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COMPLEX FORMATION OF APOENZYME, COENZYME AND SUBSTRATE OF D-AMINO ACID OXIDASE

V. CHANGE IN CONFORMATION OF THE PROTEIN BY FORMING A MODEL OF ENZYME–SUBSTRATE COMPLEX

KUNIO YAGI, TAKAYUKI OZAWA AND TATSUO OOI

Department of Biochemistry, School of Medicine, and Department of Physics, Faculty of Science, Nagoya University, Nagoya (Japan)

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SUMMARY

1. The conformations of the apoenzyme, the holoenzyme and a model of enzyme–substrate complex of D-amino acid oxidase (D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3), were determined by using β-function obtained from the combination of hydrodynamic properties, together with the results of light-scattering and of optical-rotatory-dispersion measurements.

2. The results indicate that remarkable changes in conformation of the molecule (from random ellipsoid to rigid sphere) occur with the change from apoenzyme to enzyme–substrate model.

3. The above changes are discussed in connection with the mechanism of the enzymic catalysis.

INTRODUCTION

In previously published papers[1,2] of the present series, it has been reported that a complex of D-amino acid oxidase (D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3) and benzoate, a "substrate-substitute", was obtained as a crystal, and the hydrodynamic parameters of the complex (an *ES* model tentatively named as an artificial Michaelis complex) were compared with those of the holoenzyme or the apoenzyme. Remarkable changes in the hydrodynamic parameters were observed among the samples.

In the present paper, β-function obtained from the hydrodynamic parameters, together with the results of light-scattering and optical-rotatory-dispersion measurements, make it possible to assume the conformation of the protein molecules, the change in which seems to be very important in connection with the enzymic reaction.

For this purpose, molecular weight, size, and shape were estimated for each protein.

A preliminary note for a part of this paper has appeared[3].

MATERIALS

The apoenzyme, holoenzyme and an *ES* model of D-amino acid oxidase were prepared as reported previously[1,2,4]. The FAD which had a purity greater than 92%, and

contained no other flavins, nucleic acids or metals, was prepared by the method of YAGI *et al.*[5].

METHODS

Light-scattering measurement

The measurements were carried out by a Shimazu light-scattering photometer. Two wave lengths of 436 mμ and 546 mμ from the mercury arc were used as the incident beam. The samples were dissolved in 14 mM Tris–HCl buffer (pH 8.0) which was prepared using redistilled dust-free water. Immediately before the measurements were taken, the solutions were clarified by centrifugation in a Spinco (Type L) ultracentrifuge at 110 000 × *g* for 120 min.

Reduced intensities at 90° (R_{90}) of the *ES* model, and of the apoenzyme, were measured over various concentrations at room temperature. The dissymmetry factor *(Z)* of each sample was obtained by measuring the ratio of the intensity at 45° and that at 135°.

Optical-rotatory dispersion

Measurements were carried out by using a Rudolph Type 80 S polarimeter at 25°. The samples were dissolved in the same buffer as was used in the light-scattering measurements. The measurement was made in a cell having a light path of 10 cm.

RESULTS

Light-scattering measurements

Plots of Kc/R_{90} against the concentration of the protein are shown in Fig. 1. Curve I represents the *ES* model and Curve II, the apoenzyme. Both plots give straight lines, which are in parallel with the axis of abscissa. The values of the intercepts at infinite dilution are $0.86 \cdot 10^{-5}$, and $0.81 \cdot 10^{-5}$, for the *ES* model, and for the apoenzyme, respectively.

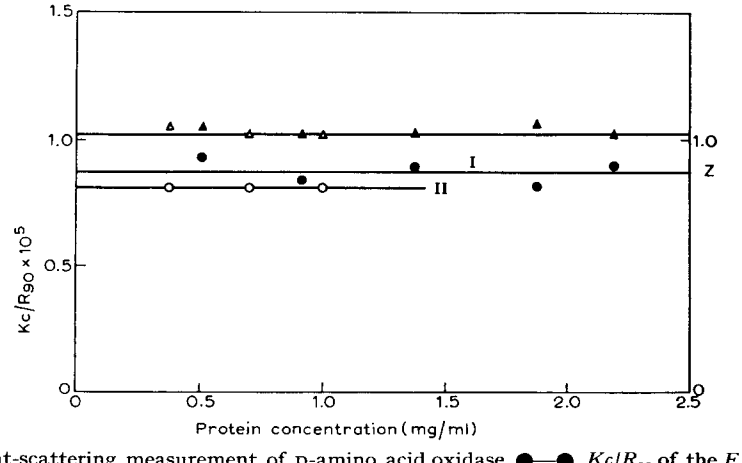

Fig. 1. Light-scattering measurement of D-amino acid oxidase. ●—●, Kc/R_{90} of the *ES* model at 5460 Å; ○—○, Kc/R_{90} of the apoenzyme at 4360 Å; ▲—▲, the dissymmetry factor *(Z)* of the *ES* model at 5460 Å; △—△, the dissymmetry factor of the apoenzyme at 4360 Å. Samples were dissolved in 14 mM Tris–HCl buffer (pH 8.0) and measured at 25.0°.

From the reciprocals of the intercepts, the molecular weight of the *ES* model was found to be 116 000 and that for the apoenzyme, 123 000. Both lines have no slope, indicating that the apparent second virial coefficients are zero.

The dissymmetry factor *(Z)* of the solutions of the *ES* model had a value of 1.02, which was the same as that of the apoenzyme as shown in Fig. 1.

Optical-rotatory dispersion

According to the Moffitt equation, $(\lambda^2 - \lambda_0^2)\,[\alpha]/\lambda_0^2$ was plotted against $\lambda_0^2/(\lambda^2 - \lambda_0^2)$ for the apoenzyme as shown in Fig. 2. The plots are on the straight line in parallel with the axis of abscissa, a result indicating that b_0 is zero, *i.e.* the molecule contains no helix. The intercept is $-425°$.

In the case of both the *ES* model and the holoenzyme, the measurement was disturbed by the absorption over the region of visible wave length.

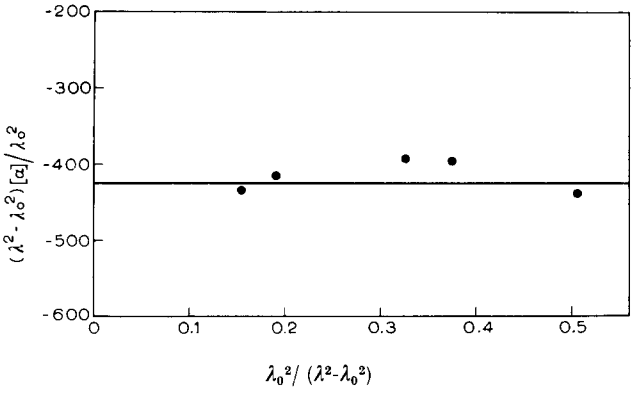

Fig. 2. Moffitt-plot of rotatory dispersion of the apoenzyme of D-amino acid oxidase. The apoenzyme of D-amino acid oxidase was dissolved in 14 mM Tris–HCl buffer (pH 8.0) at a concentration of 1.4 mg/ml and measured at 25.0° (λ_0 = 220 mμ).

Calculation of the major and minor axes of the protein molecules

Calculation was carried out by the method of SCHERAGA AND MANDELKERN[6]. Assuming that each enzyme molecule has an ellipsoidal shape, the semi-axis of revolution is designated as a, the equatorial radius as b and the axial ratio as $p = b/a$. Then the effective volume is given by $V_e = 4\pi ab^2/3$.

The quantity β, a function of p, was calculated by using hydrodynamic parameters reported in the previous paper[2]. β is given by the following equations:

$$\beta = \frac{D\,[\eta]^{1/3}\,M^{1/3}\,\eta_0}{kT} = \gamma\,F\,\nu^{1/3} \qquad (1)$$

$$\beta = \frac{Ns[\eta]^{1/3}\,\eta_0}{M^{2/3}\,(1 - \bar{v}\varrho)} = \gamma\,F\,\nu^{1/3} \qquad (2)$$

where D = diffusion constant, M = molecular weight, $[\eta]$ = intrinsic viscosity, $\gamma = N^{1/3}/(16\,200\,\pi^2)^{1/3}$, F = a function of p, ν = a shape factor, N = Avogadro's number, s = sedimentation coefficient and $\bar{v}$ = partial specific volume.

As for the molecular weights of the apoenzyme, the holoenzyme and the *ES* model, the value of 115 000 ± 500 was obtained for each protein from sedimentation and diffusion data or from diffusion and viscosity data[2]. This value was supported by the results of light-scattering measurements. Therefore, it was reasonably concluded that no aggregation was present in the solutions.

From the data described in the previous paper[2], the values of β-function are as follows;

For the ES model: $D = 6.8 \cdot 10^{-7}$ cm²/sec, $[\eta] = 1.75 \cdot 10^{-2}$ dl/g, $\eta_0 = 0.01006$ g/per cm/sec, $k = 1.38 \cdot 10^{-16}$ erg/degree, $T = 293$ K, $M = 115\,000$,

$\beta = 2.12 \cdot 10^{6}$ from Eqn. 1

and $\bar{v} = 0.655$ ml/g, $s = 11.0 \cdot 10^{-13}$ sec, $\varrho = 0.998$ g/ml, $N = 6.02 \cdot 10^{23}$,

$\beta = 2.12 \cdot 10^{6}$ from Eqn. 2.

For the holoenzyme: $D = 6.0 \cdot 10^{-7}$ cm²/sec, $[\eta] = 2.6 \cdot 10^{-2}$ dl/g, $s = 8.0 \cdot 10^{-13}$ sec, and $\bar{v} = 0.718$ ml/g,

$\beta = 2.14 \cdot 10^{6}$ from Eqn. 1 and

$\beta = 2.14 \cdot 10^{6}$ from Eqn. 2.

For the apoenzyme: $D = 4.5 \cdot 10^{-7}$ cm²/sec, $[\eta] = 6.2 \cdot 10^{-2}$ dl/g, $s = 4.5 \cdot 10^{-13}$ sec, and $\bar{v} = 0.789$ ml/g,

$\beta = 2.14 \cdot 10^{6}$ from Eqn. 1 and

$\beta = 2.15 \cdot 10^{6}$ from Eqn. 2.

The values of β are listed in Table I.

TABLE I

DIMENSIONS CALCULATED FOR THE APOENZYME, HOLOENZYME AND AN *ES* MODEL OF D-AMINO ACID OXIDASE FROM β

	Apoenzyme	*Holoenzyme*	*ES model*
$\beta \cdot 10^{-6}$	2.15	2.14	2.12
$1/p = a/b$	2.5	2.2	1
ν	3.297	3.167	2.500
$1/F$	1.078	1.066	1.000
$f \cdot 10^{8}$	8.99	6.73	5.97
$V_e \cdot 10^{19}$ (cm³)	3.60	1.57	1.34
a (Å)	81.6	56.6	31.8
b (Å)	32.5	25.8	31.8

There are two possibilities for the shape of the molecules, *viz.*, prolate ellipsoid and oblate ellipsoid.

As pointed out by SCHERAGA AND MANDELKERN, the value of β varies with p significantly only for a prolate, being relatively insensitive to p for an oblate ellipsoid. For example, $1/p$ for the apoenzyme is computed to be 2.5, assuming that the shape is prolate ellipsoid, and to be 15–20, if an oblate ellipsoidal shape be assumed. However, the dissymmetry factor for the apoenzyme, obtained by light-scattering measurement, was 1.02, a value which suggests an almost spherical

shape. This would indicate that the axial ratio around 15–20 requisite for an oblate ellipsoid is inconsistent with the experiment, and the assumption that the shape is prolate ellipsoid becomes quite reasonable.

Therefore, the values of p and ν for the prolate ellipsoid were calculated for each sample as listed in Table I.

The effective volume of an individual particle, V_e, was calculated by the following equation:

$$[\eta_{sp}/c]_{c \to 0} = [\eta] = (N/100) \cdot (V_e/M)\nu \tag{3}$$

The values of V_e are as follows (Table I): $V_e = 1.34 \cdot 10^{-19}$ cm^3 for the *ES* model, $V_e = 1.57 \cdot 10^{-19}$ cm^3 for the holoenzyme, and $V_e = 3.60 \cdot 10^{-19}$ cm^3 for the apoenzyme.

The effective volume is also computed by using the equation,

$$f = (162\,\pi^2)^{1/3}\,(V_e)^{1/3}\,\eta_0/F \tag{4}$$

Since the values of f and $1/F$ listed in Table I are obtained by the equations, $f = kT/D$, and $F = (p^{2/3}/\sqrt{1-p^2}) \cdot \ln[(1+\sqrt{1-p^2})/p]$, we can calculate the values of V_e again, that is: $V_e = 1.34 \cdot 10^{-19}$ cm^3 for the *ES* model, $V_e = 1.58 \cdot 10^{-19}$ cm^3 for the holoenzyme, and $V_e = 3.55 \cdot 10^{-19}$ cm^3 for the apoenzyme. Therefore, the values of a and b for the samples can be derived from the equations $V_e = 4\,\pi\,ab^2/3$ and $p = b/a$, as listed in Table I.

The resulting vertical sections of the protein molecules are shown in Fig. 3.

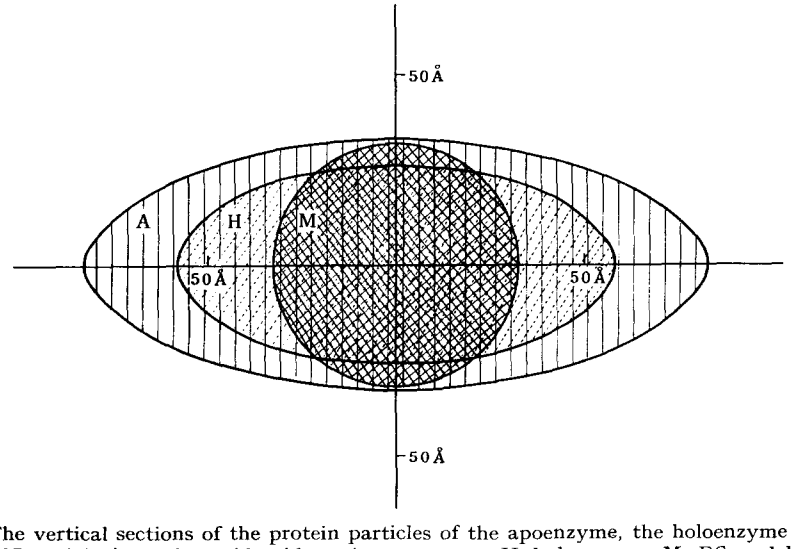

Fig. 3. The vertical sections of the protein particles of the apoenzyme, the holoenzyme and the *ES* model of D-amino acid oxidase. A, apoenzyme, H, holoenzyme, M, *ES* model.

DISCUSSION

With the conclusion of this series of papers[1,2,4,7], the following considerations should be emphasized: (a) FAD binds with the apoenzyme through the AMP and FMN

parts and positive interaction between two binding-sites of the protein occurs; (b) as a result of the binding of the protein to FAD, the red shift of the spectrum of FAD occurs, and the specific shoulder, at 490 mμ, is caused by further combination of benzoate, a substrate-substitute, with the FMN part of FAD; (c) the complex of apoenzyme, FAD and benzoate is isolated as a crystal, and is composed of equimolar amount of apoenzyme, FAD, and a substrate-substitute which is driven off by a real substrate. These findings led us to consider this complex as an *ES* model.

Since large differences in physico-chemical properties were observed between the apoenzyme, the holoenzyme and the *ES* model, and since these differences all point to a change in configuration of the protein, more detailed study was carried out on this problem.

In the previous paper[2], the α-helix content of the apoenzyme was assumed to be zero. This assumption has been confirmed by the present results derived from optical-rotatory dispersion of the apoenzyme.

Since a molecular weight of 115 000 found for the apoenzyme, the holoenzyme and the *ES* model was also confirmed by light-scattering measurements, it was concluded that no aggregation of the protein occurs by either liberation or combination of the coenzyme and a substrate-substitute.

From the light-scattering measurements, together with the calculated axial ratios of the samples, their molecular shapes were deduced. The molecular shape of the apoenzyme or the holoenzyme is prolate ellipsoid, whereas that of the *ES* model is almost spherical.

Considering also the α-helix content of the sample reported in the present paper as well as in the previous one[2], our results can be summarized as follows; denoting the apoenzyme by *A*, FAD by *F* and benzoate by *B*,

$$A + F \rightleftharpoons E \qquad E + B \rightleftharpoons EB$$

$$\text{random ellipsoid} \rightleftharpoons \text{rigid sphere}$$

Recently, we succeeded in isolating a true Michaelis complex of the oxidase[8], in which the substrate is activated by the enzyme and the FAD is turned into semiquinoid form, denoted by *E'S'*.

The sedimentation coefficient of the complex was found to be 10.4 S (see ref. 9), which is more similar to that of the *ES* model, 11.0 S, than that of the holoenzyme, 8.0 S.

Thus, the conformation of the *ES* model described in this paper points presumably to that of true *ES* as follows:

$$E + S \rightleftharpoons ES \rightleftharpoons E'S' \rightleftharpoons E + P$$
$$E + B \rightleftharpoons EB$$

From these results, the binding of the coenzyme and substrate to the apoenzyme is considered to cause the mutual interaction between the apoenzyme, the FAD and the substrate. The interactions mentioned above, especially the change of the protein conformation, would presumably play an essential role in the enzymic catalysis.

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