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# MECHANISM OF ENZYME ACTION

## VII. KINETIC ANALYSIS OF THE REACTION OF D-AMINO-ACID OXIDASE WITH D-ARGININE

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SUMMARY

1. D-Amino-acid oxidase [D-amino-acid:$O_2$ oxidoreductase (deaminating), EC 1.4.3.3], upon anaerobic mixing with D-arginine, was found to be converted from the oxidized to the fully reduced state, but the transient appearance of a purple intermediate, which is characteristic of the reaction with neutral amino acids, was not observed by stopped-flow spectrophotometry. The rate of reduction of the enzyme with D-arginine was directly proportional to the concentration of the amino acid, and the second-order rate constant was calculated to be 390 $M^{-1} \cdot s^{-1}$ at pH 8.3 and 20 °C. The rate was reduced to about one-fourth by substitution of the $\alpha$-H of the substrate for deuterium, indicating the rate-limiting removal of the $\alpha$-H of the substrate.

2. In the reaction of the fully reduced enzyme with $O_2$, no long-wavelength absorbing intermediate was observed. The second-order rate constant of the reaction was $1.9 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at pH 8.3 and 20 °C.

3. The kinetic analysis of the overall reaction of the enzyme-catalyzed oxidation of D-arginine, examined at various concentrations of the substrate and $O_2$, indicated the involvement of a first-order step in the reaction mechanism in addition to the reductive and oxidative steps.

## INTRODUCTION

The purple intermediate of D-amino acid oxidase [D-amino-acid:$O_2$ oxidoreductase (deaminating), EC 1.4.3.3], obtained in crystalline form in this laboratory [1] is considered to be a "key complex" to elucidate the mechanism of action of this enzyme. In the study on this enzyme, neutral amino acids such as D-alanine were mostly used as substrate, and the investigation on the reaction with basic amino acids has been fragmentary. When basic amino acids such as D-arginine were used as substrate, the purple intermediate was not observed by a stopped-flow technique [2, 3]. The difference between the mode of reaction with neutral and basic amino acids is dealt with in the present study, since this difference was thought to aid the understanding of the purple intermediate as well as that of the basal part of the reaction mechanism of this enzyme.

## MATERIALS AND METHODS

DL-Arginine was purchased from Nakarai Chemicals, Kyoto, and D-arginine from Sigma, St. Louis. L-Arginine was found to have no inhibitory effect on the oxidation of D-alanine catalyzed by D-amino-acid oxidase and DL-arginine gave essentially the same reaction rate with D-arginine for the anaerobic reduction of the oxidized enzyme. Therefore, DL-arginine was used in the study on kinetic isotope effect. DL-[$\alpha$-$^2$H]Arginine was prepared by the reaction of DL-arginine with salicylaldehyde and $CuSO_4$ in $^2H_2O$ by applying the method described by Tamiya and Oshima [4]. The purity of this preparation was checked by paper chromatography and NMR, and it was found that the isotopic substitution of the $\alpha$-H of the amino acid for deuterium was almost complete. Other chemicals were of reagent grade.

D-Amino-acid oxidase was prepared by the method described by Yagi et al. [5]. The molar concentration of the enzyme was expressed in terms of the enzyme-bound FAD. Glucose oxidase was purchased from Boehringer, Mannheim. $O_2$ and its mixture with $N_2$ were the products of Takachiho Kagaku Kogyo, Tokyo.

To attain anaerobiosis for the reaction in a stopped-flow apparatus, 10 ml of 0.017 M pyrophosphate buffer, pH 8.3, containing glucose ($5 \cdot 10^{-3}$ M) was bubbled vigorously with Ar for 10 min, then 0.5 ml of a solution containing the enzyme and glucose oxidase was mixed. The final concentration of glucose oxidase was $5 \cdot 10^{-7}$ M. The solution was incubated over 20 min, during which time the traces of dissolved $O_2$ were consumed by the catalytic oxidation of glucose by glucose oxidase. The solution of DL-arginine was made anaerobic in the same way using the glucose–glucose oxidase system.

The fully reduced enzyme ($4.2 \cdot 10^{-5}$ M) was prepared by vigorously bubbling Ar for 10 min through 10 ml of pyrophosphate buffer, pH 8.3, containing $3 \cdot 10^{-4}$ M D-arginine, and by adding 0.5 ml of a solution of D-amino-acid oxidase. The enzyme was converted to the fully reduced state after the traces of dissolved $O_2$ in the enzyme solution were consumed by the catalytic oxidation of D-arginine.

Absorbance measurements were carried out with a Beckman DK-2A or a Shimadzu QV-50 spectrophotometer. The rapid reaction studies were performed using a Yanaco SPS-1 stopped-flow spectrophotometer. $O_2$ consumption was measured in a closed 3-ml cell by use of a Kyusui Kagaku Bioxygraph.

All measurements were carried out in 0.017 M pyrophosphate buffer, pH 8.3, at 20 °C.

## RESULTS

*Spectral change and kinetics of the reduction of* D-*amino-acid oxidase with* D-*arginine*

When D-amino-acid oxidase was mixed with an excess of D-arginine under anaerobic conditions, absorbance at 455 nm of the enzyme decreased in accord with the previous data [2, 3] (Fig. 1). The time course of the absorbance change is obviously biphasic; it consists of a rapid phase (a $\rightarrow$ b) and a slow phase (b $\rightarrow$ c). This biphasic change is analogous to that observed in the purple intermediate formation with neutral amino acids [6], and could be explained in terms of the presence of the dimeric and monomeric forms of the enzyme (see Discussion).

The changes in absorption spectrum at fixed time intervals, obtained from the

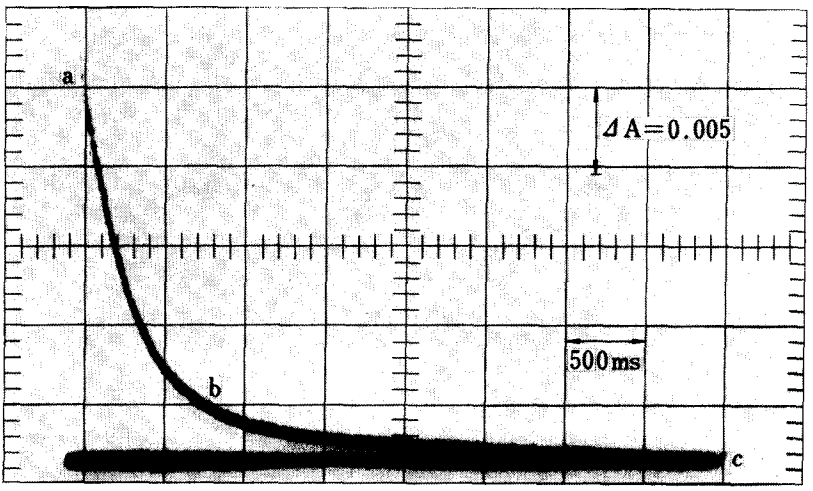


Fig. 1. Oscilloscope trace for the reaction of D-amino-acid oxidase with D-arginine under anaerobic conditions. Equal volumes of the anaerobic solutions of the enzyme ($4.6 \cdot 10^{-5}$ M) and of D-arginine ($1.8 \cdot 10^{-2}$ M) were mixed. The reaction at pH 8.3 at 20 °C was followed by decrease of the difference absorbance against the fully reduced enzyme at 455 nm with a stopped-flow spectrophotometer (light-path length, 2 mm). The solutions were made anaerobic by bubbling of Ar, followed by consuming the traces of $O_2$ by the addition of glucose oxidase ($5 \cdot 10^{-7}$ M) and glucose ($5 \cdot 10^{-3}$ M) (see text).

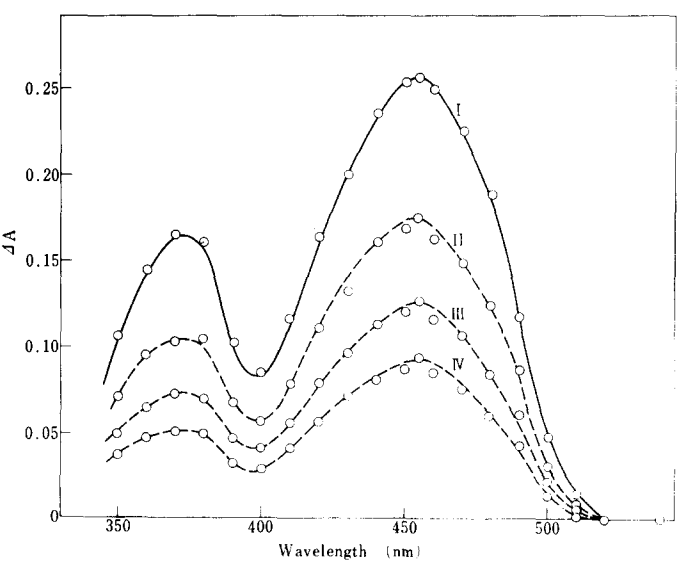


Fig. 2. Spectral change in the anaerobic reaction of D-amino-acid oxidase with D-arginine. The conditions were the same as those specified in Fig. 1, except that the final concentrations of the enzyme and D-arginine were $2.4 \cdot 10^{-5}$ M and $5 \cdot 10^{-3}$ M, respectively, and that the reaction traces were obtained at various wavelengths. The circles indicate the difference absorbance against the fully reduced enzyme obtained from the stopped-flow traces, which are normalized to the light-path length of 10 mm. The solid line indicates the difference spectrum of the oxidized enzyme against the fully reduced enzyme at the same enzyme concentration as that of the stopped-flow experiment. I, immediately after mixing; II, 100 ms after mixing; III, 200 ms after mixing; IV, 300 ms after mixing.

stopped-flow experiment, are shown in Fig. 2. The total absorbance changes developed between zero time and 10 s coincided with the difference spectrum of the oxidized enzyme against the fully reduced enzyme. It is clear from these results that in the reaction with D-arginine the enzyme is reduced to the fully reduced state without appearance of any long-wavelength absorbing intermediate. This feature differs from that of the reaction with neutral amino acids such as D-alanine, in which the purple intermediate appears prior to the formation of the fully reduced enzyme.

The rapid phase of the absorbance change at 455 nm in the reduction of the enzyme with D-arginine followed first-order kinetics. The pseudo first-order rate constants ($k_{obs}$) of the rapid phase of the reaction at various concentrations of D-arginine were obtained. The plot of the reciprocal of $k_{obs}$ versus the reciprocal of the concentration of D-arginine gave a straight line intersecting the ordinate at a point indistinguishable from the origin (Fig. 3, Curve I) in accord with the previous results [2, 3]. This indicates that the concentration of any complex, if formed, is marginal during the time course of the reduction of the enzyme with D-arginine. The second-order rate constant ($k_{red}$) calculated from Fig. 3, Curve I is 390 $M^{-1} \cdot s^{-1}$.

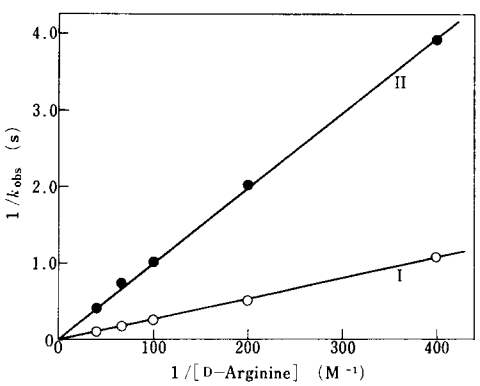


Fig. 3. Double reciprocal plot of the rate of reduction of the enzyme and D-arginine concentration. The reduction of the enzyme (final concentration, $8.8 \cdot 10^{-6}$ M) with D-[α-H]- or D-[α-$^2$H]arginine (concentrations indicated in the figure) under anaerobic conditions was followed by monitoring the absorbance change at 455 nm using a stopped-flow apparatus with 2-mm cell. From the reaction traces, pseudo first-order rate constants were obtained and their reciprocal values ($1/k_{obs}$) were plotted against the reciprocal values of the substrate concentration. Curve I, with DL-[α-H]arginine; Curve II, with DL-[α-$^2$H]arginine.

In order to see whether the reaction involves rate-limiting cleavage of the α-CH bond of D-arginine, the effect of substitution of the α-H of the amino acid for deuterium on the reduction of the enzyme was examined (Fig. 3, Curve II). The second-order rate constant was calculated to be 98 $M^{-1} \cdot s^{-1}$. This indicates that the substitution of the α-H of the substrate for deuterium reduces the rate to about one-fourth of the original.

*Spectral change and kinetics of the oxidation of the fully reduced* D-*amino-acid oxidase with* $O_2$

The above results suggest that the fully reduced enzyme is an intermediate in

the catalytic oxidation of D-arginine. Thus, the reaction of the fully reduced enzyme with $O_2$ was studied. When the fully reduced enzyme, produced by anaerobic reduction with a little excess of D-arginine, was mixed with the buffer containing $O_2$, it was converted into the oxidized enzyme. In this case, however, the reaction trace was essentially monophasic, differing from that of the reductive process. It followed first-order kinetics. The stopped-flow traces of the reaction at various wavelengths did not show any long-wavelength absorbing intermediate. The double reciprocal plot of the rate of the reaction ($k_{obs}$) and the concentration of $O_2$ gave a straight line which intersects the ordinate at a point indistinguishable from the origin (Fig. 4), indicating

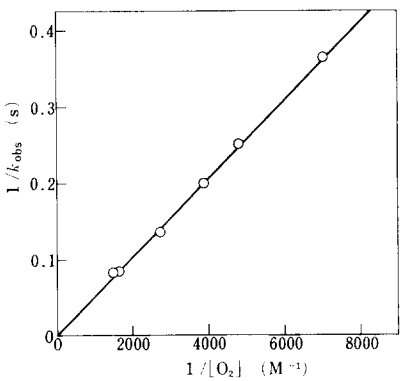


Fig. 4. Double reciprocal plot of the rate of oxidation of the fully reduced enzyme and $O_2$ concentration. A solution of the fully reduced enzyme (final concentration, $2.1 \cdot 10^{-5}$ M) was mixed with solution of $O_2$ (concentrations indicated in the figure) in a stopped-flow apparatus with 2-mm cell and the absorbance change at 455 nm was recorded. The concentration of $O_2$ was measured with a Kyusui Kagaku Bioxygraph. The reciprocal values of pseudo first-order rate constants obtained from the stopped-flow traces were plotted against the reciprocal values of $O_2$ concentration.

that the concentration of any species of complex, if formed, must be very low during the time course of the reaction. The second-order rate constant ($k_{ox}$) calculated from the plot was $1.9 \cdot 10^4$ $M^{-1} \cdot s^{-1}$. These observations indicate that the mechanism of the oxidation of D-arginine by D-amino-acid oxidase involves the fully reduced enzyme as an intermediate which reacts with $O_2$.

*The overall reaction*

To examine further the reaction mechanism, kinetic study of the overall reaction was carried out. Since D-amino-acid oxidase exists in monomer–dimer equilibrium [7–9] and the rate of the overall reaction differs between the monomer and dimer [10, 11], the reaction was examined at such a high enzyme concentration that the enzyme existed mainly in the dimeric form. $O_2$ consumption due to the catalytic oxidation of D-arginine was measured at different initial concentrations of $O_2$ and the substrate. When the reciprocal values of the velocity of $O_2$ consumption ($e/v$) were plotted against the reciprocal values of the D-arginine concentration (1/[S]) at fixed concentrations of $O_2$, a series of straight lines were obtained (Fig. 5A). They are essentially in parallel with each other. From the intercepts of the lines on the ordinate, the apparent maximum velocities per mole of the enzyme-bound FAD ($V_{app}/e$)

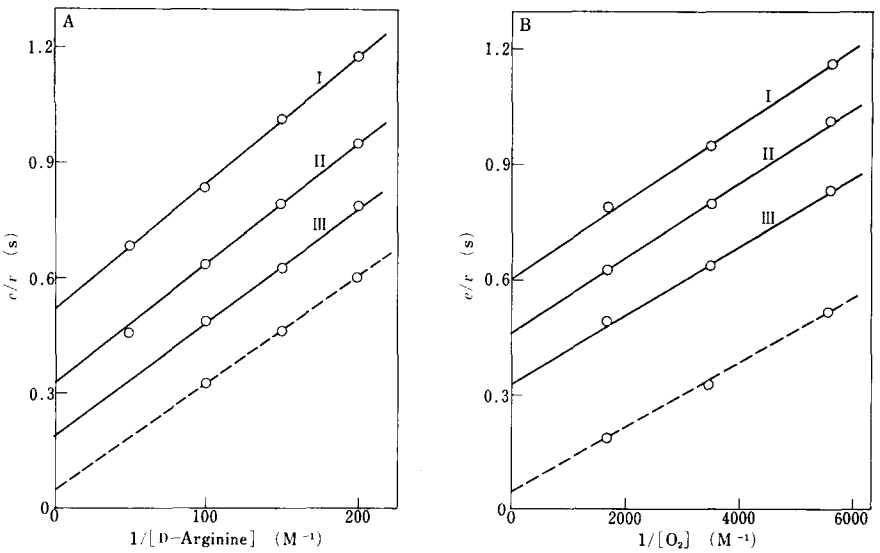


Fig. 5. Double reciprocal plot of the catalytic oxidation of D-arginine by D-amino-acid oxidase. $O_2$ consumption was measured at 20 °C with reaction mixtures containing the enzyme ($1.3 \cdot 10^{-6}$ M), FAD ($2 \cdot 10^{-5}$ M), D-arginine and $O_2$ in 0.017 M pyrophosphate buffer (pH 8.3). Final concentrations of D-arginine were $5.0 \cdot 10^{-3}$ M (B-I), $6.7 \cdot 10^{-3}$ M (B-II) and $1.0 \cdot 10^{-2}$ M (B-III), and those of $O_2$ were $1.80 \cdot 10^{-4}$ M (A-I), $2.84 \cdot 10^{-4}$ M (A-II) and $5.82 \cdot 10^{-4}$ M (A-III). $O_2$ consumption was recorded by use of a Kyusui Kagaku Bioxygraph with a closed 3-ml cell. The broken lines in A and B represent the reciprocal of the apparent maximum velocity ($e/V_{app}$) at the infinite concentration of $O_2$ and D-arginine, respectively.

for D-arginine at known concentrations of $O_2$ were obtained. The double reciprocal plot of $V_{app}/e$ and the $O_2$ concentration gave a straight line which intersects the ordinate at a finite value as indicated by the broken line in Fig. 5B, and the maximum velocity of 21 $s^{-1}$,* was obtained from the intercept of the figure. Straight lines were also obtained when the $e/v$ values were plotted against the reciprocal values of $O_2$ concentrations at fixed D-arginine concentrations (Fig. 5B). They are also essentially in parallel with each other. Similarly, when the reciprocal of $V_{app}/e$ value was plotted against the reciprocal of the D-arginine concentration, a straight line was obtained as indicated by the broken line in Fig. 5A. Therefore, the following equation could be deduced from the data described above:

$$e/v = A/[S] + B/[O_2] + C \qquad (1)$$

where $A$, $B$ and $C$ are constants. The values of $1/A$, $1/B$ and $1/C$ were calculated to be 385 $M^{-1} \cdot s^{-1}$, $1.3 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ and 21 $s^{-1}$, respectively. The value of $1/A$ is in good agreement with that of $k_{red}$, and the value of $1/B$ with that of $k_{ox}$.

* Since the double treatment of the kinetic data multiplies the experimental errors, this value obtained for $1/C$ should be regarded as one involving considerable deviations. Taking this situation into account, the previous result obtained with phenazine methosulfate [12] as electron acceptor can be interpreted to involve some finite value, even though the straight line apparently passed the origin.

## DISCUSSION

The present study by stopped-flow spectrophotometry confirmed that in the reaction of D-amino-acid oxidase with D-arginine, the transient appearance of a purple coloured intermediate was not observed. In this respect, the mode of reaction of this enzyme with D-arginine is different from that with neutral amino acids such as D-alanine. The kinetic analysis of the reduction of the enzyme with D-arginine provided no evidence of the formation of an enzyme–substrate complex. In addition, the oxidation of the fully reduced enzyme with $O_2$ was second-order reaction, providing no evidence of the complex formation between the two. However, the plot of $e/V_{app}$ versus $1/[O_2]$ of the overall reaction gave a straight line intersecting the ordinate at a finite value. This indicates the occurrence of a rate-limiting complex. Accordingly, Eqn 1, which is identical with that reported by Dalziel [13], $e/v_0 = \Phi_0 + \Phi_1/[S_1] + \Phi_2/[S_2]$, should be applied, and it is clear that the reaction of this enzyme with D-arginine and $O_2$ cannot be accounted for by the simple mechanism:

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

$$E_{red} + O_2 \rightarrow E_{ox} + H_2O_2 \tag{3}$$

since this mechanism would predict no limiting catalytic $V$. The following mechanism, essentially identical with that formulated by Dalziel [13], should be applied:

$$E_{ox} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{red}\cdot P \underset{k_1'}{\overset{k_2'}{\rightleftharpoons}} E_{red} + P \tag{4}$$

$$E_{red} + O_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{ox}\cdot H_2O_2 \underset{k_3'}{\overset{k_4'}{\rightleftharpoons}} E_{ox} + H_2O_2 \tag{5}$$

and the value $\Phi_0$ $(= C)$ equals to $1/V = 1/k_2' + 1/k_4'$. Setting either $k_2'$ or $k_4'$ equals to 21 $s^{-1}$ and the other to a much larger value would account for the observed rate-limiting velocity. Considering also the previously reported result with other substrate that the rate-limiting step in catalysis is the dissociation of product from the enzyme species [14], the overall rate might indeed be controlled by $k_2'$.

The above mechanism also predicts values for $\Phi_1$ $(= A)$ and $\Phi_2$ $(= B)$. From Dalziel's formulation [13],

$$\frac{1}{\Phi_1}\left(=\frac{1}{A}\right) = \frac{k_1k_2'}{k_2 + k_2'} \tag{6}$$

If $k_2$ is zero, which might indeed be the case if $k_1$ measures simply the reduction of the enzyme by D-arginine, then $1/\Phi_1$ is simplified to $k_1$ $(= k_{red})$ as found. Similarly

$$\frac{1}{\Phi_2}\left(=\frac{1}{B}\right) = \frac{k_3k_4'}{k_4 + k_4'} \tag{7}$$

Again assuming that $k_4$ is zero, $1/\Phi_2$ is simplified to $k_3$ $(= k_{ox})$ as found.

On the other hand, it should be noted that the kinetic isotope effect for the reduction of this enzyme by substitution of the $\alpha$-H of the substrate for deuterium

was observed. This shows that the reductive reaction involves a first-order reaction step in which the transfer of electrons from the substrate to the enzyme occurs, since the association of the enzyme with the substrate is not considered to be affected by the isotopic substitution. In this case, the concentration of the enzyme–substrate complex is considered to be minute, since the kinetics of the reductive process followed second-order reaction. Thus, the reaction sequence involving the enzyme (oxidized form)–substrate complex can be written:

$$E_{ox} + S \underset{k_-}{\overset{k_{+1}}{\rightleftharpoons}} E_{ox} \cdot S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E_{red} \cdot P \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E_{red} + P \tag{8}$$

where $E_{ox} \cdot S$ and $E_{red} \cdot P$ indicate the enzyme (oxidized form)–substrate complex and the enzyme (fully reduced form)–product complex, respectively. Since a long-wavelength absorbing intermediate like the purple intermediate observed with D-alanine cannot be observed in the reduction of the enzyme with D-arginine, $k_{+2}$ represents the rate of conversion of $E_{ox} \cdot S$ to the fully reduced state, and the observed kinetic isotope effect is thought to result from the decrease in $k_{+2}$. It is noted that the extent of the kinetic isotope effect on the reduction of the enzyme with D-arginine is comparable to that obtained for the formation of the purple intermediate with D-leucine [3, 15, 16].

The stopped-flow traces shown in Fig. 1 showed a biphasic change. This feature is similar to that observed previously with the reaction of the enzyme with neutral amino acids under anaerobic conditions [6]. In the formation of the purple intermediate with D-alanine, the major portion of the enzyme reacted rapidly and the minor portion reacted slowly, and the rapidly reacting form of the enzyme was assigned to the dimer and the slowly reacting form to the monomer. Since the enzyme concentration in the present study is similar to that in the previous study and the ratio of the slowly reacting species to the rapidly reacting species in the present study is also similar to that obtained with D-alanine, it is reasonable to consider that the major rapid change corresponds to the reaction of the dimeric enzyme and the minor slow change to the reaction of the monomeric enzyme. On the other hand, the trace of oxidative process was essentially monophasic, so the rate of the oxidative process of the monomeric enzyme is considered to be similar to that of the dimeric enzyme. In the previous report from this laboratory [11], the value of $v/e$ at the infinite concentration of D-arginine ($V_{app}/e$) has been found to be increased upon dilution of the enzyme. Since the value of $V_{app}/e$ is equal to $1/(B/[O_2] + C)$ and the $1/B$ value is considered to be the same for both the reactions with the monomeric and dimeric enzyme, the increase of $V_{app}/e$ with decreasing the enzyme concentration could be explained in terms of the decrease in $C$, viz. the increase in the rate of dissociation of the enzyme–product complex.

The mechanism of the reaction of D-amino-acid oxidase with D-arginine is similar to that of glucose oxidase reaction in that the fully reduced enzyme is the intermediate which reacts with $O_2$ and the reductive and oxidative reactions of the enzyme proceed without accumulation of appreciable amounts of an enzyme–substrate and an enzyme–$O_2$ complex, respectively [17]. The similarity of this enzyme to glucose oxidase implies a common feature in the reaction mechanisms of flavoenzymes.

The fact that the purple intermediate, which is characteristic of the reaction of

neutral amino acids such as D-alanine with this enzyme, is not observed with D-arginine and can be ascribed to either the possibility that the purple intermediate is not involved or the possibility [18] that the purple intermediate is involved but the rate of its disappearance is faster than the rate of its formation. In any case, the difference must be ascribed to the positively charged guanidino group of D-arginine. Taking into account the fact that the alkyl group of neutral amino acid interacts with some hydrophobic locus of the enzyme [19], the presence of a charged group at the $\omega$-position may render the substrate repulsive from its binding site of the enzyme, resulting in a possible instability of $E_{ox} \cdot S$. On the other hand, the positively charged group may facilitate the transfer of electron(s) from the substrate to the enzyme through the local electric field due to the charge. This interpretation comes from the theoretical consideration of Yomosa [20] who indicated that the degree of charge transfer in a donor–acceptor complex may be affected critically by the local electric field. If these two kinds of effects can be expected to be provoked by the presence of the positive charge at the $\omega$-position of the substrate, the mode of reaction of this enzyme with D-arginine becomes comprehensible.

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