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

I. KINETIC ANALYSIS USING INDICATORS

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SUMMARY

1. It was found that both riboflavin-5′-monosulfate (FMS) and adenosine-5′-monosulfate (AMS) compete with FAD for binding with apo-D-amino acid oxidase but not with each other, which suggests the binding of the FMN as well as the AMP moieties of the FAD molecule with the apo-protein.

2. Using FMS and AMS as indicators, *p*-aminobenzoic acid (PABA) was found to be a competitor with the FMN part of FAD. It was considered likely that the phenylamino group affects the binding point of the FMN part of FAD with the apo-protein. The dissociation constant of the apo-protein–PABA complex calculated from the competition of PABA with FAD for the protein appeared to be $7.3 \cdot 10^{-3}$ *M*. In the presence of AMS, this dissociation constant decreased to $2.5 \cdot 10^{-3}$ *M*, *i.e.*, the affinity of PABA to the protein increased, which indicates an interaction between the two binding sites.

3. *p*-Chloromercuribenzoate (PCMB) was found to be a competitor of the AMP moiety of FAD. This action was attributed to the binding of the SH-reactive group of PCMB with the sulfhydryl group of the apo-protein, which suggests that the AMP moiety of FAD combined with sulfhydryl group of the apo-protein. The dissociation constant of the complex of PCMB and apo-protein was calculated to be $1.6 \cdot 10^{-7}$ *M*. In the presence of FMS, this constant decreased to $8.5 \cdot 10^{-8}$ *M*, also suggesting an interaction of the binding sites of the apo-protein.

INTRODUCTION

In order to elucidate the processes involved in the dynamic enzyme complex formation it is necessary to demonstrate the binding sites of apo-enzyme, coenzyme, and substrate, since these are fundamental to the mechanism of enzyme action. For the study of this subject, D-amino acid oxidase was selected as a tool. The reasons for the selection are that the apo-enzyme and coenzyme of this enzyme are instan-

Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin-5′-monophosphate; AMP, adenosine-5′-monophosphate; FMS, riboflavin-5′-monosulfate; AMS, adenosine-5′-monosulfate; PABA, *p*-aminobenzoic acid; PCMB, *p*-chloromercuribenzoate.

taneously dissociable and associable with a rather large dissociation constant, and that the coenzyme FAD has a characteristic physico-chemical nature.

Concerning the binding of D-amino acid oxidase, previous work[1–5], especially the series on the mechanism of inhibition of this enzyme[3–5], provided some evidence. It has been noted by a model experiment and kinetic analysis of enzyme action[4] that the tyrosyl group of the apo-protein is responsible for the complex formation with the 3-NH group of the isoalloxazine ring of FAD, which is analogous to the old yellow enzyme reported by THEORELL AND NYGAARD[6].

At the same time, the phenylamino group of the benzene derivative was found to affect the binding site of the apo-protein with FAD[4], but the portion affected has not been studied.

On the other hand, the importance for the enzymic activity of the sulfhydryl group in D-amino acid oxidase apo-protein has been reported by several workers[7–10] but their conclusions about the binding site of the protein-sulfhydryl group were all different. SINGER AND BARRON[7], and SINGER[8] suggested that the protein-sulfhydryl group might be involved directly in the binding of the substrate. FRISELL AND HELLERMAN[9] concluded, however, that the enzyme-sulfhydryl group is not needed for the direct binding of the substrate, D-amino acids. KUBO *et al.*[10] were of the view that the protein-sulfhydryl group binds with the 3-NH group of FAD. Thus, opinions are divided about the binding of the sulfhydryl group of the apo-protein.

The indicator method previously reported for the study of the mechanism of inhibition[5] proved to be useful for solving these problems.

Results obtained by kinetic analyses for the purpose of demonstrating of the bindings involved in D-amino acid oxidase are reported in this paper. A preliminary note has already appeared[11].

MATERIALS

D-Amino acid oxidase protein, free from FAD, was prepared by the method of NEGELEIN AND BRÖMEL[12].

FAD was prepared by the method of YAGI *et al.*[13], as described previously.

FMS and AMS, kinetic indicators, were synthesized by the method of TAKAHASHI, YAGI, AND EGAMI[14], and that of EGAMI AND TAKAHASHI[15], respectively.

DL-Alanine, PABA, and PCMB were chemically synthesized samples.

METHOD

The oxidase activity was measured as described previously[3]. The dissociation constant of FAD with the apo-protein (K_f) and that of D-alanine with the apo-protein (K_s) were calculated to be $1.1 \cdot 10^{-7}\ M$ and $3.3 \cdot 10^{-3}\ M$, respectively, using the equation of Michaelis-Menten.

Assuming that an inhibitor inhibits the oxidase activity in competition with the coenzyme, FAD, for the apo-protein, the reaction velocity (v) can be shown as

$$v = \frac{Vf}{K_f(1 + i/K_{ip}) + f} \qquad (1)$$

where K_{ip} is the dissociation constant of inhibitor–protein complex, f and i are the

concentration of FAD and inhibitor, respectively, and V is the maximum velocity obtained in the presence of a large excess of FAD.

Rearranging eqn. (1) we obtain:

$$\frac{1}{v} = \frac{K_f(1 + i/K_{ip})}{Vf} + \frac{1}{V} \tag{2}$$

K may be calculated from the slope of the straight line obtained by plotting $1/v$ against $1/f$ in the presence of a restricted amount of inhibitor (Method I).

K_{ip} may also be evaluated by the following equation, which is derived from the eqn. (1) and the Michaelis-Menten equation,

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\frac{i}{K_{ip}} \tag{3}$$

where v_0 and v are the reaction velocity in the absence and presence of the inhibitor, respectively, with a restricted amount of FAD. The slope of the straight line obtained by plotting v_0/v against i refers to $(1 - v_0/V)/K_{ip}$.

If the reaction mixture contains two inhibitors, both of which compete with the same part of FAD (Case I), the reaction velocity can be shown as follows (see the formulation in the previous paper[5]):

$$v = \frac{Vf}{K_f(1 + i_1/K_1 + i_2/K_2) + f} \tag{4}$$

where i_1 and i_2 are the concentration of two inhibitors, I_1 and I_2, and K_1 and K_2 are the dissociation constants of complexes (I_1-enzyme protein) and (I_2-enzyme protein), respectively.

On the other hand, if the reaction mixture contains two inhibitors one of which (I_1) competes with the FMN part of FAD and the other (I_2) competes with the AMP part of FAD, respectively, or in other words, inhibitors compete with FAD but not with each other, the reaction velocity can be shown as

$$v = \frac{Vf}{K_f(1 + i_1/K_1 + i_2/K_2 + i_1i_2/K_1K_3) + f}$$

or

$$v = \frac{Vf}{K_f(1 + i_1/K_1 + i_2/K_2 + i_1i_2/K_2K_4) + f} \tag{5}$$

where K_3 is the dissociation constant of (I_1, I_2-enzyme protein) $\rightleftharpoons I_2$ + (I_1-enzyme protein), and K_4 is that of (I_1, I_2-enzyme protein) $\rightleftharpoons I_1$ + (I_2-enzyme protein). If no interaction takes place between the affinities of I_1-enzyme protein and that of I_2-enzyme protein, namely $K_3 = K_2$ and $K_4 = K_1$, eqn. (5) can be represented as

$$v = \frac{Vf}{K_f(1 + i_1/K_1 + i_2/K_2 + i_1i_2/K_1K_2) + f} \tag{6}$$

From the eqns. (4), (5), or (6) and the Michaelis-Menten equation, the following equations can be derived

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2}\right) \tag{7}$$

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1i_2}{K_1K_3}\right)$$

or

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_2 K_4}\right) \quad (8)$$

$$\frac{v_0}{v} = 1 + \left(1 - \frac{v_0}{V}\right)\left(\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_1 K_2}\right) \quad (9)$$

These equations show that the plots of v_0/v against the concentration of inhibitors give a straight line in Case I (eqn. (7)) and a second-order curve in Case II (eqn. (8) or (9)). Accordingly, if plots of v_0/v against i are on a straight line in the presence of both the inhibitor and FMS, and those are on a second-order curve in the presence of both the inhibitor and AMS, it may be concluded that the inhibitor competes with the FMN part of FAD. On the contrary, if plots of v_0/v are on a second-order curve in the presence of both the inhibitor and FMS, and on a straight line in the presence of both the inhibitor and AMS, it may be concluded that the inhibitor competes with the AMP part of FAD.

In this way, the behaviour of an inhibitor in its competition with FAD can be analyzed and the binding site of the apo-protein can also be studied.

RESULTS

Inhibitory action of FMS and AMS on D-*amino acid oxidase and their interaction on the oxidase protein*

The values of v_0/v plotted against the concentration of FMS or AMS were on a straight line as shown by Curves I and II in Fig. 1.

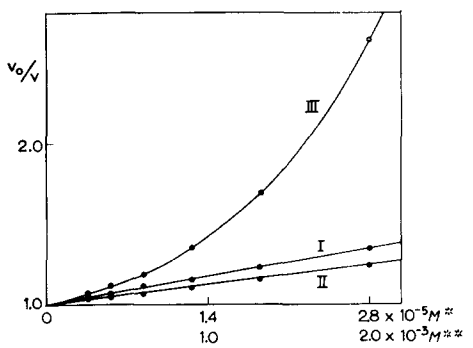

Fig. 1. Inhibitory action of FMS and AMS on D-amino acid oxidase in competition with FAD. The reaction mixture contained 15 μg of the oxidase protein, an excess of DL-alanine (0.15 M, final concentration), a restricted amount of FAD ($1.7 \cdot 10^{-7}$ M, final concentration) and graduated concentrations of inhibitors.

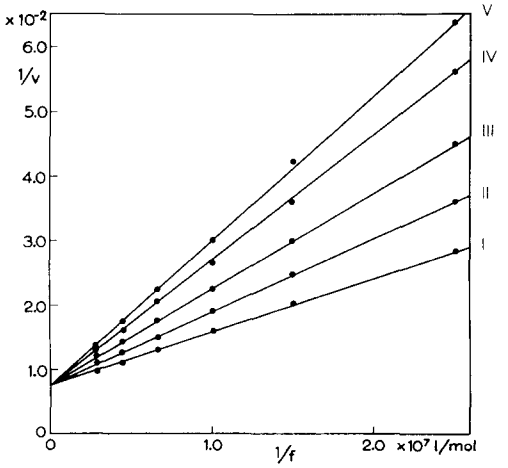

Fig. 2. Inhibitory action of FMS, AMS, PABA and PCMB on D-amino acid oxidase in competition with FAD. The reaction mixture contained 15 μg of the oxidase protein, an excess of DL-alanine (0.15 M, final concentration), graduated concentrations of FAD as indicated in the figure, and a concentration of inhibitor as shown below. I: in the absence of inhibitor; II: in the presence of $1.25 \cdot 10^{-5}$ M (final concentration) of FMS; III: in the presence of $2.5 \cdot 10^{-3}$ M (final concentration) of AMS; IV: in the presence of $1.0 \cdot 10^{-2}$ M (final concentration) of PABA; V: in the presence of $2.8 \cdot 10^{-7}$ M (final concentration) of PCMB.

* Indicates the concentration of FMS, and ** that of AMS. I: in the presence of FMS; II: in the presence of AMS; III: in the presence of both FMS and AMS. V was 132 μl O_2 uptake/30 min and v_0 was 80 μl O_2 uptake/30 min.

This result indicates that the inhibition is of single mechanism involving FAD. Method I was applied and the plots of $1/v$ against $1/f$ gave a straight line with the same intercept as that obtained by the plots of the experiment without any inhibitor, as shown in Fig. 2. From these results, it may be concluded that both FMS and AMS compete with FAD. The dissociation constant of the complex of FMS and the apo-protein, and that of AMS and the apo-protein were calculated to be $3.2 \cdot 10^{-5}$ M and $3.1 \cdot 10^{-3}$ M, respectively, from the slope of Curve I and Curve II in Fig. 1, and Curve II and Curve III in Fig. 2.

The presence of both FMS and AMS causes the plots of v_0/v to be second-order curve, as shown by Curve III in Fig. 1. Curve III shows that FMS and AMS do not compete with each other, but with the FMN and AMP part of FAD, respectively. By substituting the values of v_0/v into eqn. (8), K_3 and K_4 were calculated to be $6.6 \cdot 10^{-4}$ M and $6.7 \cdot 10^{-6}$ M, respectively. These results indicate that a positive interaction occurred between the binding portion of the apo-protein with the FMN part of FAD and that of the apo-protein with the AMP part of FAD, so that the affinity of each binding ($1/K_4$, $1/K_3$) becomes five times larger than the original affinity ($1/K_1$, $1/K_2$).

Analysis of inhibition by PABA of the oxidase, using kinetic indicators

As reported previously[4], it was demonstrated by methods I and II that PABA inhibits the oxidase in competition with FAD. The results are shown in Fig. 2, Curve IV, and in Fig. 3, Curve I. From these curves K_{ip} was calculated to be $7.3 \cdot 10^{-3}$ M.

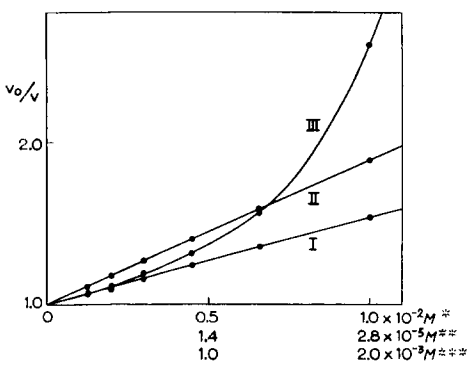

Fig. 3. Analysis of the inhibitory action of PABA on D-amino acid oxidase in competition with FAD, using kinetic indicators. The reaction mixture contained 15 μg of the oxidase protein, an excess of DL-alanine (0.15 M, final concentration), a restricted amount of FAD ($1.7 \cdot 10^{-7}$ M, final concentration) and graduated concentration of inhibitors.

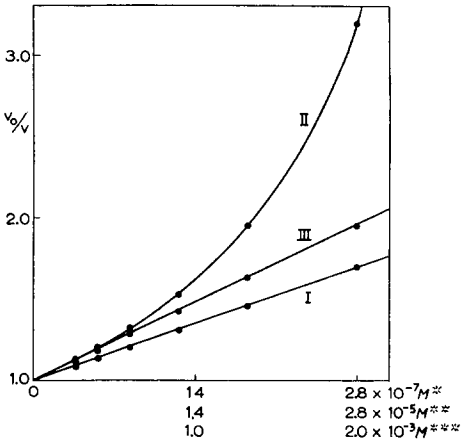

Fig. 4. Analysis of the inhibitory action of PCMB on D-amino acid oxidase in competition with FAD, using kinetic indicators. The reaction mixture contained 15 μg of the oxidase protein, an excess of DL-alanine (0.15 M, final concentration), a restricted amount of FAD ($1.7 \cdot 10^{-7}$ M, final concentration) and graduated concentration of inhibitors.

* Indicates the concentration of PABA, ** that of FMS, and *** that of AMS. I: in the presence of PABA; II: in the presence of both PABA and FMS; III: in the presence of both PABA and AMS. V was 132 μl O_2 uptake/30 min and v_0 was 80 μl O_2 uptake/30 min.

* Indicates the concentration of PCMB, ** that of FMS, and *** that of AMS. I: in the presence of PCMB; II: in the presence of both PCMB and FMS; III: in the presence of both PCMB and AMS. V was 132 μl O_2 uptake/30 min and v_0 was 80 μl O_2 uptake/30 min.

In the presence of FMS and PABA, plots of v_0/v are on a straight line, as shown in Fig. 3, Curve II, which indicates that FMS and PABA compete with each other. On the other hand, plots of v_0/v in the presence of AMS and PABA are on a second-order curve, as shown in Fig. 3, Curve III, which indicates that AMS and PABA do not compete with each other. From these results, it is concluded that PABA competes with the FMN part of FAD.

Analysis of inhibition by PCMB of the oxidase, using kinetic indicators

It was observed through spectrophotometry and fluorimetry that PCMB does not form a complex with FAD. Method I in the presence of $2.8 \cdot 10^{-7}$ M of PCMB, a rate-limiting concentration of FAD, and excess DL-alanine (0.15 M in its final concn.) gave a straight line with the intercept $1/V$ as shown in Fig. 2, Curve V. From the slope of this line, K_{ip} was calculated to be $1.6 \cdot 10^{-7}$ M. Plots of v_0/v against concn. of PCMB (Method II) were on a straight line with the intercept 1, as shown in Fig. 4, Curve I. The value of K_{ip} obtained from the slope of this line agreed with that mentioned above.

Plots of v_0/v against concns. of PCMB and FMS gave a second-order curve as shown in Fig. 4, Curve II, whereas in the presence of PCMB and AMS they gave a straight line as shown in Fig. 4, Curve III.

From these results, it may be concluded that PCMB combines with the apo-protein in competition with the AMP part of FAD.

DISCUSSION

It was demonstrated that both FMS and AMS compete with FAD, but not with each other. In view of their chemical structure, it is reasonable to expect that FMS and AMS would compete specifically with the FMN and AMP part of FAD, respectively, and they can be adopted as the specific kinetic indicators as already mentioned.

In a previous report from this laboratory[4], it was demonstrated that PABA competes with both FAD and the substrate for D-amino oxidase protein, and that phenylamino and phenylcarboxyl groups are responsible for competitive inhibition with FAD and with the substrate, respectively. Since a large excess of the substrate was present in the reaction mixture used in the present experiments, PABA competed only with FAD and this action is attributed to its phenylamino group.

As to the behaviour of the inhibitory action of PABA, kinetic analysis using indicators demonstrated that the inhibitor competes with the FMN part of FAD. In view of the chemical structure of FMN, it was assumed that the competition occurred between the phenylamino group of PABA and the 3-NH group of the isoalloxazine ring.

The inhibitory action of PCMB was also examined and it was found that PCMB competes with FAD to combine with the apo-protein, and that it competes with the AMP part of FAD.

Concerning the mechanism of inhibitory action of PCMB, SINGER AND BARRON[7] reported competitive inhibition of PCMB with the substrate and they suggested the combination of the protein-sulfhydryl group with the substrate.

However, the previous paper from this laboratory[4] demonstrated that the inhibitory action of benzoate or its derivatives in competition with the substrate

can be attributed to their phenylcarboxyl group and that this type of inhibition is independent of other radicals in the benzene ring. Therefore, the inhibitory action of the phenylcarboxyl group of PCMB in competition with the substrate is thought to be involved in the inhibitory action of PCMB observed by SINGER AND BARRON[7]. On the other hand, FRISELL AND HELLERMAN[9] reported that the inhibitory action of phenylmercuric acetate is not in competition with the substrate. From these results, it may be considered that the competitive inhibition of PCMB with the substrate may be attributed to the phenylcarboxyl group of the inhibitor.

As to the competition of PCMB with FAD, it may be attributed to its sulfhydryl-reactive group, since it was made clear that the phenylcarboxyl group does not take part in the competition with FAD and that the competition of PCMB with FAD was found to be of a single mechanism. Moreover, the competition of PCMB was attributed to its competition with the AMP part of FAD as mentioned before.

Thus, the conclusion deduced from the above considerations that the sulfhydryl group of D-amino acid oxidase apo-protein combines with the AMP part of FAD may solve the confusion[7–10] regarding the binding site of the sulfhydryl group of the apo-protein.

From these results, it should also be noticed that the binding between FAD and the apo-protein of D-amino acid oxidase occurs at least in two portions.

Furthermore, it was noticed that there is an important interaction between the binding sites of the apo-protein with FMS and AMS. The present results indicated that the attachment of AMS to the apo-protein increases the affinity of the apo-protein for FMS, and that the binding of FMS increases the affinity of the apo-protein for AMS. These positive interactions were also observed between the binding site of FMS and that of PCMB, and the binding site of AMS and that of PABA.

These demonstrations of positive interaction are of special interest in the study of the combination of the apo-protein with FAD, and consequently, of the mechanism of action of this enzyme.

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