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REACTIVITY OF D-AMINO ACID OXIDASE WITH ARTIFICIAL ELECTRON ACCEPTORS

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SUMMARY

1. The fully reduced form of D-amino acid oxidase [D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3] reacted with a number of electron acceptors and the order of reactivity was: benzoquinone > phenazine methosulfate (PMS) > oxygen > 2,6-dichlorophenolindophenol (DCIP) > methylene blue > ferricyanide. The stoichiometric reaction between the fully reduced enzyme and PMS was established.

2. A mechanism for the catalytic oxidation of D-arginine by the enzyme using PMS as electron acceptor involved the fully reduced enzyme which was reoxidized by PMS.

3. When D-alanine was used as substrate, PMS, ferricyanide, DCIP and methylene blue served as electron acceptor. Analyzing the kinetic data of the catalytic oxidation of D-alanine as well as D-α-aminobutyric acid when PMS was used as acceptor, it was revealed that the purple intermediate formed through the reaction of the enzyme with the substrate was involved in the mechanism of the oxidation. The rate of reaction of PMS with the intermediate was fairly small compared with that of PMS with the fully reduced enzyme.

4. PMS reacted stoichiometrically with the semiquinoid form of the enzyme at a faster rate than with the fully reduced enzyme.

INTRODUCTION

The fully reduced flavins are reoxidized rapidly by a large number of compounds. Flavin enzymes, on the other hand, exhibit remarkable specificity in their interaction with the electron acceptors. It has often been observed that the very rapid reaction of free flavins is considerably hindered by their binding to apoproteins.

Abbreviations: PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol.

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Attention has recently been drawn to this aspect of flavoprotein-acceptor specificity[1-3].

The reaction of D-amino acid oxidase [D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3] has been extensively investigated using oxygen as acceptor. However, little information is available on the reaction of this enzyme with artificial acceptors. During the catalytic oxidation of neutral amino acids such as D-alanine, a purple intermediate[4-8], assigned to a strong charge transfer complex ("inner complex") of the flavin and substrate moieties[7,8], has been observed. This intermediate reacts rapidly with oxygen to produce the oxidized enzyme, and can be distinguished from either the fully reduced or the semiquinoid enzyme. The fully reduced enzyme occurs in the catalytic cycle and reacts with oxygen, when basic amino acids such as D-arginine are used as substrates[9]. The occurrence of these two different types of intermediates, the purple intermediate and the fully reduced form, in the reaction sequence of the same enzyme depending on the type of substrate used, should be taken into account to study the specificity of acceptors in the reoxidation of the enzyme. In this paper, we report the oxidation of the fully reduced enzyme and the purple intermediate by artificial electron acceptors. The reactivity of the semiquinoid enzyme is also reported, even though the semiquinoid enzyme is not involved in the reaction sequence of this enzyme.

MATERIALS AND METHODS

Enzyme

The holoenzyme of D-amino acid oxidase was prepared according to the method of Yagi *et al.*[10]. The concentration of the enzyme was expressed in terms of the enzyme-bound FAD.

Chemicals

Phenazine methosulfate (PMS), methylene blue, 2,6-dichlorophenolindophenol (DCIP), benzoquinone, potassium ferricyanide, D-alanine, D-α-aminobutyric acid, DL-arginine and all other chemicals of analytical grade were obtained from Nakarai Chemicals, Kyoto. L-Arginine does not have any inhibitory effect on the enzyme (unpublished result from this laboratory).

Apparatus used for absorbance measurement

Absorbance was measured in a Beckman DK-2A spectrophotometer or in a Shimadzu QV-50 spectrophotometer. The transmittance change caused by mixing equal volumes of the two reactant solutions was measured using a Yanaco SPS-1 stopped-flow spectrophotometer. The details of this apparatus were described elsewhere[11]. The light-path of the observation chamber was either 2 mm or 10 mm.

Assay for the reaction of the fully reduced enzyme with acceptors

The fully reduced enzyme was prepared by vigorously bubbling argon gas for 10 min through 10 ml of sodium pyrophosphate buffer, pH 8.3, containing 150 μM D-arginine, and by adding 0.5 ml of D-amino acid oxidase. The final enzyme concentration of the solution was approximately 20 μM. This addition introduced a small amount of dissolved oxygen into the system which was consumed by the oxidation of the excess substrate present. The production of the fully reduced enzyme was

indicated by the bleaching of the yellow color of the enzyme. The solution of the acceptor was made anaerobic by vigorously bubbling argon gas. To maintain anaerobiosis during the experiment, the gas phase of the reservoir syringes of the stopped-flow apparatus was flushed continuously with argon gas. The argon gas used in all the experiments was passed through an alkaline pyrogallol solution. After mixing equal volumes of the fully reduced enzyme and acceptor, the reactions were followed by measuring the transmittance change at 455 nm (*i.e.* measuring the reoxidation of the reduced enzyme) for all the acceptors except DCIP. The transmittance change at 600 nm was measured when DCIP was used.

In the experiment for determining the effect of pH on the reaction of the fully reduced enzyme with PMS or oxygen, the fully reduced enzyme prepared in 20 mM Na_2HPO_4 or 20 mM $NaHCO_3$ was mixed with an equal volume of a solution of above salts of different pH values, containing PMS or oxygen. The pH value of the reaction mixture was determined by mixing separately equal volumes of the two solutions.

Assay for the reaction of $FMNH_2$ with PMS

$FMNH_2$ solution was prepared by irradiating FMN with visible light in anaerobic sodium pyrophosphate buffer (17 mM, pH 8.3), containing 1.0 mM EDTA[12]. The reaction of $FMNH_2$ with PMS was monitored in a stopped-flow apparatus by measuring the transmittance change at 450 nm.

Demonstration of the reaction of the purple intermediate with PMS

For the demonstration of the reaction of the purple intermediate with PMS, the reaction of the enzyme with D-alanine was carried out in the absence and presence of PMS under aerobic conditions. The appearance of the intermediate was followed by measuring the increase in absorbance at 550 nm and the reduction of PMS by the decrease in absorbance at 388 nm in the stopped-flow apparatus.

Determination of the rate constant for the conversion of the purple intermediate to the fully reduced form

It was determined by measuring the decrease in absorbance at 550 nm on anaerobic mixing of the enzyme with the substrate, D-alanine or D-α-aminobutyric acid. The final concentrations of the substrates and enzyme were 2.5 mM and 8.1 μM, respectively.

Assay for the catalytic oxidation of substrates using artificial electron acceptors

In the assay of the catalytic oxidation of substrates using acceptors, anaerobic solutions of D-amino acid oxidase *plus* 10 μM FAD were mixed with anaerobic solutions of D-arginine, D-alanine or D-α-aminobutyric acid containing different concentrations of acceptors. The transmittance change was recorded at appropriate wavelength for each acceptor. The following molar extinction coefficients[2] for the acceptors were used: PMS, 22 000 $M^{-1} \cdot cm^{-1}$ for the oxidized form at 388 nm and 1500 $M^{-1} \cdot cm^{-1}$ for the reduced form at 388 nm; DCIP, 21 000 $M^{-1} \cdot cm^{-1}$ at 600 nm; ferricyanide, 1020 $M^{-1} \cdot cm^{-1}$ at 420 nm; and methylene blue, 41 000 $M^{-1} \cdot cm^{-1}$ at 610 nm.

Demonstration of the reaction of the semiquinoid enzyme with acceptors

The semiquinoid form of the enzyme was obtained by irradiating with visible light an anaerobic solution of the oxidized enzyme in 17 mM pyrophosphate buffer, pH 8.3, containing 50 mM EDTA in a Thunberg tube, until the solution turned red in color[13]. This solution was transferred anaerobically into the stopped-flow apparatus, and was irradiated further in the driving syringe to obtain maximum amount of the semiquinoid enzyme. The semiquinoid enzyme was mixed with an anaerobic solution of PMS, and the transmittance change at 530 nm was measured.

The semiquinoid enzyme was similarly prepared in a 3 ml anaerobic cuvette. A small volume (3 μl) of PMS solution was introduced into the cuvette with a microsyringe. The spectra were recorded in a Beckman DK-2A spectrophotometer.

Unless otherwise stated, all assays were performed in 17 mM sodium pyrophosphate buffer, pH 8.3 and at 20 °C. During all these experiments, extreme care was taken to avoid exposure to light.

RESULTS

Reactivity of the fully reduced D-*amino acid oxidase*

Rates of reaction with artificial electron acceptors

The time course of the reaction of the fully reduced enzyme with all the acceptors followed essentially pseudo-first-order kinetics. The pseudo-first-order rate constant was calculated by plotting the logarithm of the absorbance change against time. The rate constants at different concentrations of acceptors are given in Table I.

TABLE I

PSEUDO-FIRST-ORDER RATE CONSTANT FOR THE REACTION OF THE FULLY REDUCED D-AMINO ACID OXIDASE WITH ACCEPTORS

A solution of the fully reduced enzyme (18.6 μM) prepared as described in Materials and Methods, was mixed with an equal volume of anaerobic solution of acceptor in the stopped-flow apparatus. Final concentrations of acceptors are indicated in the table. The reaction traces of the transmittance change at 455 nm, in the case of all acceptors except for DCIP, were recorded. The transmittance change at 600 nm was recorded for DCIP. From these traces, pseudo-first-order rate constants were calculated. The reactions were carried out in 17 mM sodium pyrophosphate buffer pH 8.3, and at 20 °C.

Acceptor	*Concn (μM)*	*Redox potential* E'_0 (V)*	k_{obs} (s^{-1})
Benzoquinone	60	+0.24	25
	120		51
	200		73
PMS	115	+0.08	8.0
	450		32
DCIP	500	+0.16	2.5
	1000		4.4
Methylene blue	100	0	0.2
Ferricyanide	1100	+0.43	0.24
	2200		0.71
	3300		0.99
Oxygen**	140	+0.8	2.7
	710		12

* From ref. 3.

** From unpublished results of Yagi, Nishikimi, Ohishi and Takai.

The order of reactivity was: benzoquinone > PMS > oxygen > DCIP > methylene blue > ferricyanide. It is clear that no positive correlation exists between the redox potentials of these acceptors and the reactivity of the reduced enzyme. In the case of ferricyanide, the transient and small absorbance increase at 530 nm was observed, indicating the occurrence of the semiquinoid enzyme. This form of the enzyme absorbs light at the longer wavelengths[13,14] (see Fig. 7, Curve II).

The reactivity with acceptors of the fully reduced enzyme was compared with that of the fully reduced flavin. The reaction of $FMNH_2$ (14 μM) and PMS (47 μM) was completed within the mixing time of the stopped-flow apparatus (2.5 ms). Therefore, it is noted that the reduced free flavin reacted with PMS more rapidly than the reduced enzyme-bound flavin.

Reaction of the fully reduced enzyme with PMS

Although benzoquinone was the most reactive of the acceptors tested, its instability at alkaline pH prevented us from examining the reaction with this acceptor in detail. On the other hand, PMS is stable at alkaline pH and is widely used to couple flavoprotein enzymes to other acceptors including oxygen. Hence, PMS was used to investigate in detail the mechanism of the reaction of artificial electron acceptor with this enzyme.

In order to establish stoichiometry of the reaction, the difference spectrum

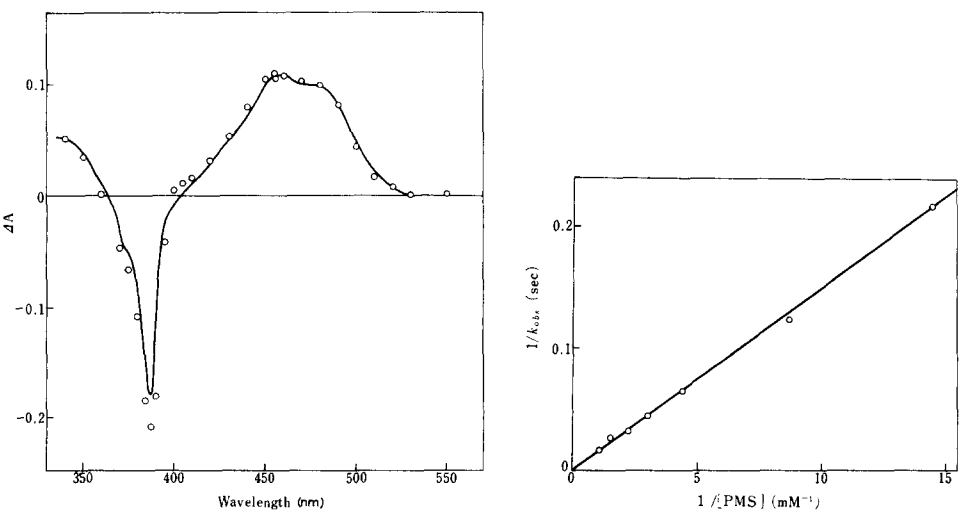

Fig. 1. Spectral change on mixing the fully reduced D-amino acid oxidase with PMS. A solution of the fully reduced enzyme (20 μM) in 17 mM sodium pyrophosphate buffer, pH 8.3, was mixed at 20 °C with an equal volume of anaerobic solution of PMS (160 μM) in the same buffer in the stopped-flow apparatus with a 2-mm cell. The transmittance change (between zero time and 1.5s) at wavelengths indicated in the figure was recorded on a storage oscilloscope. The absorbance change calculated was normalized to light path of 10 mm, and was plotted in the figure. The solid line represents the calculated difference spectrum obtained by assuming equimolar reaction of the fully reduced enzyme with PMS.

Fig. 2. Double-reciprocal plot for the reaction of the fully reduced D-amino acid oxidase with PMS. The pseudo-first-order rate constant was determined from the reaction trace at 455 nm obtained upon mixing the fully reduced enzyme (18.6 μM) with an equal volume of various concentrations of PMS in the stopped-flow apparatus. The reaction was carried out in 17 mM sodium pyrophosphate buffer, pH 8.3 and at 20 °C.

due to the reaction of the fully reduced enzyme (prepared by reducing the oxidized enzyme with excess D-arginine) with PMS was examined. The progress of the reaction monitored at 388 nm showed an initial rapid phase followed by a slow phase. Slow phase could be ascribed to the catalytic reduction of PMS by the substrate used in excess to produce the fully reduced enzyme. The rapid phase was investigated by measuring the difference spectrum between zero time and 1.5 s (Fig. 1). The solid line indicates the change calculated assuming that the oxidation of the fully reduced enzyme and the reduction of PMS occur in equimolar amounts. It can be seen from the figure that the calculated and the observed difference spectra are similar, indicating stoichiometric reaction between the fully reduced enzyme and PMS. The time course of absorbance change at all wavelengths was essentially similar and followed first-order kinetics.

When the reciprocal of the pseudo-first-order rate constant was plotted against the reciprocal of PMS concentration, a straight line passing through the origin was obtained (Fig. 2). This indicated that the reaction was second-order. From the plot, the second-order rate constant was estimated to be $6.7 \cdot 10^4$ $M^{-1} \cdot s^{-1}$.

The pH dependence of the pseudo-first-order rate constant for the reaction with PMS or oxygen is shown in Table II. From the table, it is evident that the rate constant with both the acceptors is independent of pH in the range from 7.3 to 10.1.

TABLE II

EFFECT OF pH ON THE RATE OF REACTION OF THE FULLY REDUCED D-AMINO ACID OXIDASE WITH PMS OR OXYGEN

A solution of the fully reduced enzyme (18.6 μM) was mixed with an equal volume of anaerobic solution of PMS (280 μM) or air-saturated buffer. Sodium phosphate buffer (20 mM) was used to obtain pH 7.3 and 7.9. Sodium carbonate buffer (20 mM) was used to obtain pH 8.4, 8.8 and 10.1. The reaction was carried out at 20 °C.

pH	*Pseudo-first-order rate constant* k_{obs} (s^{-1})	
	PMS	*Oxygen*
7.3	10	2.3
7.9	10	2.4
8.4	10	2.3
8.8	10	2.3
10.1	11	2.1

Catalytic oxidation of D-*arginine using PMS as electron acceptor*

When the reciprocal of the velocity of the overall reaction (e/v) measured using PMS as acceptor was plotted against the reciprocal of the D-arginine concentration ($1/[S]$) at fixed concentrations of PMS, a series of parallel straight lines were obtained (Fig. 3A). From the intercepts of the lines on the ordinate, the apparent maximum velocities per mole of the enzyme-bound FAD (V_{app}/e) for D-arginine at known concentrations of PMS were obtained. When the reciprocal of V_{app}/e value was plotted against the reciprocal of PMS concentration, a straight line passing through the origin was obtained as indicated by broken line in Fig. 3B. Straight parallel lines were also obtained when e/v values were plotted against the reciprocal values of PMS concentrations at fixed D-arginine concentrations (Fig. 3B). Similarly, when the reciprocal

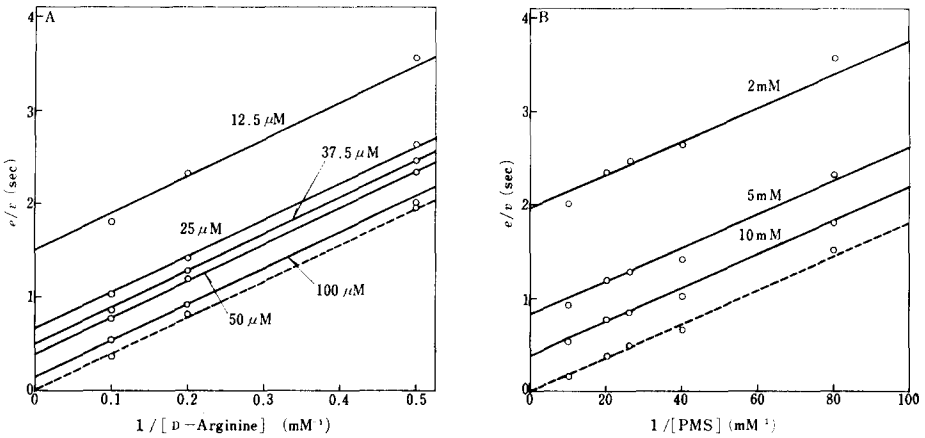

Fig. 3. Kinetics of the catalytic oxidation of D-arginine using PMS as electron acceptor. An anaerobic solution, containing the enzyme (1.74 μM), FAD (20 μM) and PMS was mixed with an equal volume of anaerobic solution of D-arginine in the stopped-flow apparatus. Final concentrations of PMS and D-arginine are indicated in A and B, respectively. The transmittance change at 388 nm was measured at 20 °C in 17 mM sodium pyrophosphate buffer, pH 8.3. The initial velocity was expressed in moles of PMS reduced per s per mole of the enzyme. The broken lines in A and B represent the reciprocal of maximum velocity (e/V_{app}) at infinite concentration of PMS and D-arginine, respectively (see the text).

of V_{app}/e value was plotted against the reciprocal of D-arginine concentration, a straight line passing through the origin was obtained as indicated by broken line in Fig. 3A.

The following equation was deduced from the data described above;

$$e/v = A/[S] + B/[\text{PMS}] \quad (1)$$

where A and B are constants. The values of $1/A$ and $1/B$ were calculated to be $2.5 \cdot 10^2$ $M^{-1} \cdot s^{-1}$ and $5.4 \cdot 10^4$ $M^{-1} \cdot s^{-1}$, respectively (Table III).

TABLE III

KINETIC PARAMETERS OBTAINED FROM THE OVERALL REACTION OF D-ARGININE OR D-α-AMINOBUTYRIC ACID

Substrate	$1/A$ $(M^{-1} \cdot s^{-1})$	$1/B$ $(M^{-1} \cdot s^{-1})$
D-Arginine	$2.5 \cdot 10^2$	$5.4 \cdot 10^4$
D-α-Aminobutyric acid	—*	$9.0 \cdot 10^2$

* As the lines in Fig. 6 were almost parallel to the abscissa, the slope could not be estimated.

Reactivity of the purple intermediate of D-*amino acid oxidase*

Anaerobic oxidation of D-*alanine by the enzyme using artificial electron acceptors*

It is well known that during the oxidation of D-alanine by D-amino acid oxidase a purple intermediate is produced rapidly and under anaerobic conditions it is slowly converted to the fully reduced enzyme[4,6,8]. It was, therefore, of interest to examine the reaction of artificial acceptors with the purple intermediate. Table IV summarizes

TABLE IV

RATES OF THE CATALYTIC OXIDATION OF D-ALANINE USING ARTIFICIAL ELECTRON ACCEPTORS

An anaerobic solution of D-amino acid oxidase (7.6 μM) was mixed with an equal volume of anaerobic solution of acceptor and D-alanine (50 mM). Final concentrations of acceptors are indicated in the table. The transmittance changes with time at the following wavelengths were measured, 600 nm for DCIP, 610 nm for methylene blue, 420 nm for ferricyanide and 388 nm for PMS. The initial rates (v/e) were expressed as moles of acceptor reduced per s per mole of the enzyme. The reactions were performed in 17 mM sodium pyrophosphate buffer, pH 8.3 and at 20 °C.

Acceptor	*Concn (μM)*	*v/e (s^{-1})*
DCIP	50	0.069
	250	0.12
	500	0.16
Methylene blue	50	0.018
Ferricyanide	50	0.21
	250	0.20
PMS	115	0.10

the rates of reaction with a number of electron acceptors when the enzyme was mixed with D-alanine under anaerobic conditions.

The rate of conversion of the purple intermediate to the fully reduced form was determined to be 0.012 s^{-1}. Since the values shown in Table IV were larger than 0.012 s^{-1}, it was considered that the acceptors are reacting with the purple intermediate. To study this reaction in detail, the experiments specified below were carried out using PMS as acceptor.

Demonstration of the reaction of the purple intermediate with PMS

When D-alanine was mixed with the enzyme under aerobic conditions, the purple intermediate was formed after consumption of the dissolved oxygen in the reaction medium. The intermediate was gradually converted to the fully reduced

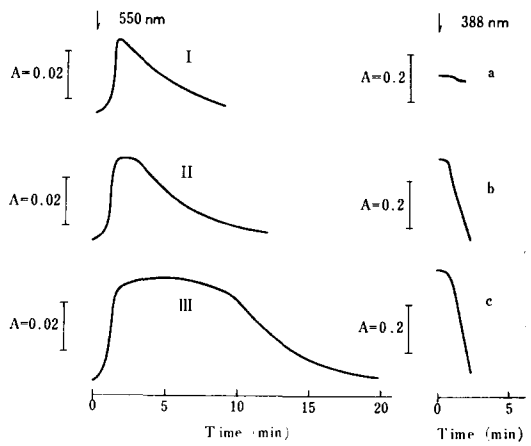

Fig. 4. Effect of PMS on the accumulation of the purple intermediate. An aerobic solution of D-amino acid oxidase (18.6 μM) was mixed with an equal volume of aerobic solution of D-alanine (1.0 mM) containing no (I, a), 42 μM (II, b) or 200 μM (III, c) PMS in the stopped-flow apparatus. The reaction was carried out in 17 mM sodium pyrophosphate buffer, pH 8.3 and at 20 °C using a 10-mm cell. The transmittance changes at 550 nm and 388 nm were measured. The absorbance changes with time are shown in the figure. The arrows indicate the time of mixing.

form as shown by Curve I in Fig. 4. This result is consistent with previous studies[5,6]. Upon addition of PMS to the reaction system, the steady-state occurrence of the purple intermediate was achieved. The time taken before the start of the disappearance of the intermediate increased with increasing amounts of PMS added (Fig. 4, Curves II and III). During the aerobic phase of the reaction, there was no decrease in the absorbance at 388 nm. The rapid decrease in the absorbance at 388 nm (Fig. 4, Curves b and c) was observed after maximum accumulation of the intermediate. The absorbance change due to the formation of the intermediate in the absence of PMS was minute (Fig. 4, Curve a). These observations indicate that the purple intermediate reacted with PMS and the enzyme was converted to the oxidized form and recycled until all the PMS molecules were reduced.

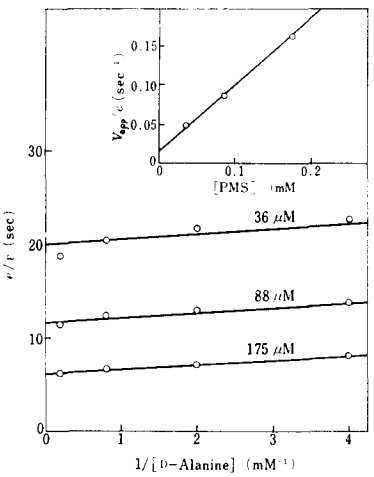

Fig. 5. Kinetics of the catalytic oxidation of D-alanine using PMS as electron acceptor. An anaerobic solution, containing the enzyme (8.2 μM), FAD (20 μM) and PMS was mixed with an equal volume of anaerobic solution of D-alanine in the stopped-flow apparatus, as described in Fig. 3. Final concentrations of PMS are indicated in the figure. The insert shows the plot of apparent maximum velocity calculated from the figure against the concentration of PMS.

Catalytic oxidation of D-*alanine using PMS as electron acceptor*

In order to establish the above interpretation, the anaerobic reaction of D-alanine in the presence of PMS was investigated in detail. The effect of increasing concentration of D-alanine on the rate of reaction at different fixed concentrations of PMS is shown in Fig. 5. A set of parallel lines were obtained, when the reciprocal of the initial velocity per mole of enzyme (v/e) at fixed concentrations of PMS was plotted against the reciprocal of D-alanine concentration. It was evident that the rate of reaction increased with increasing concentrations of PMS (36–175 μM). The value of the apparent maximum velocity, V_{app}/e, varied from 0.05 to 0.16 s^{-1}.

Since the rate of conversion of the purple intermediate to the fully reduced form (0.012 s^{-1}) is comparable to the reaction rate observed with D-alanine at low concentrations of PMS, it seems that depending on the concentration, PMS might interact with both the purple intermediate and the fully reduced form of the enzyme.

Catalytic oxidation of D-α-*aminobutyric acid using PMS as electron acceptor*

Yagi *et al.*[8] demonstrated that when D-α-aminobutyric acid was used as sub-

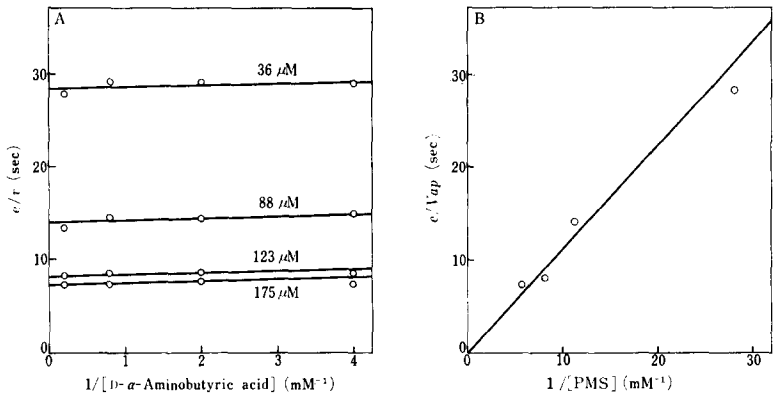

Fig. 6. Kinetics of the catalytic oxidation of D-α-aminobutyric acid using PMS as electron acceptor. An anaerobic solution, containing the enzyme (8.2 μM), FAD (20 μM) and PMS was mixed with an equal volume of anaerobic solution of D-α-aminobutyric acid in the stopped-flow apparatus, as described in Fig. 3. Concentrations of D-α-aminobutyric acid were varied at different fixed concentrations of PMS (A). The reciprocal of apparent maximum velocity per mole of the enzyme-bound FAD (e/V_{app}) obtained from Fig. 6A was plotted against the reciprocal of PMS concentration (B).

strate, the purple intermediate produced was very slowly converted to the fully reduced form. The rate of the reaction was determined to be 0.0017 s^{-1}. This value was considerably smaller than that determined for the reaction using D-alanine (0.012 s^{-1}). Therefore, the reactivity of PMS with the purple intermediate seemed to be more easily determined with D-α-aminobutyric acid than with D-alanine. With D-α-aminobutyric acid, it was found that the reaction was almost independent of substrate concentration and a series of parallel lines at different concentrations of PMS were obtained (Fig. 6A). When the intercept, e/V_{app} was plotted against 1/[PMS], a straight line passing through the origin was obtained (Fig. 6B). The kinetics of the reaction can be explained by Eqn 1. The value of $1/A$ could not be evaluated precisely, since the slope was very small. The value of $1/B$ was calculated from Fig. 6B to be $9.0 \cdot 10^2$ $M^{-1} \cdot s^{-1}$. This value is 1/60 of that for the reaction with the fully reduced enzyme produced by D-arginine (Table III).

Reaction of the semiquinoid enzyme with PMS

Semiquinoid flavin intermediates are involved in the catalysis of some flavoproteins[15,16]. Although this type of intermediate is not involved in the catalysis of D-amino acid oxidase, it was considered valuable to establish the mode of interaction of the semiquinoid enzyme with PMS. When the semiquinoid enzyme (44 μM, Curve II in Fig. 7) was mixed with PMS (30 μM, Curve IV), the spectrum shown by Curve III was obtained. The spectrum indicates the oxidation of the semiquinoid enzyme. Subtraction of the spectrum of the oxidized enzyme (Curve I) from Curve III gave the summation of the spectra of the reduced form and remaining oxidized form of PMS. From these spectral changes, stoichiometry of 2 moles of the semiquinoid enzyme oxidized per mole of PMS reduced was calculated.

When the reaction was studied in the stopped-flow apparatus, the pseudo-first-order rate constants, at 52 and 130 μM of PMS, were determined to be 12 and

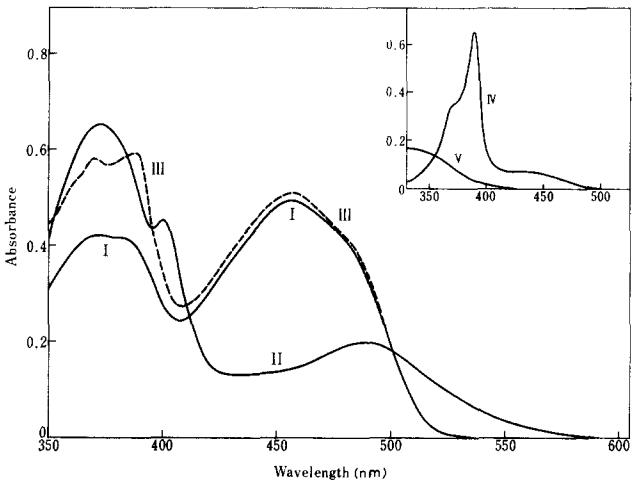

Fig. 7. Spectral change due to the oxidation of the semiquinoid enzyme by PMS. The semiquinoid enzyme was prepared by irradiating the enzyme (44 μM) in sodium pyrophosphate buffer (17 mM), pH 8.3, in the presence of 50 mM EDTA, under anaerobic conditions. PMS was added to a final concentration of 30 μM. The spectra were recorded in a Beckman DK-2A spectrophotometer. I, the spectrum of the oxidized enzyme; II, the spectrum of the semiquinoid enzyme; III, the spectrum obtained upon the addition of PMS to the semiquinoid enzyme; IV, the spectrum of PMS (30 μM); V, the spectrum of reduced PMS (30 μM) obtained by adding solid $NaBH_4$.

30 s^{-1}, respectively. The mean value of second-order rate constants calculated from these data was $2.3 \cdot 10^5$ $M^{-1} \cdot s^{-1}$. This value is greater than that for the reaction of the fully reduced enzyme with PMS.

DISCUSSION

The evidence for the reaction of flavin enzymes with a wide range of acceptors is fragmentary. It was reported that D-amino acid oxidase did not react with a number of acceptors[3,17]. The results presented in Table IV showed that, contrary to the observation of Dixon and Kleppe[17], the enzyme interacted with PMS and ferricyanide besides methylene blue and DCIP when D-alanine was used as substrate. When D-arginine was used as substrate, the enzyme reacted rapidly with the artificial electron acceptors (Table I). Examination of the order of reactivity of acceptors showed no correlation between the redox potential of the acceptor and the rate of reaction with the fully reduced enzyme.

The reoxidation of the fully reduced enzyme by PMS was stoichiometric and followed second-order kinetics with rate constant of $6.7 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at pH 8.3. The double reciprocal plot, shown in Fig. 2, gave a straight line passing through the origin. This indicated that the concentration of PMS-reduced enzyme complex, if formed, might always be very small during the time course of the reaction. The rate of the reaction was not affected by changing pH of the solution. It was also the case when oxygen was used as acceptor (Table II). These results indicated that ionization of the protein might have no significant role in this reaction in the pH range 7.3–10.1.

The earlier results[9] suggested that the reduction of the enzyme by D-arginine

obeyed second-order rate equation with $k_{red} = 3.1 \cdot 10^2\ M^{-1} \cdot s^{-1}$, permitting the following equation to be written.

$$E_{ox} + S \xrightarrow{k_{red}} E_{red} + P \tag{2}$$

The results discussed above allow us to write the equation for the reaction of the fully reduced enzyme with PMS.

$$E_{red} + PMS \xrightarrow{k_{ox}} E_{ox} + PMSH_2 \tag{3}$$

where E_{ox}, E_{red}, S and P are the oxidized enzyme, the fully reduced enzyme, D-arginine and the product, respectively. Applying the steady-state treatment, we obtain the following equation.

$$e/v = \frac{1}{k_{red}[S]} + \frac{1}{k_{ox}[PMS]} \tag{4}$$

Comparison of Eqns 1 and 4 shows that $1/A = k_{red}$ and $1/B = k_{ox}$. The values of $1/A$, $2.5 \cdot 10^2\ M^{-1} \cdot s^{-1}$ and k_{red}, $3.1 \cdot 10^2\ M^{-1} \cdot s^{-1}$; and $1/B$, $5.4 \cdot 10^4\ M^{-1} \cdot s^{-1}$ and k_{ox}, $6.7 \cdot 10^4\ M^{-1} \cdot s^{-1}$, are in reasonable agreement. The mechanism shown by Eqns 2 and 3 satisfactorily describes the oxidation of D-arginine by the enzyme when PMS is used as electron acceptor.

Taking into account the earlier reports[5,6] on the mechanism of D-amino acid oxidase, the following reaction sequence can be written for the oxidation of D-alanine or D-α-aminobutyric acid using PMS as electron acceptor.

$$E_{ox} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{ox} \cdot S \tag{5}$$

$$E_{ox} \cdot S \xrightarrow{k_2} E' \cdot S' \tag{6}$$

$$E' \cdot S' \xrightarrow{k_3} E_{red} + P \tag{7}$$

$$E' \cdot S' + PMS \xrightarrow{k_4} E_{ox} \cdot P + PMSH_2 \tag{8}$$

$$E_{ox} \cdot P \xrightarrow{k_5} E_{ox} + P \tag{9}$$

$$E_{red} + PMS \xrightarrow{k_6} E_{ox} + PMSH_2 \tag{10}$$

where $E_{ox} \cdot S$, $E' \cdot S'$ and $E_{ox} \cdot P$ are the oxidized enzyme–substrate complex, the purple intermediate and the oxidized enzyme–product complex, respectively. Using the steady-state approximation for the calculation of initial velocity of the reduction of PMS, the following rate equation is derived.

$$e/v = \frac{k_{-1} + k_2}{k_1 k_2} \cdot \frac{1}{[S]} + \frac{1}{(k_4[PMS] + k_3)} \left(1 + \frac{k_3}{k_6[PMS]} + \frac{k_4[PMS]}{k_5}\right) + \frac{1}{k_2} \tag{11}$$

For the reaction with D-α-aminobutyric acid, the rate of disappearance of the purple intermediate (Reaction 7) is very small ($k_3 = 0.0017\ s^{-1}$) compared to other reactions in the sequence, and can be neglected. Assuming that k_2 and k_5 are much larger than $k_4[PMS]$, a simplified equation, which is the sum of $1/[S]$ and $1/[PMS]$ terms, can be obtained.

$$e/v = \frac{k_{-1} + k_2}{k_1 k_2} \cdot \frac{1}{[S]} + \frac{1}{k_4[PMS]} \tag{12}$$

The plot of e/V_{app} *vs* $1/[PMS]$ gave a straight line passing through the origin, justifying the assumptions made above. The value of $1/B$ calculated from the plot, $9.0 \cdot 10^2$ $M^{-1} \cdot s^{-1}$, gives the value of k_4. The observation that the rate is not very much influenced by the substrate concentration can be explained by the fact that in Eqn 12 the second term is larger than the first term under the conditions investigated.

When D-alanine is used as substrate, rates of Reactions 7 and 8 can be similar as described in the Results. Substituting in Eqn 11 the following values for: $k_5 = 8.3$ s^{-1} obtained from ref. 6, $k_3 = 0.012$ s^{-1}, $k_6 = 6.7 \cdot 10^4$ M^{-1} s^{-1} both determined in this study, and assuming that the value of k_4 is similar to that obtained with D-α-aminobutyric acid, we find that in the second term of Eqn 11, the values of $k_3/k_6[PMS]$ and of $k_4[PMS]/k_5$ are far less than 1 at the concentrations of PMS investigated. Since Reaction 6 is very fast ($k_2 \simeq 10^3$ s^{-1} obtained from refs 6 and 8), the term $1/k_2$ in Eqn 11 can be neglected. Hence, Eqn 11 is simplified as

$$e/v = \frac{k_{-1} + k_2}{k_1 k_2} \cdot \frac{1}{[S]} + \frac{1}{k_4[PMS] + k_3} \tag{13}$$

At infinite concentration of substrate, Eqn 13 is reduced to

$$V_{app}/e = k_4 [PMS] + k_3 \tag{14}$$

where V_{app}/e represents the apparent maximum velocity per mole of the enzyme-bound FAD. When the values of V_{app}/e obtained from Fig. 5 are plotted against the concentrations of PMS, a straight line was obtained (insert of Fig. 5). From the slope, the value of k_4 is estimated to be $8.0 \cdot 10^2$ $M^{-1} \cdot s^{-1}$, and the value of the intercept is 0.015 s^{-1}, which reasonably agreed with the value of k_3 determined independently in this study. The value of k_4 is very similar to that obtained for the reaction with D-α-aminobutyric acid ($9.0 \cdot 10^2$ $M^{-1} \cdot s^{-1}$).

The investigation emphasizes the difference between the interaction of PMS with the purple intermediate and that with the fully reduced enzyme, and therefore the importance of an investigation of the reactivity of artificial electron acceptors with the enzyme intermediates produced during flavoprotein catalysis.

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