COENZYME-INDUCED SUBUNIT ASSOCIATION OF THE FLAVOENZYME D-AMINO ACID OXIDASE: A KINETIC LIGHT SCATTERING STUDY

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1. Introduction

Hog kidney D-amino acid oxidase [EC 1.4.3.3] contains a single FAD per 35 000–40 000 MW monomer [1–4], and undergoes self-association [5–12]. Many ligands, such as competitive inhibitor, product, and substrate, influence the association process of the oxidase [11,13–16]. The holoenzyme has a higher apparent MW than the apoenzyme at a given enzyme concentration [6,9–11]. Recently, Yagi et al. [17,18] analyzed the binding property of FAD in D-amino acid oxidase according to monomer-dimer equilibrium and demonstrated that the affinity of the dimer for the coenzyme FAD is about 70 times higher than that of the monomer. These thermodynamic studies indicate that the coenzyme induces association of the subunits in the equilibrium state of the oxidase. However, there is no direct evidence yet available concerning the reaction mechanism of FAD-induced subunit association. In this communication, we demonstrate the subunit association subsequent to FAD binding to the apoenzyme and explore the rate-limiting step of this reaction by the kinetic light scattering method.

2. Materials and methods

Hog kidney D-amino acid oxidase [4,15] and its apoenzyme [19] were purified as described previously. The concentration of the apoenzyme was determined by either the method of Lowry et al. [20] or the $A^{1\%}_{1\,cm}$ value of 22.5 for the apoenzyme at 280 nm [3]. Commercial FAD (Sigma) was purified chromatographically [21,22].

Laser light scattering measurements were performed with a Union light scattering photometer LS-601. Light source was 5 mW He/Ne laser, 632.8 nm wavelength. A cylindrical cell of 8 mm in diameter was used. All solutions were dialyzed overnight against 0.1 M sodium pyrophosphate buffer, pH 8.3, to maintain the same chemical potential of each diffusible component in the solution and the solvent [23,24]. Dialyzed solutions and solvents were clarified by the membrane filters of 0.2 or 0.45 μm pore size (Sartorius-Membranfilter GmbH). Each reaction was initiated by mixing FAD dissolved in the dialyzate with the apoenzyme solution, and then the mixture was directly filtrated into the cell. The calibrations of the instrument were performed by distilled benzene. As the molecular dimensions of the oxidase are less than 5% of the wavelength of the incident light, all measurements were performed at 90° of the direction of the incident light [25].

Optical absorption measurements were made using a Cary 219 spectrophotometer and fluorescence was measured with a Hitachi MPF-4 spectrofluorometer.

We adopted half-time as a measure of the reaction rate. In light scattering and optical absorption, it was defined as the time giving half of the maximum signal change. In fluorescence, it was evaluated from the slope of slower phase in the first-order plot.

3. Results and discussion

As shown in fig.1, the addition of FAD to the apoenzyme caused a time-dependent increase in scattering intensity and after about 15 min the intensity reached the final constant value. Since the pro-

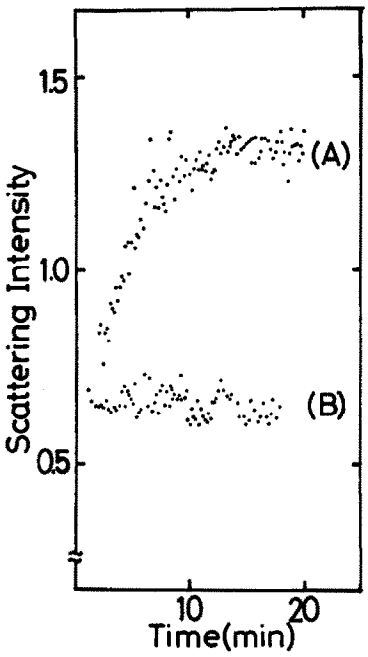

Fig.1. Change of light scattering from D-amino acid oxidase solution induced by FAD at pH 8.3, 20°C. The enzyme concentration was 0.25 mg/ml with 40 μM FAD (A) and without FAD (B). The ordinate is excess scattering intensity ($\Delta I_{90}/I_0$) over the solvent, where I_{90} denotes photon counts per 5 sec of the scattered light measured at right angle and I_0 the digitized photocell output monitored to eliminate the fluctuation of the laser light.

tein concentration used (0.25 mg/ml) was low enough to neglect the contribution of the second virial coefficient to the change in the scattering intensity [26], this finding indicates that the increase in the apparent weight-average MW by the addition of FAD was attributed to the subunit association of the oxidase. In contrast, the scattered intensity from the apoenzyme solution at the same concentration without FAD did not change within the same time scale (fig.1), indicating that no aggregation occurs.

Changes in the scattering intensity after mixing the apoenzyme with FAD were too slow if they had proceeded in a diffusion-controlled manner [27]. Indeed, the relaxation rate (<1 sec) for the subunit dissociation of the holoenzyme [8] is too rapid to consider the binding steps of subunits as the rate-determining steps of this association reaction. The half-life of this reaction was practically constant in the protein concentration from 0.1 to 3.8 mg/ml with excess FAD. It can, therefore, be deduced that the

Table 1
Comparison of half-times for light scattering with those for spectroscopic methods

Methods	Half-time (min)		
	20°C	10°C	5.5°C
Light scattering	3.3	7.1	14.0
Absorbance change at 493 nm	3.3 (2.1[a])	6.9[a]	13.2[a]
Fluorescence quenching of the protein	3.8	–	–

[a] These values were calculated from the data of [19]

association process involves the unimolecular isomerization steps(s). Massey and Curti [19] proposed a two-step mechanism for the binding of FAD to the apoenzyme in which the rapid binding of FAD precedes the slower conformational change of the protein,

$$A + F \rightleftharpoons H^* \rightleftharpoons H$$

where A, F, H^*, and H represent the apoenzyme, FAD , the holoenzyme in the transient state, and the holoenzyme, respectively. The slower step can be monitored by the increase in absorbance at 493 nm or by quenching of flavin or protein fluorescence in the slower phase [19]. As shown in table 1, the time-course of the increase in scattering intensity agrees well at each temperature with those of the changes detected by the above-mentioned spectroscopic observations. These results indicate that the rate-limiting step in the process of the subunit association triggered by FAD binding is that of the conformational change of the protein.

The extrapolated value of scattering intensity of the apoenzyme with FAD to zero time was nearly identical to that of the apoenzyme solution without FAD (fig.1). This observation can be interpreted thermodynamically as follows. Suppose that both steps of FAD binding to the apoenzyme (e.g., $A + F \rightleftharpoons H^*$) and association of subunits (e.g., $2H^* \rightleftharpoons H_2^*$) before the conformational changes in the protein are in rapid equilibrium. The rapid association processes of H^* subunit species (e.g., $2H^* \rightleftharpoons H_2^*$) scarcely contribute to the increase in the observed intensity of scattered light during the dead time, viz., the time interval between mixing and measurement. Hence, the intrinsic binding constants of FAD in the reactions, $A + F \rightleftharpoons H^*$, $A_2 + F \rightleftharpoons AH^*$, $AH^* + F \rightleftharpoons H_2^*$, etc. do

not differ largely from each other. Consequently, in a protein concentration range where the apoenzyme exists almost exclusively as its monomer [8], this reaction proceeds mainly along the pathway, $A \rightleftharpoons H^* \rightleftharpoons H \rightleftharpoons H_2 \rightleftharpoons$ polymers.

Compared with the rate of subunit association of 0.52 mg/ml apoenzyme plus 0.08 mM FAD system, the inclusion of 0.48 mM benzoate, a competitive inhibitor, accelerated the rate about twice ($t_{1/2}$ = 1.7 min at 20°C). This phenomenon was also observed in the absorbance change at 493 nm ($t_{1/2}$ = 1.1 min at 20°C). As benzoate binds weakly, if at all, with the apoenzyme [28], these results suggest that benzoate binds with the conformer (H^*) of the holoenzyme and accelerates the rate of the conformational change of the protein.

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