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MECHANISM OF ENZYME ACTION. IV ISOLATION BY CRYSTALLIZATION OF THE FULLY REDUCED FORM OF D-AMINO ACID OXIDASE

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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), which was produced by anaerobic reduction of the enzyme with excess D-alanine, has been isolated by crystallization. The color of the crystals was pale yellow and their shape was a hexagonal prism or hexagonal prism with bipyramids.

2. The absorption spectra of the crystals and their mother liquor were essentially identical with the spectrum of the enzyme fully reduced with sodium dithionite. The crystals were practically diamagnetic The mother liquor showed a slight anomaly in optical rotatory dispersion, but it was far less than that found in the purple intermediate complex of the enzyme with the substrate

3. The crystals were found to be composed of equimolar amounts of the fully reduced enzyme and the intact substrate, D-alanine, indicating the exchange of the product for the intact substrate; a direct evidence for the turnover of substrate on the surface of the enzyme molecule

4. By mixing the fully reduced enzyme with excess pyruvate and ammonia, the purple intermediate complex was formed On eliminating the pyruvate in the purple intermediate solution by treatment with hydrogen peroxide, the purple color faded to pale yellow This indicates the reversible conversion between the purple complex and the fully reduced form

INTRODUCTION

In the previous paper[1], the purple intermediate complex of D-amino acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1 4 3 3) was crystallized, and it was characterized as a diamagnetic "inner complex" (ref. 2) between the enzyme and the substrate[3,4].

In the final step of the anaerobic reaction, however, the enzyme should be fully reduced by receiving two electrons from the substrate. The main purpose of the present

Abbreviations ORD, optical rotatory dispersion, ESR, electron spin resonance

study is, therefore, to characterize the fully reduced enzyme through its isolation by crystallization Another purpose is to elucidate the interrelationship between the intermediate and the fully reduced states. A part of this study has been preliminarily reported[5]

MATERIALS AND METHODS

The holoenzyme of D-amino acid oxidase was prepared according to the method of YAGI *et al.*[6]

D-Alanine, sodium benzoate and other chemicals were of reagent grade Protein content in the enzyme preparation was estimated by the usual biuret method[7].

For the analyses of the constituents of the crystals, a washing technique, which had been found to be available for the estimation of the constituents of some crystalline preparations[1,4,8], was adopted

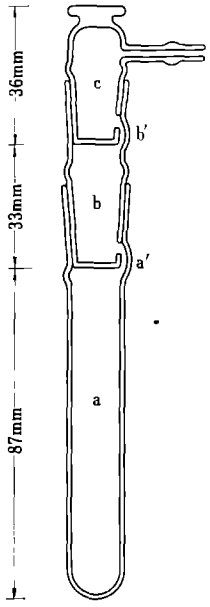

Fig 1 Apparatus for washing the crystals Tubes (a), (b) and (c) were made from Pyrex glass and they are connected by ground joints with each other The chambers (a), (b) and (c) can be interconnected through apertures (a') and (b')

To wash the crystals under anaerobic conditions, a special glass apparatus (Fig 1) was devised The crystalline suspension in tube (a) stopped with tube (b) was centrifuged with an angle centrifuge at 3000 × *g* for 10 min to separate crystals from the supernatant After the chamber (b) was evacuated by using stopcock (c), the supernatant in chamber (a) was sucked out into chamber (b) by opening a small aperture (a'). Then aperture (a') was closed, the supernatant transferred into chamber (b) was removed, and chamber (b) was washed with chilled pyrophosphate buffer ($1.67 \cdot 10^{-2}$ M, pH 8.3) three times A definite amount of the chilled buffer was put into chamber (b) and made oxygen-free by evacuating and flushing argon gas alterna-

tively with the aid of stopcock (c) The oxygen-free chilled buffer was then transferred into chamber (a) by opening aperture (a'), and the crystalline sediment was washed with the chilled buffer using a small magnetic stirrer The supernatant was removed as mentioned above The same procedure was repeated

The amounts of low molecular weight substances in each washing and in the solution of the crystals were analyzed after deproteinization with trichloroacetic acid The amount of D-alanine was estimated by the usual ninhydrin technique[9] The estimation of pyruvate was made colorimetrically by use of 2,4-dinitrophenylhydrazine[10]

Absorption spectra were recorded using a Beckman DK-2A spectrophotometer To measure the absorption spectrum of the crystal, an Olympus microspectrophotometer was used Measurements of optical rotatory dispersion (ORD) were made by use of a JASCO ORD/UV recorder Electron spin resonance (ESR) spectra were recorded using a JES 3B X-band ESR spectrometer of Japan Electron Optics Laboratory Co

All operations were carried out at room temperature. For the anaerobic experiments, a Thunberg-type cuvette was used throughout

RESULTS

1 *Isolation by crystallization of the fully reduced form of* D-*amino acid oxidase*

As it was known (*e.g.*, ref 11) that this enzyme could be fully reduced with an excess amount of its substrate, D-alanine, an attempt was made to crystallize the fully reduced form of the enzyme after the reduction with this substrate

Six ml of the enzyme solution ($6.7 \cdot 10^{-4}$ M with respect to FAD) were mixed with triturated D-alanine ($3 \cdot 10^{-2}$ M in final concentration) under anaerobic conditions The amino acid was quickly brought into the solution with gentle shaking. The yellow color of the solution changed rather rapidly into purple and then gradually turned to pale yellow, indicating the enzyme was fully reduced To this pale yellow solution, powdered ammonium sulfate was added to a final concentration of $2 \cdot 10^{-1}$ M and the resulting solution was stored at 5° under anaerobic conditions After standing over a week, a crop of pale yellow crystals appeared. Microscopic observation revealed that the shape of the crystals was a hexagonal prism (see Fig 2a), like the crystals of the purple intermediate[12] Sometimes, crystals having a hexagonal prism shape with

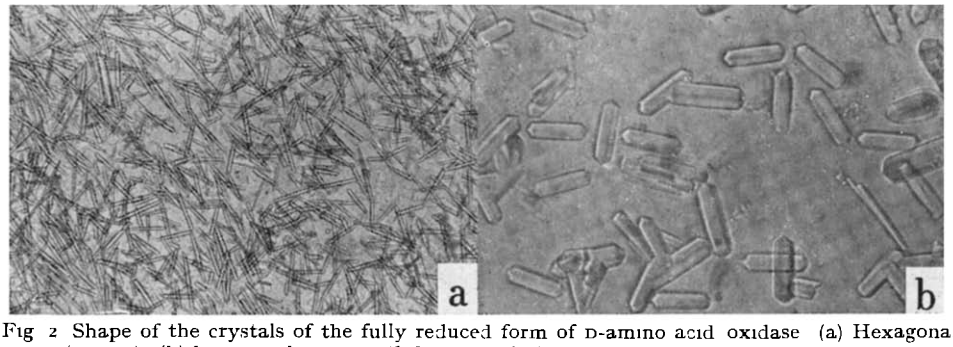

Fig 2 Shape of the crystals of the fully reduced form of D-amino acid oxidase (a) Hexagonal prism (× 200), (b) hexagonal prism with bipyramids (× 750)

bipyramids appeared as shown in Fig 2b. This shape was also observed when the crystallization of the purple intermediate had been achieved[1].

To analyze the constituents of the crystals, they were washed 5 times with 0 5 ml of chilled pyrophosphate buffer under anaerobic conditions, and the remaining crystals were dissolved in the same buffer After the deproteinization by adding trichloroacetic acid (5% final concentration), the low molecular weight substances liberated in the supernatant of each washing and of the solution of the crystals were determined The analytical results on D-alanine and pyruvate indicated that the molar ratio of alanine to FAD ([Ala]/[FAD]) in the washing drew nearer to unity with increasing numbers of washings, whereas that of pyruvate to FAD ([pyruvate]/[FAD]) fell much less than 0 1. In the solution of the washed crystals, equimolar amounts of D-alanine and FAD were found These results would indicate that the present crystalline preparation is a 1.1 complex between the fully reduced enzyme and the intact substrate, D-alanine

2. Physicochemical properties

Absorption spectra of the crystals and their mother liquor are shown by Curves I and II, respectively, in Fig. 3. No absorption peak was found in the visible range (400–600 mμ). These spectra are rather similar to the spectrum of the enzyme fully reduced with dithionite (Curve III in Fig 3), but different apparently from that of the oxidized, purple intermediate or half-reduced (semiquinoid) state of the enzyme (*cf* ref 3)

ESR measurements indicated that the crystalline sample gives no significant ESR signal

The ORD pattern of the mother liquor is shown by Curve I in Fig. 4 This pattern is essentially identical with that of the fully reduced enzyme reported by AKI *et al.*[13]

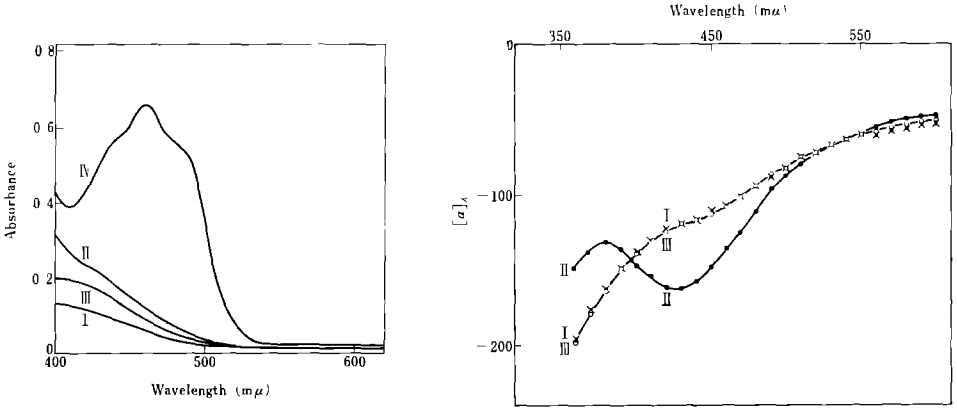

Fig 3. The absorption spectra of the fully reduced form of D-amino acid oxidase I, crystal of fully reduced form, II, mother liquor of I, III, the enzyme (7 0 10^{-5} M with respect to FAD) fully reduced with excess dithionite, IV, after the aeration of II A part of II was diluted to an appropriate concentration, and was bubbled with oxygen

Fig 4 The optical rotatory dispersion patterns of the fully reduced enzyme and its related state I, the enzyme ($1\ 7 \cdot 10^{-4}$ M with respect to FAD) fully reduced with D-alanine ($5\ 0 \cdot 10^{-2}$ M), II, I was mixed with lithium pyruvate ($1\ 0\ 10^{-1}$ M) and ammonium sulfate ($5\ 0\ 10^{-2}$ M), III, the enzyme ($1\ 7 \cdot 10^{-4}$ M with respect to FAD) fully reduced with sodium dithionite ($3\ 0 \cdot 10^{-3}$ M)

A slight anomaly found is probably due to the interaction between the apoenzyme and the fully reduced coenzyme, because the enzyme fully reduced with sodium dithionite, in which no enzyme–substrate interaction exists, showed the same ORD pattern (Curve III in Fig. 4)

3 Reactivity of the fully reduced enzyme

The fully reduced enzyme prepared here reacted with molecular oxygen. On introducing oxygen into the mother liquor, its pale yellow color turned, for a moment, to purple and soon returned to pale yellow Repeating this procedure, the color of the solution finally became deep yellow. The absorption spectrum of this yellow solution is shown by Curve IV in Fig. 3, which is identical with that of the enzyme–pyruvate complex[12] Similar changes were observed in the case of the crystals, indicating that the enzymatic oxidation takes place even in the crystals.

Although MASSEY AND GIBSON[14] reported that the purple intermediate of D-amino acid oxidase could be prepared by mixing the fully reduced enzyme (reduced with substrate) with excess amounts of pyruvate and ammonia, the conversion from the fully reduced form to the purple form was investigated in more detail to confirm the reversible reaction between the two

As shown in Figs 4 and 5, changes in the absorption spectrum and in ORD were noticeable when the enzyme fully reduced with D-alanine was mixed with excess triturated lithium pyruvate and ammonium sulfate A broad absorption band emerged in the vicinity of 550 mμ (Curve II, in Fig 5), which is obviously identical with that

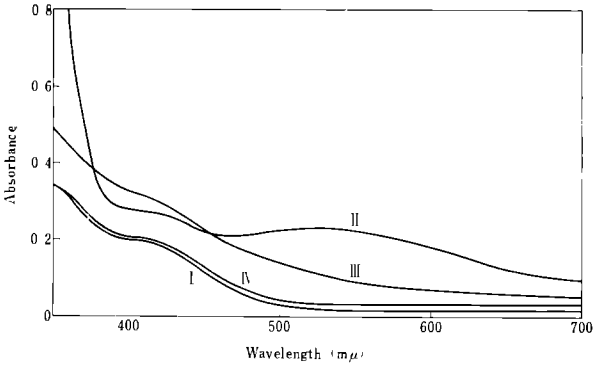

Fig 5 Reversibility of the conversion from the fully reduced state to the intermediate state I, the enzyme ($7.0 \cdot 10^{-5}$ M with respect to FAD) fully reduced with D-alanine ($5.0 \cdot 10^{-2}$ M), II, I was mixed with lithium pyruvate ($1.0 \cdot 10^{-1}$ M) and ammonium sulfate ($5.0 \cdot 10^{-2}$ M), III, II was then mixed with hydrogen peroxide ($1.0 \cdot 10^{-1}$ M), IV, I was mixed with sodium benzoate ($1.0 \cdot 10^{-1}$ M)

of the purple intermediate[3]. Moreover, an extrinsic negative Cotton effect appeared in the ORD spectrum having a trough at 430 mμ, an inflexion point close to 400 mμ and a peak at 380 mμ (Curve II, in Fig. 4) This Cotton effect is also identical with that of the purple intermediate reported previously[3] In addition, very slow ionization of the complex, which could be traced by measuring the increase in paramagnetic susceptibility[3], was observed even without exciting light These facts may indicate

the formation of an inner complex of the enzyme with substrate moiety through the reverse reaction

On the other hand, when pyruvate presented in the purple solution was eliminated by the addition of an appropriate amount of hydrogen peroxide, the charge transfer band[3] having a peak at 550 mμ gradually diminished and the trough amplitude at 430 mμ in ORD became smaller As shown by Curve III in Fig 5, the shape of the resulting spectrum was in good accord with that of the fully reduced enzyme, indicating that the two electrons shared by the enzyme and substrate moieties were completely transferred to the enzyme. These results suggest that the interconversion between the intermediate and the fully reduced states is governed by the concentration of the substrate and the final products in the medium.

This reversibility was further confirmed by demonstrating the relation between the concentration index (negative logarithm of concentration) of pyruvate added to the mother liquor under anaerobic conditions and the absorbance of the resulting mixture at 550 mμ, which is an indication of the amount of the purple intermediate As shown by Fig 6, the plot thus obtained appeared to fit a sigmoid curve, and an inflexion point was found at p[pyruvate] = 1 7. From this pK', the dissociation constant of the purple intermediate into the fully reduced enzyme and pyruvate was determined to be $2.0 \cdot 10^{-2}$ M

When the concentration of the substrate in the medium was decreased to one-tenth, the spectrum changed from Curve III to Curve IV in Fig 7 upon increasing the concentration of pyruvate, indicating that in this case the excess pyruvate expelled

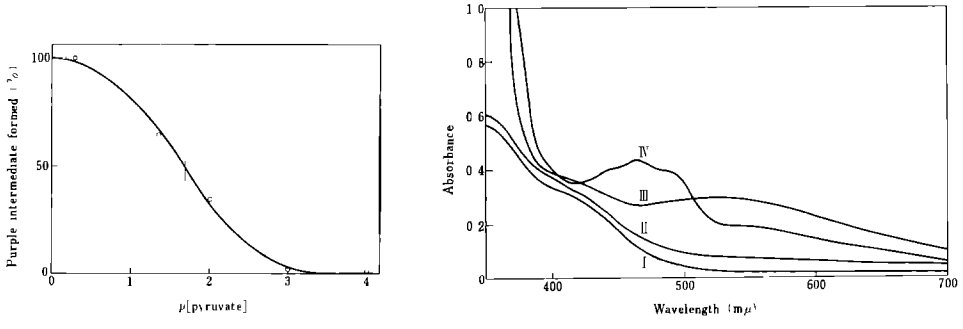

Fig 6 Dependence of enzymatic equilibria on the concentration of a product, pyruvate Open circles demonstrate the observed amount (%) of the purple intermediate formed from the fully reduced enzyme by the addition of pyruvate indicated, in the presence of ammonium sulfate ($5\ 0\ 10^{-2}$ M) and D-alanine ($5\ 0 \cdot 10^{-2}$ M) The enzyme concentration was $8\ 2\ 10^{-5}$ M with respect to FAD The amount of the intermediate was calculated from the absorbance at 550 mμ, based upon the assumption that all of the enzyme in the media forms the purple complex when the absorbance at 550 mμ shows its maximal value, in this case, it reached the maximum when the concentration of pyruvate was elevated to $4\ 0\ 10^{-1}$ M The solid line represents the theoretical curve of the following proposed equation

$$\text{p[pyruvate]} = \text{p}K' + \log \frac{\text{[fully reduced enzyme]}}{\text{[purple intermediate]}}$$

where pK' is assumed to be 1 7

Fig 7 The change in the absorption spectrum of the enzyme with different concentrations of the substrate and the products I, the enzyme ($1\ 1\ 10^{-4}$ M with respect to FAD) fully reduced with D-alanine ($5\ 0 \cdot 10^{-3}$ M), II, I was mixed with lithium pyruvate ($1\ 0\ 10^{-2}$ M) and ammonium sulfate ($5\ 0\ 10^{-2}$ M) The concentration of lithium pyruvate was increased to $1\ 0\ 10^{-1}$ M (Curve III) and to $4\ 0\ 10^{-1}$ M (Curve IV)

the substrate moiety of the purple intermediate complex to form the enzyme–pyruvate complex Similar phenomena should be observed by adding benzoate to the purple intermediate. In fact, the substrate moiety of the purple intermediate was expelled by the benzoate to form the oxidized enzyme–benzoate complex as occurred previously[3]. However, the fully reduced species was not changed to the oxidized enzyme–benzoate complex upon addition of benzoate (see Curve IV, Fig 5)

As reported previously[15], rhodoflavin is formed when the diamagnetic purple intermediate or semiquinoid enzyme is mixed with trichloroacetic acid. In the case of the fully reduced enzyme, however, rhodoflavin was not found in the degradates, no ESR signal was detected.

DISCUSSION

In the classical studies[16–18], it had been considered that the enzyme is capable of removing two hydrogen atoms from its substrate and subsequently transferring them to molecular oxygen to form hydrogen peroxide The direct product of the reaction had been supposed to be α-amino acid[19], which is decomposed to α-keto acid and ammonia through spontaneous hydrolysis The reactions that had been supposed to be involved are

$$RCH(NH_2)COOH + E \rightarrow RC(=NH)COOH + EH_2 \quad (1)$$

$$RC(=NH)COOH + H_2O \rightarrow RCOCOOH + NH_3 \quad (2)$$

$$EH_2 + O_2 \rightarrow E + H_2O_2 \quad (3)$$

where E represents the enzyme.

The present data on crystalline, fully reduced enzyme indicate the formation of the complex of the fully reduced enzyme with D-alanine This may be direct evidence of "turnover" of the substrate The reactions could be expressed as

$$SH_2 + E \rightleftharpoons \text{purple intermediate} \rightleftharpoons EH_2\text{–}S \quad (4)$$

$$EH_2\text{–}S + SH_2 \rightleftharpoons EH_2\text{–}SH_2 + S \quad (5)$$

where SH_2 equals the substrate, S, the product Hence, the formula (1) is considered to be confirmed by the present results so far as the anaerobic reaction is concerned

In the anaerobic reaction, the species EH_2–SH_2 should be situated at the final stage of the reaction sequence However, EH_2–SH_2 could be changed to the purple intermediate by the addition of the products and could be again returned to EH_2–SH_2 by the elimination of pyruvate, indicating the reversibility between the fully reduced and the purple intermediate states The relation between the concentration index of pyruvate and the concentration of the purple intermediate indicates that the oxidation–reduction state of the enzyme is governed by the concentrations of the coexisting substrate and products It is noted that the decrease of the substrate concentration and the increase of the pyruvate concentration yield the enzyme–pyruvate complex[12] from EH_2–SH_2 These results would confirm the reversibility included in the whole process in formulae (4) and (5)

It is obvious that the fully reduced enzyme prepared here reacts with molecular oxygen However, this does not always indicate the direct participation of the fully reduced enzyme in the aerobic oxidation of the substrate, because there are several

lines of evidence[14,20] indicating that oxygen is capable of receiving electrons at an intermediate state of the enzyme.

Although many electronic structures of the enzyme–substrate complex could be postulated as intermediates, the spectroscopically demonstrable intermediate before the fully reduced state in the anaerobic reaction is undoubtedly the purple complex Therefore, the reactivity of oxygen with the purple intermediate stage of the enzyme–substrate complex, where two frontier electrons of the substrate moiety are not completely transferred to the enzyme, should be elucidated in a forthcoming paper

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