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EXCHANGE OF FREE AND BOUND COENZYME OF FLAVIN ENZYMES STUDIED WITH [^{14}C]FAD

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Summary

The exchange of bound FAD for free FAD was studied with D-amino acid oxidase (D-amino acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3) and β-D-glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4). For a simple measurement of the reaction rate, equimolar amounts of the enzyme and [^{14}C]FAD were mixed.

The exchange occurred very rapidly in the holoenzyme of D-amino acid oxidase at 25°C, pH 8.3 (half life of the exchange: 0.8 min), but slowly in the presence of the substrate or a competitive inhibitor, benzoate. It also occurred slowly in the purple complex of D-amino acid oxidase.

In the case of β-D-glucose oxidase, however, the exchange occurred very slowly at 25°C, pH 5.6, regardless of the presence of the substrate or *p*-chloromercuribenzoate.

On the basis of these findings, the turnover of the coenzymes of flavin enzymes in mammals is discussed.

Introduction

In the previous paper, it was reported that the turnover rate of flavins in mouse liver, expressed as biological half-life, was determined to be 5—6 days when 80 μg of [^{14}C]riboflavin per mouse was administered per os daily [1]. Yang and McCormick [2] reported that the biological half-life of flavins obtained with the whole body of rat is longer (16 days) when a lesser amount of riboflavin (55 μg of [^{14}C]riboflavin/100 g of body weight) was injected daily. These results indicate that the turnover rate depends on the amount of administered riboflavin.

In mammals, administered riboflavin is synthesized to coenzyme and the coenzyme thus formed is exchanged for the coenzyme bound to enzyme

protein in the cell. The exchange rate of coenzyme of individual flavin enzyme may depend on the amount of free coenzyme coexisting and the rate constants of dissociation and association of the flavin enzyme. For a better understanding of the turnover of flavins in vivo, the present paper deals with the exchange rate of free FAD for bound FAD of D-amino acid oxidase and of β-D-glucose oxidase.

Although the rate constants have usually been obtained with a rapid scanning spectrophotometric, a fluorometric or a manometric technique [3—6], a new method using a trace amount of [^{14}C]FAD was devised for the present study.

Materials

Enzyme. D-Amino acid oxidase was prepared from hog kidney according to the method of Yagi et al. [7,8]. It contains a single component with a molecular weight of 39 000, when checked by sodium dodecyl sulfate-disc electrophoresis

The lyophilized preparation of purified β-D-glucose oxidase was kindly donated by Dr. K. Kusai of Nagase Co. Ltd., Osaka. Its purity was checked by measuring the ratio of absorbance of the peaks and the enzymic activity [9,10].

The concentrations of the enzymes were expressed in terms of the enzyme-bound FAD determined spectrophotometrically. The molar extinction coefficients adopted were 11.3 $mM^{-1} \cdot cm^{-1}$ at 455 nm for D-amino acid oxidase [7] and 10.8 $mM^{-1} \cdot cm^{-1}$ at 460 nm for β-D-glucose oxidase [11].

[2-^{14}C]FAD. [2-^{14}C]FAD was prepared from rat liver and kidney as follows. [2-^{14}C]Riboflavin (0.53 μmol, $3 \cdot 10^6$cpm/mol) was injected daily into each of five rats fed on bread for 3 days, and on the 5th day the rats were decapitated. The liver and kidney were homogenized in ten volumes of hot water (80°C). Flavins were extracted at 80°C for 15 min twice. The combined extract was saturated with ammonium sulfate. The denatured protein was then filtered off, flavins in the filtrate were extracted with phenol, and finally a small amount of water and excess ether were added. Through these steps, flavins in phenol were transferred into a small amount of water. Flavins in the water layer were applied on filter paper and separated by paper chromatography using solvent A (*n*-butanol/acetic acid/water, 4 : 1 : 5), solvent B (5% sodium phosphate, dibasic) and solvent C (*n*-butanol/*n*-propanol/water, 2 : 2 : 1), successively. The purity of [2-^{14}C]FAD was checked by the ratios of absorbances at 260, 375 and 450 nm. These values agreed with the data reported by Whitby [12]. The specific radioactivity of [2-^{14}C]FAD was $1.35 \cdot 10^6$—$1.62 \cdot 10^6$ cpm/μmol of FAD. The yield was 300 μg.

Theoretical

In a closed two-compartment system, there occur the following relations:

$$A^* \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} B^* \qquad (1)$$

where A^* represents concentration of isotopic 'A' species and B^* that of iso-

topic 'B' species. When starting with labelled 'B' species and non-labelled 'A' species, the initial concentration of isotopic 'B' species (B_0^*) equals the total concentration of the isotopic species.

$$A^* + B^* = \text{constant } (B_0^*) \tag{2}$$

The half-life of the exchange reaction ($t_{1/2}$) can be expressed as

$$t_{1/2} = \frac{0.693}{k_{BA} + k_{AB}} \tag{3}$$

On the other hand, the dissociation and association of flavin enzyme can be expressed by the following equation:

$$EF \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E + F \tag{4}$$

where EF, E and F represent holoenzyme, apoenzyme *, and flavin, respectively; k_1 and k_2 represent the rate constants of dissociation and association of flavin enzyme. In Eqn. 4, the values of dissociation constant ($K = k_1/k_2$), concentration of holoenzyme ([EF]), concentration of apoenzyme ([E]), and concentration of flavin ([F]) are constant. Then, if F is replaced by radioactive F*, this system can be considered to be the same as the above two-compartment system, and the exchange rate constants correspond as follows:

$$k_{AB} = k_1 \tag{5}$$

$$k_{BA} = k_2[E] \tag{6}$$

$$K = \frac{[E][F^*]}{[EF]} \tag{7}$$

In Eqn. 4, the time course of the concentration of free labelled FAD is expressed by the following equation,

$$\frac{[F^*] - [F_\infty^*]}{[F_0^*] - [F_\infty^*]} = e^{-(k_1 + k_2[E])t} \tag{8}$$

where F_0^*, F_∞^*, and F^* represent the initial radioactivity of free labelled FAD, the final radioactivity at equilibrium, and the radioactivity during the exchange reaction at time t, respectively. If [EF] is equal to [F*],

$$[E] = K \tag{9}$$

Consequently, from Eqn. 5, 6, 9 and $K = k_1/k_2$,

$$k_{BA} = k_2[E] = k_2K = k_1 = k_{AB} \tag{10}$$

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* The apoenzyme is considered to have the same conformation as that accomplished by combining with FAD. If we start with the apoenzyme preparation, the following equilibrium holds.

$$E + FAD \rightleftharpoons E \cdot FAD \rightleftharpoons E' \cdot FAD$$

where E is the apoenzyme preparation and E′ is the apoenzyme of which conformation is modified by complex formation with FAD. In our case, the species of the apoenzyme is considered to correspond to E′. However, if it is not the case, our dissociation constant obtained becomes an apparent one of the above equilibrium.

Under the condition, [EF] = [F*], radioactivity of [EF] becomes equal to that of [F*] in equilibrium, so the counting error of radioactivities in both [EF] and [F] can be minimized. In order to satisfy the condition that [EF] is equal to [F], the same amount of radioactive free FAD as that of FAD in [EF] was added to the reaction mixture, since the dissociation constants of the holoenzyme of D-amino acid oxidase and β-D-glucose oxidase are considerably small.

If the half life ($t_{1/2}$) of the exchange reaction can be measured under the condition of [EF] = [F], k_1 and k_2 would be obtained from the following equations:

$$t_{1/2} = \frac{0.693}{K_{BA} + K_{AB}} = \frac{0.693}{2k_1} = \frac{0.346}{k_1} \tag{11}$$

$$k_1 = \frac{0.346}{t_{1/2}} \tag{12}$$

$$k_2 = \frac{k_1}{K} = \frac{0.346}{t_{1/2}K} \tag{13}$$

Therefore, in the above system ([EF]) = [F*]), the following relation holds:

$$\frac{[FAD^*(t)]}{[FAD^*(t_\infty)]} = 1 + e^{-(0.693/t_{1/2})t} \tag{14}$$

where $[FAD^*(t)]$ and $[FAD^*(t_\infty)]$ represent the specific radioactivity of free FAD at a definite incubation time and that of free FAD at the infinite time, viz., the time when the specific radioactivity of free FAD in the reaction mixture reaches 50% of the initial specific radioactivity of free FAD. After the exchange reaction, the decreased value of the specific radioactivity of free FAD is equal to the increased value of the specific radioactivity of FAD bound to the enzyme.

Determination of dissociation constant of β-D-glucose oxidase

From the study on the recombination of the apoenzyme and FAD [10,13], β-D-glucose oxidase is considered to have a very small dissociation constant. Therefore, the dissociation constant was determined by equilibrium dialysis. At equilibrium, EF ⇌ E + F, the dissociation constant (K) is represented as follows:

$$K = \frac{[E][F]}{[EF]} = \frac{(C\alpha)^2}{C(1-\alpha)}, \quad C = [EF] + [E] = [EF] + [F]$$

When the degree of dissociation is much lower than 1 and ($C\alpha$) stands for free FAD, i.e., [F],

$$K = \frac{[F]^2}{C} \tag{15}$$

Thus, the dissociation constant (K) can be calculated from only the concentration of free FAD and the initial concentration of the enzyme.

Experimental

Measurement of enzyme activity. The enzyme activity of D-amino acid oxidase [7], and that of β-D-glucose oxidase [9] were measured by oxygen uptake. Oxygen concentration was estimated polarographically in a Beckman Oxygen Sensor.

Exchange reaction. The reaction system (usually 10 ml) for D-amino acid oxidase consisted of the enzyme solution (50 μM with respect to FAD) and free FAD (50 μM) in the presence and absence of the substrate (0.14 M D-alanine) or a competitive inhibitor, benzoate (7 mM).

The reaction system for the purple complex of D-amino acid oxidase consisted of D-amino acid oxidase (80 μM with respect to FAD), free FAD (80 μM), DL-alanine (0.1 M), lithium pyruvate (0.1 M) and ammonium sulfate (0.05 M) [14]. The reaction was carried out in a Thunberg tube filled with nitrogen gas.

In the case of β-D-glucose oxidase, the reaction mixture consisted of the enzyme solution (50 μM with respect to FAD) and free FAD (50 μM), in the presence and absence of the substrate, D-glucose (0.11 M), or an inhibitor, *p*-chloromercuribenzoate (0.1 mM). The exchange reaction in the presence of the substrate was carried out under both aerobic and anaerobic conditions.

After equilibrium was reached, a trace amount of [^{14}C]FAD solution (total radioactivity: approx. 10 000 cpm) was added to each 10 ml of the reaction mixture.

The reaction mixture was continuously agitated with a magnetic stirrer at 37, 25 or 15°C for D-amino acid oxidase, and at 25°C for β-D-glucose oxidase. In the case of β-D-glucose oxidase at 5°C, no stirring was carried out. After a definite time of incubation, an aliquot (1.8 ml) of the reaction mixture was taken into a small test tube containing ammonium sulfate to be 30% saturation for D-amino acid oxidase reaction mixture and 90% saturation for β-D-glucose oxidase reaction mixture at 0°C. The mixture was centrifuged to precipitate the holoenzyme and filtered through a glass filter to obtain a clear sample solution.

In the case of D-amino acid oxidase, the FAD content in the filtrate was determined spectrophotometrically. An aliquot (0.5 ml) of the filtrate was subjected to scintillation spectrophotometry for the measurement of the radioactivity of [^{14}C]FAD. In the case of β-D-glucose oxidase, FAD in the filtrate containing a large amount of ammonium sulfate was extracted with 1 ml of phenol. The phenol layer was mixed with excess ether, and from this mixture FAD was extracted with 1.8 ml of water. The aqueous extract was washed with 3 ml of ether four times to eliminate phenol. FAD in the water layer was subjected to the measurement of concentration and radioactivity as in the case of D-amino acid oxidase. The specific radioactivity was calculated from the FAD concentration and radioactivity of the aliquot.

The exchange rate between free FAD and FAD bound to the enzyme was calculated from the specific radioactivity of free FAD after the exchange reaction.

Determination of dissociation constant of β-D-glucose oxidase. 2 ml of β-D-glucose oxidase (50.7 μM with respect to FAD) was dialyzed against 28 ml of

0.1 M sodium acetate buffer (pH 5.6) at 5°C for 30 h while being continuously stirred with a magnetic stirrer. Then free FAD in the dialysate was determined by the lumiflavin fluorescence method [15], and the dissociation constant was calculated by Eqn. 15.

Results

Exchange reaction of FAD in D-amino acid oxidase

The rate of exchange of free FAD for bound FAD of D-amino acid oxidase at 37°C was found to be 69% at 1 min and 97% at 5 min after the addition of [^{14}C]FAD to the reaction mixture. On the other hand, when the substrate, D-alanine, was added to the holoenzyme, the rate of exchange was only 5% after 1 h incubation. It increased very slowly with incubation time and reached only 9% after 2 h incubation. When the exchange reaction was carried out in the purple complex under anaerobic conditions, only 5% was exchanged after 2 h incubation. In the presence of the competitive inhibitor, benzoate, the exchange rate was 17% after 2 h incubation.

The values of the half-life of the exchange reaction of D-amino acid oxidase at 15, 25 and 37°C were determined graphically to be 1.0 min, 0.8 min and 0.7 min, respectively (Fig. 1).

Rate constants of dissociation and association of D-amino acid oxidase were calculated from the value of the half-life of the exchange reaction described above and the dissociation constant which was estimated from the fluorescence polarization degree [16,17]. The results are summarized in Table I.

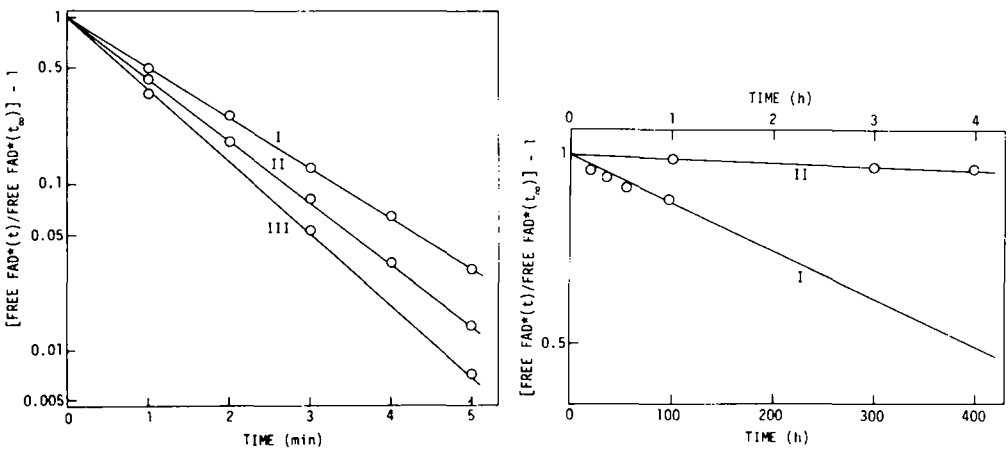

Fig. 1. Graphic determination of half-life of the exchange reaction of FAD in D-amino acid oxidase. The mixture of exchange reaction contained $5 \cdot 10^{-5}$ M each of D-amino acid oxidase and free FAD in 0.017 M pyrophosphate buffer (pH 8.3). At a definite time (t) after the addition of a trace amount of [^{14}C]FAD, the specific radioactivity was measured and expressed as free FAD*(t). The value of free FAD*(t_∞) is 50% of the initial specific radioactivity of free FAD (see text). The value of [freeFAD*(t)/free FAD*(t_∞)] − 1 on a log scale was plotted against time. From the slope of the curve, the value of half-life was calculated. I: 15°C; II: 25°C; III: 37°C.

Fig. 2. Graphic determination of the half-life of the exchange reaction of FAD in β-D-glucose oxidase. The conditions were the same as those described in Fig. 1, except that β-D-glucose oxidase was used and the exchange reaction was carried out in 0.1 M sodium acetate buffer (pH 5.6). I: 5°C; bottom time scale; II: 25°C; top time scale.

TABLE I

VARIOUS PARAMETERS OF THE EXCHANGE REACTION OF FREE AND BOUND FAD IN FLAVIN ENZYME

Enzyme	Temperature (°C)	$t_{1/2}$	K * (M)	k_1 (min^{-1})	k_2 ($M^{-1} \cdot min^{-1}$)
D-Amino acid oxidase	37	0.7 min	$2.1 \cdot 10^{-7}$	$5.0 \cdot 10^{-1}$	$2.4 \cdot 10^{6}$
	25	0.8 min	$1.2 \cdot 10^{-7}$	$4.3 \cdot 10^{-1}$	$3.6 \cdot 10^{6}$
	15	1.0 min	$2.2 \cdot 10^{-7}$	$3.5 \cdot 10^{-1}$	$1.5 \cdot 10^{6}$
β-D-Glucose oxidase	5	400 h	$1.3 \cdot 10^{-10}$	$1.4 \cdot 10^{-5}$	$1.1 \cdot 10^{5}$

* Dissociation constants for D-amino acid oxidase at various temperatures were obtained by fluorescence polarization at the concentration of $4 \cdot 10^{-5}$ M [16,17]. Dissociation constant for β-D-glucose oxidase was obtained from equilibrium analysis.

Exchange reaction of FAD in β-D-glucose oxidase

FAD in the holoenzyme of β-D-glucose oxidase was exchanged for free FAD very slowly and the rate of the exchange was not changed significantly by the anaerobic or the aerobic addition of the substrate or by the addition of the inhibitor, *p*-chloromercuribenzoate. It was found that only 3—5% of the FAD was exchanged after 5 h incubation at 25°C and 12% of bound FAD was exchanged after 70 h incubation at 5°C. The half-life of exchange reaction was calculated to be 400 h at 5°C from the slope of curve I in Fig. 2.

The dissociation constant of β-D-glucose oxidase was determined to be $1.3 \cdot 10^{-10}$ M by the method described in Theoretical. From this value and the value of the half-life, rate constants of dissociation and association of β-D-glucose oxidase were calculated as listed in Table I.

Discussion

The data obtained in the present study show that the coenzyme FAD of D-amino acid oxidase could rapidly be exchanged for free FAD, and that in the presence of the substrate or the inhibitor, benzoate, the exchange rate decreased remarkably.

As for the exchange reaction in the presence of the substrate (S) or the competitive inhibitor (I), the following equilibrium system could be considered:

$$\begin{array}{ccc} E + F & \rightleftharpoons & EF \\ & & + \\ & & S \text{ (or I)} \rightleftharpoons EFS \text{ (or EFI)} \end{array}$$

From this equation, it is clear that the amount of EF decreases in the presence of the substrate or the inhibitor. Although the substrate gives a complicated effect because of its turnover, it obviously shifts the equilibrium to the right. Consequently, the exchange of F in the system is restrained.

If such an effect of the substrate on the enzyme, as seen in the case of D-amino acid oxidase, occurs in the living body, the coenzyme will be exchanged more slowly in the presence of the substrate, and vice versa in the deficiency of the substrate, i.e., on starvation.

For the value of k_2 of D-amino acid oxidase at 15°C, $1.5 \cdot 10^{6}$ $M^{-1} \cdot min^{-1}$

was obtained. It is noted that this value is ten times larger than that obtained by Massey and Curti, $1.3 \cdot 10^5\ M^{-1} \cdot min^{-1}$ at 15.9°C [5]. This difference might be ascribed to the difference in the reaction system: we used the holoenzyme, whereas they used the apoenzyme in the assay system (see footnote on page 247).

In the case of β-D-glucose oxidase, the exchange of bound FAD for free FAD was very slowly, and the half-life of the reaction was calculated to be 400 h. The exchange rate of FAD in the holoenzyme was so slow that the effect of the substrate or the inhibitor on the exchange reaction was not clear.

The great difference in the half-life between the two enzymes is attributed mainly to the values of k_1 as shown in Table I: the association rates of FAD and the apoenzymes in both enzymes are almost equal, but the dissociation rates of the holoenzymes are greatly different.

It may be emphasized that our new method using radioactive coenzyme for determination of rate constants of association and dissociation of flavin enzymes can be applied to the crude enzymes and that it might be useful in clarifying the mechanism of exchange of other coenzymes.

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