxidase and barbiturates or the related compounds. (A) I, the holoenzyme of D-amino acid oxidase (72 μM) in 17 mM pyrophosphate buffer (pH 8.3); II, I + phenobarbital (10 mM); III, I + 1-methyl-5-bromouracil (10 mM). (B) I, the holoenzyme (65 μM) in 17 mM pyrophosphate buffer (pH 8.3); II, I + 9-ethyladenine (20 mM); III, I + 1-methyl-4-thiouracil (10 mM).

charge-transfer-like absorption has already been observed upon addition of another sulfur containing compound, thioproline[10]. The changes in the absorption spectrum of D-amino acid oxidase upon addition of barbiturates or the related compounds indicate their interaction with the enzyme. The fact that the changes were different from one another suggests that these compounds come close to the isoalloxazine moiety of FAD when bound to the enzyme. However, uridine, 5-bromouridine and adenosine did not cause any change.

The CD spectrum of the holoenzyme of D-amino acid oxidase, shown by Curve I in Fig. 2A, is essentially identical with that previously reported[11,12]. Upon addition of an excess of phenobarbital to a solution of the enzyme, remarkable increases in molar ellipticity at 385 and 460 nm were observed (Curve II in Fig. 2A). The feature of the spectral change, however, somewhat differs from that observed with benzoate or *o*-aminobenzoate[11]. The change is rather similar to that observed with straight-chain fatty acids like *n*-valerate[12]. An essentially identical change was also observed upon addition of barbital. However, barbituric acid did not give any change.

1-Methyl-5-bromouracil also produced a modification of the CD spectrum of the enzyme (Curve III in Fig. 2A). The increase in molar ellipticity at 460 nm is slightly weaker than that observed with phenobarbital. 1-Methyluracil gave a similar but slight change. 9-Ethyladenine also produced a slight change in the CD spectrum (Curve II in Fig. 2B). The modification of the CD spectrum caused by 1-methyl-4-thiouracil was as remarkable as that of the absorption spectrum. A broad CD band extending to 700 nm corresponding to the diffuse absorption was observed in addition to the increases in ellipticity at 460 and 385 nm (Curve III in Fig. 2B). It is noticed that the differences in the CD changes caused by the above-mentioned compounds

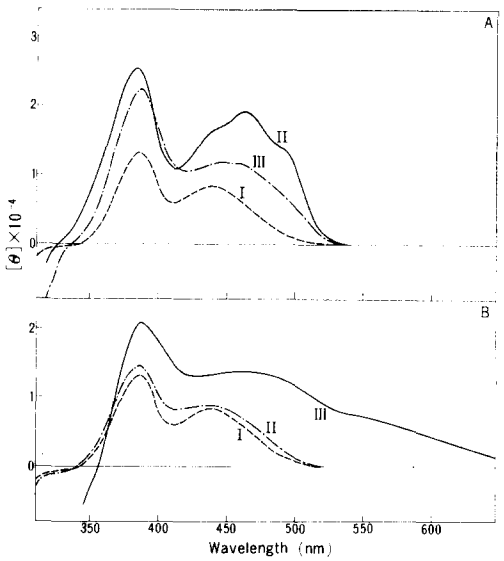

Fig. 2. CD spectra of the mixtures of D-amino acid oxidase and barbiturates or the related compounds. (A) I, the holoenzyme of D-amino acid oxidase (100 μM) in 17 mM pyrophosphate buffer (pH 8.3); II, I + phenobarbital (10 mM); III, I + 1-methyl-5-bromouracil (10 mM). (B) I, the holoenzyme (100 μM) in 17 mM pyrophosphate buffer (pH 8.3); II, I + 9-ethyladenine (20 mM); III, I + 1-methyl-4-thiouracil (10 mM).

were greater than those in the changes in the absorption spectrum. On the other hand, uridine, 5-bromouridine and adenosine did not give any change in accord with the results obtained by the absorbance measurement. The changes in molar ellipticity at 460 nm of D-amino acid oxidase caused by the addition of these compounds are listed in Table I.

Effects of these compounds on the fluorescence of D-amino acid oxidase were complicated. Phenobarbital and barbital increased intensity of the fluorescence, while other compounds decreased or did not change it (see Table I). A modification of the fluorescence spectrum produced by phenobarbital observed in our experiments was identical with that reported by Giuditta and Casola[6].

In order to see whether the spectroscopically-determined interaction discussed above really affects the activity of D-amino acid oxidase or not, the oxygen consumption due to the catalytic oxidation of D-alanine at pH 8.3 was examined in the presence and absence of barbiturates or the related compounds. All of the compounds that produce modifications of the absorption and CD spectra inhibited the enzyme. In all the cases, the double-reciprocal plots of the reaction velocity *versus* substrate concentration represented a typical competitive type of inhibition. Obtained inhibition constants are given in Table I.

At pH 8.3 the molecules of phenobarbital exist in the equilibrium of the partially ionized and non-ionized forms, since its pK_a is 7.5 (ref. 13). To examine the inhibitory action of the non-ionized form, the pH dependence of the inhibition constant was measured. As shown in Fig. 3, the pK_i ($-\log K_i$) remained almost constant below pH 8. It seems, therefore, that the non-ionized form of phenobarbital effectively inhibits the enzyme. This view is supported by the inhibitory action of

TABLE I

COMPARISON OF PROPERTIES OF BARBITURATES AND RELATED COMPOUNDS IN RELATION TO THEIR COMPLEX FORMATION WITH D-AMINO ACID OXIDASE

Compounds	$\Delta[\theta] \times 10^{-4}$ *at 460 nm**	*Fluorescence***	K_i *(mM)****	pK_a†
Phenobarbital	1.3	3	2.6	7.45 (ref. 13)
Barbital	1.3	3	3.1	7.97 (ref. 13)
Barbituric acid	0	1	N.I.	4.02 (ref. 14)
1-Methyl-5-bromouracil	0.6	0.5	6.9	7.8 (ref. 15)
5-Bromouridine	0			
1-Methyl-4-thiouracil	0.8	0.4	2.9	8.2 (ref. 16)
1-Methyluracil	0.25	0.7	3.3	9.71 (ref. 17)
Uridine	0		N.I.	9.4 (ref. 17)
9-Ethyladenine	0.1	1	9.7	

* The increase in molar ellipticity at 460 nm of D-amino acid oxidase upon addition of the compound. The concentration of the compounds was 10 mM except for 9-ethyladenine (20 mM).

** The change in fluorescence intensity of D-amino acid oxidase (10 μM) upon addition of the compound. The concentration of the compounds was 10 mM except for 9-ethyladenine (20 mM). Excitation was at 375 nm. Emission at 520 nm was compared, taking the fluorescence intensity of the enzyme as 1.

*** The inhibition constant for D-amino acid oxidase at pH 8.3. N.I. signifies that no inhibition was observed at the concentration of 10 mM.

† The first dissociation constant. The values are quoted from the literature. The value in ref. 16 is for 4-thiouridine, and is assumed to be the same as that for 1-methyl-4-thiouracil.

9-ethyladenine, which is not ionized at pH 8.3, where the inhibitory effect was observed. Thus, it is reasonable to consider that the non-ionized form of 1-methyluracil can form the complex with the enzyme, since its pK_a is 9.7 and most of the molecules exist in the non-ionized form[17]. This situation is also the case for other inhibitors examined in the present study. Therefore, it is not surprising that there is no correlation between the values of K_i and pK_a of barbiturates and uracil derivatives (see Table I). The pK_i of phenobarbital decreased with an increase of pH above 9 (Fig. 3). This feature is similar to that observed with other inhibitors such as benzoate, indicating the presence of an ionizing group in the enzyme protein which influences the binding of inhibitors[18].

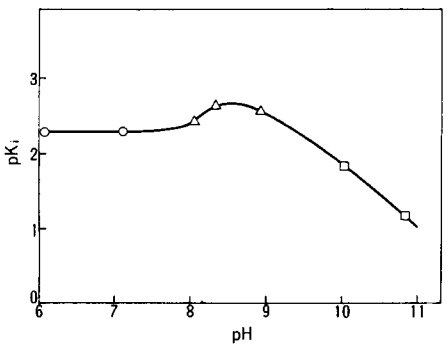

Fig. 3. Dependence of the inhibition constant of phenobarbital on pH in the oxidation of D-alanine by D-amino acid oxidase. The inhibition experiments were carried out in 50 mM sodium phosphate buffer (○), 17 mM sodium pyrophosphate buffer (△), and 50 mM sodium carbonate buffer (□).

The inhibition mechanism of these compounds seems to be different from that of benzoates and other carboxylates whose carboxyl group is totally ionized above pH 6. For the inhibitory effect of the carboxylates, the COO^- group is essential[8,9]. It is apparent that such an ionized carboxyl group competitively binds to the site where substrate amino acid does through its COO^- group. However, a hydrophobic interaction seems to be important for barbiturates in their complex formation with this enzyme, as deduced from comparison of the behavior of phenobarbital and barbital with that of barbituric acid in the complex formation. In connection with this view, it should be recalled that a hydrophobic interaction is also required for the complex formation between carboxylate-type inhibitors and the enzyme[9]. It can be expected that the compounds which have bulky, hydrophilic side groups hardly interact with the enzyme. Thus, it is reasonable that uridine, 5-bromouridine and adenosine did not bind to D-amino acid oxidase.

Although the hydrophobic interaction is one of the important factors for barbiturates and the related compounds to combine with the enzyme in competition with the substrate as mentioned above, the other interaction might be involved in the complex formation; the interaction between these compounds and the adenine moiety of FAD might be involved, since it has been found that they form molecular complexes with adenine derivatives through hydrogen bonds[19–22].

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