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Circular dichroic spectra of D-amino-acid oxidase and its complexes

As reported in our previous communications[1-3], extrinsic Cotton effects in ORD appeared when D-amino-acid oxidase (D-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) formed complexes with the substrates or inhibitors. These Cotton effects were considered to be due to the substrate or inhibitor binding which provokes or intensifies the optical activity in the coenzyme chromophore[4]. In support of this view, CD spectra of the enzyme and its complexes are reported herein.

The holoenzyme of D-amino-acid oxidase was prepared from the enzyme–benzoate complex according to the method of YAGI *et al.*[5]. This preparation was completely free from benzoate. The purple intermediate complex was prepared by mixing the enzyme with D-alanine in the presence of excess pyruvate and NH_4^+ as described in a previous paper[4].

The visible and ultraviolet absorption spectra of the enzyme and its complexes were recorded using a Beckman DK-2A spectrophotometer. The CD spectra were measured at room temperature by use of a JASCO-ORD/UV recorder with a CD attachment.

As indicated by Curve I in Fig. 1A, the holoenzyme showed two positive bands

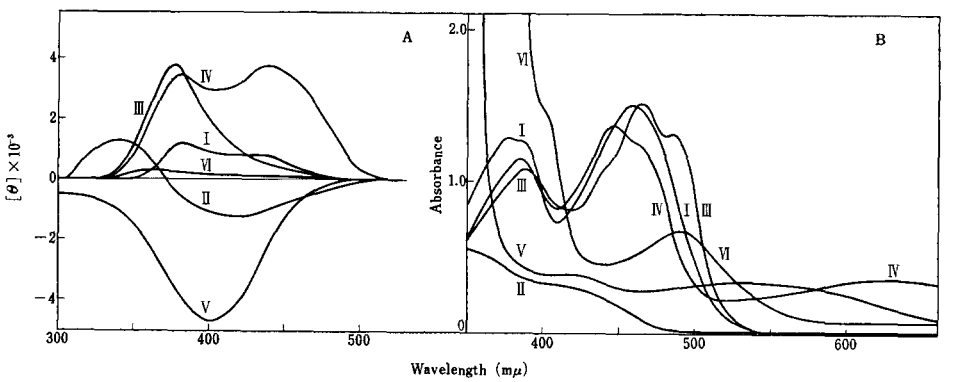

Fig. 1. Circular dichroic and absorption spectra of D-amino-acid oxidase and its complexes. (A) Circular dichroic spectra. (B) Absorption spectra. I, holoenzyme; II, fully reduced enzyme (the enzyme was reduced with $5 \cdot 10^{-2}$ M D-alanine); III, benzoate complex (the enzyme was mixed with $1 \cdot 10^{-3}$ M benzoate); IV, enzyme complex with Δ^1-piperidine-2-carboxylate (the enzyme was mixed with $1 \cdot 10^{-3}$ M of this compound); V, purple intermediate (the enzyme was mixed with $5 \cdot 10^{-2}$ M D-alanine in the presence of $1 \cdot 10^{-1}$ M pyruvate and $5 \cdot 10^{-2}$ M ammonium sulfate); VI, semiquinoid enzyme complex with products[12] (the enzyme was half-reduced with sodium dithionite and was mixed with $3 \cdot 10^{-2}$ M lithium pyruvate and $1 \cdot 10^{-2}$ M ammonium sulfate). The enzyme concentration was fixed at $1.33 \cdot 10^{-4}$ M with respect to FAD. Measurements were made at room temperature. Unit of $[\Theta]$: degree·cm²/dM.

which correspond to the two absorption bands of the coenzyme chromophore (see Curve I in Fig. 1B). AKI and his co-workers[6,7] reported a CD pattern different from that obtained by us, but their enzyme preparation seemed to be contaminated with some amount of benzoate which is known to form a complex with this oxidase[8-10], because their holoenzyme was prepared according to the procedure of MASSEY,

Abbreviations: ORD, optical rotatory dispersion; CD, circular dichroism.

PALMER AND BENNETT[11]. The absorption spectrum of their preparation reported was considered to be rather similar to that of the benzoate complex of the enzyme (Curve III in Fig. 1B).

The CD spectrum of the fully reduced enzyme showed a broad negative band centered at 420 mμ and a positive band at 340 mμ (Curve II in Fig. 1A), being in good agreement with that of AKI *et al.*[6].

The benzoate complex of this enzyme showed only a high ellipticity centered at 377 mμ as indicated by Curve III in Fig. 1A. The CD spectrum reported by AKI *et al.*[6] on their D-amino-acid oxidase preparation showed relatively high ellipticity at the same wavelength, which indicates that their preparation contained the enzyme–benzoate complex as suggested above.

Similar CD patterns were obtained when the enzyme was mixed with fatty acids such as *n*-valerate. These facts indicate that this type complexing intensifies the optical activity mainly at 370 mμ absorption band. This agrees well with the previously reported result of difference ORD between the benzoate complex and the holoenzyme, in which a positive Cotton effect was found having an inflexion point only at about 380 mμ (ref. 2).

Besides the above-mentioned benzoate type complex, MASSEY AND GANTHER[9] reported the formation of another type complex, the complex between the enzyme and n-donor such as Δ^1-piperidine-2-carboxylate. This type complex has a diffuse absorption band in the longer wavelength region due to a charge transfer complexing (see Curve IV in Fig. 1B). The CD spectrum of the enzyme complex with Δ^1-piperidine-2-carboxylate was shown by Curve IV in Fig. 1A. Differing from the benzoate-type complexing, this interaction strongly influenced the optical activity of FAD; this complex clearly showed two intensified positive bands at 380 mμ and 440 mμ, which correspond to the two absorption bands of the coenzyme.

In a previous paper[3], formation of a green complex spectrophotometrically identical with that between the enzyme and Δ^1-piperidine-2-carboxylate was found when the enzyme reacted aerobically with D-lysine. This green complex possesses two positive Cotton effects at wavelengths corresponding to 370 mμ and 450 mμ absorption bands of FAD, respectively. This result is consistent with the present observations; this green complex has been recently identified as a complex of the enzyme with Δ^1-piperidine-2-carboxylate* which was formed from the oxidized product of D-lysine, ε-amino-α-oxo-caproate through spontaneous cyclization.

A similar CD spectrum was obtained when the enzyme was mixed with another n-donor such as *o*-aminobenzoate.

The CD spectra of these enzyme–inhibitor complexes were not changed when they were aged in the dark, or even illuminated.

In contrast to these inhibitor complexes, a large negative band centered at 400 mμ was found in the CD spectrum of the purple intermediate (Curve V in Fig. 1A). This fact is in good agreement with the previous ORD measurements, in which was noted an inflexion point of a negative Cotton effect at 400 mμ[1,4]. Such a drastic change in the optical activity of the coenzyme chromophore is considered to be due to the strong charge transfer interaction between the enzyme and its substrate. In this case, it may be worth noting the spontaneous decrease in the molecular ellipticity

* K. YAGI, K. OKAMURA, A. TAKAI AND A. KOTAKI, unpublished results.

TABLE I

CHANGES IN THE ABSORPTION SPECTRUM, IN THE ELLIPTICITY OF CIRCULAR DICHROISM AND IN THE PARAMAGNETIC SUSCEPTIBILITY OF THE PURPLE INTERMEDIATE DURING THE STORAGE IN THE DARK OR THE ILLUMINATION

Illumination was performed by use of a Mazda daylight lamp (30 W) at a distance of 20 cm at 5°.

Time of aging (day) or illumination (h)	$[\Theta] \times 10^{-3}$ *(degree·cm²/dM)*	D_{492}/D_{550}	*Free radical* content (%)*
0 (day)	−4.4	0.95	0
28 (day)	−3.2	1.32	11.5
0 (h)	−4.4	0.95	0
10 (h)	−3.0	1.36	13.0

* Calculated from ESR measurements.

of the purple intermediate when it was aged in the dark at 5° or illuminated at the same temperature. With time of aging in the dark or illumination, the CD spectrum changed, with a decrease of its negative ellipticity, and finally became similar to the spectrum of the semiquinoid form of the enzyme (Curve VI in Fig. 1A). Furthermore, the decrease in the molecular ellipticity at 400 mμ was accompanied by the increase in the electron paramagnetic susceptibility and that in the absorbance at 492 mμ as shown in Table I. As pointed out previously[4,12], the purple complex gradually changes into a paramagnetic species in the dark, and light accelerates this conversion; this change is in fact accompanied by the decrease in the trough amplitude of ORD at 430 mμ. Therefore, the present results are essentially in accord with previous observations, supporting further the interpretation that the purple intermediate is an inner complex[13] formed between the enzyme and the substrate.

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