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MECHANISM OF ENZYME ACTION. III. CRYSTALLIZATION OF THE SEMIQUINOID FORM OF D-AMINO-ACID OXIDASE

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SUMMARY

1. The semiquinoid form of D-amino-acid oxidase (D-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) has been isolated by crystallization from the solution obtained by illuminating the purple complex of the enzyme with the substrate, D-alanine, under anaerobic conditions. The occurrence of this species could be explained by the photo-accelerated separation of two electrons shared by the enzyme and the substrate moieties in the complex.

2. Spectroscopic observations indicated that the present crystalline preparation was a mixture of the semiquinoid enzyme–substrate (D-alanine) complex, and the complex of the semiquinoid enzyme with the products, pyruvate and ammonia.

3. The semiquinoid enzyme could neither be reduced further by the substrate nor be oxidized by the products. It formed blue-colored complexes with benzoate and substituted benzoates, except ortho-substituted ones, without any influence upon its paramagnetic susceptibility. Benzoate could compete with D-alanine and with pyruvate in combining with the semiquinoid enzyme.

4. Although the oxidized enzyme could be converted into its semiquinoid form by irradiation with strong light under anaerobic conditions or by reduction with sodium dithionite, the complex with benzoate, an enzyme–substrate complex model, was very resistant to photo-reduction, and was converted directly into the fully-reduced state by reduction with sodium dithionite.

INTRODUCTION

It was reported that the crystalline preparation of the purple intermediate of the anaerobic reduction of D-amino-acid oxidase (D-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) with its substrate contains equimolar amounts of the coenzyme and the substrate moiety, and showed only a very small paramagnetic susceptibility[1]. However, freshly prepared purple complex was found to be diamag-

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Abbreviations: ESR, electron spin resonance; ORD, optical rotatory dispersion.

netic. On aging, this diamagnetic complex gradually changed into a paramagnetic species even in the dark, and light seemed to accelerate this conversion[2]. In contrast with the ordinary enzymatic process, this spontaneous charge transfer was a very slow reaction and it occurred only under conditions which permitted the accumulation of the purple intermediate. This reaction included the complete transfer of one electron from the substrate to the coenzyme moiety. One of the end-products should therefore be the semiquinoid enzyme. The main purpose of the present study was to isolate this semiquinoid enzyme in a crystalline form and to show some of its properties. It was expected that such studies would make it possible to elucidate further the nature of the purple intermediate. Some parts of this study have been preliminarily reported[3].

MATERIALS AND METHODS

Crystalline D-amino-acid oxidase was prepared according to YAGI *et al.*[4]. The purple intermediate was prepared as described previously[1]. Free semiquinoid enzyme was prepared by reducing the enzyme with sodium dithionite[5] or by photo-reducing it under anaerobic conditions[6]. The latter method required a strong light; in our case the oxidized enzyme was exposed for 30 min at 5° to a light condensed from a Mazda tungsten lamp (1 kW) through a quartz lens.

FAD and protein contents were determined as reported previously[4]. The amount of D-alanine was estimated by the usual ninhydrin technique[7]. The estimation of pyruvate was made colorimetrically by use of 2,4-dinitrophenylhydrazine[8].

Absorption and ESR spectra, and optical rotatory dispersion (ORD) were recorded as reported previously[2]. The diffuse reflectance spectrum of the crystalline

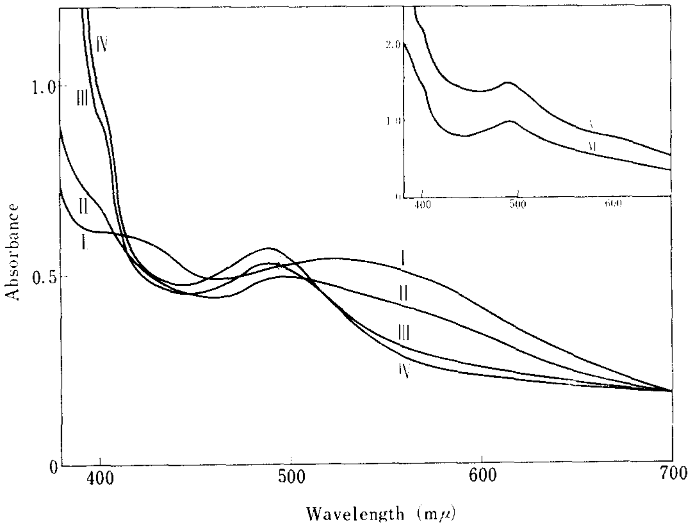

Fig. 1. Absorption and reflectance spectra of the semiquinoid form of D-amino-acid oxidase prepared from the purple intermediate by illumination. I, absorption spectrum of the purple intermediate ($1.86 \cdot 10^{-4}$ M, in respect to FAD); II, after 12 h illumination of I, illumination was made with a Mazda 30-W daylight lamp at a distance of 20 cm at 5°; III, after 24 h illumination of I; IV, after 48 h illumination of I; V, reflectance spectrum of the crystalline suspension obtained from IV; VI, absorption spectrum of its mother liquor.

suspension was recorded by a Perkin–Elmer Model 450 spectrophotometer reflectance accessory.

RESULTS

Crystallization of the semiquinoid form of D*-amino-acid oxidase and its characterization*

Since the spontaneous conversion from the purple intermediate into the semiquinoid enzyme was found to be accelerated by illumination, an attempt was made to crystallize the pure semiquinoid form of this oxidase from the illuminated solution of the purple intermediate.

By illuminating the purple complex (Fig. 1, Curve I) with a Mazda 30-W daylight lamp at a distance of 20 cm at 5°, a new absorption peak appeared at 492 mμ with a decrease in absorbance at longer wavelengths as shown in Fig. 1, Curves II–IV. Then, triturated $(NH_4)_2SO_4$ was added to a concentration of 0.2 M under anaerobic conditions. After storing the mixture in a light, cold room (5°) for 2 days, reddish crystals appeared. As shown in Fig. 2, its shape appears to be a hexagonal prism with bipyramids, like the purple complex crystals[1].

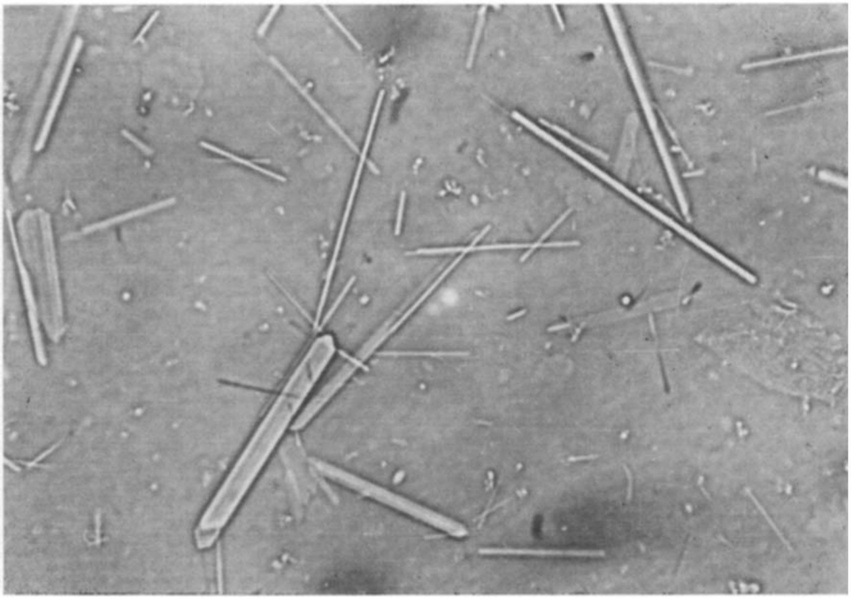

Fig. 2. Crystals of the semiquinoid form of D-amino-acid oxidase (400 times).

However, significant ESR signals were found both in the crystals and in the mother liquor, quantitatively, indicating that the enzyme in both the crystals and the mother liquor is in the semiquinoid form.

The visible ORD pattern of the mother liquor is shown in Fig. 3, Curve I. Its shape is fairly similar to that of the photo-reduced semiquinoid enzyme[6] (Curve II, in Fig. 3), except that some deviation is found around 600 mμ.

The diffuse reflectance spectrum of the crystalline suspension is shown by Curve

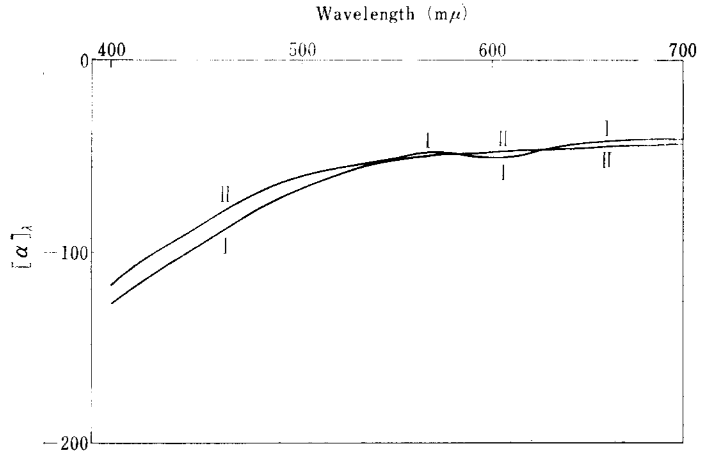

Fig. 3. Optical rotatory dispersions of the semiquinoid forms of D-amino-acid oxidase induced from the purple complex and from the oxidized enzyme by illumination. I, the semiquinoid enzyme prepared by illuminating the purple complex solution ($1.14 \cdot 10^{-4}$ M, in respect to FAD) with a Mazda daylight lamp (30 W) at a distance of 20 cm for 48 h at 5°; II, the semiquinoid form of the enzyme prepared by illuminating the oxidized enzyme ($1.14 \cdot 10^{-4}$ M, in respect to FAD) with a Mazda tungsten lamp (1 kW) at a distance of 20 cm for 30 min at 5°.

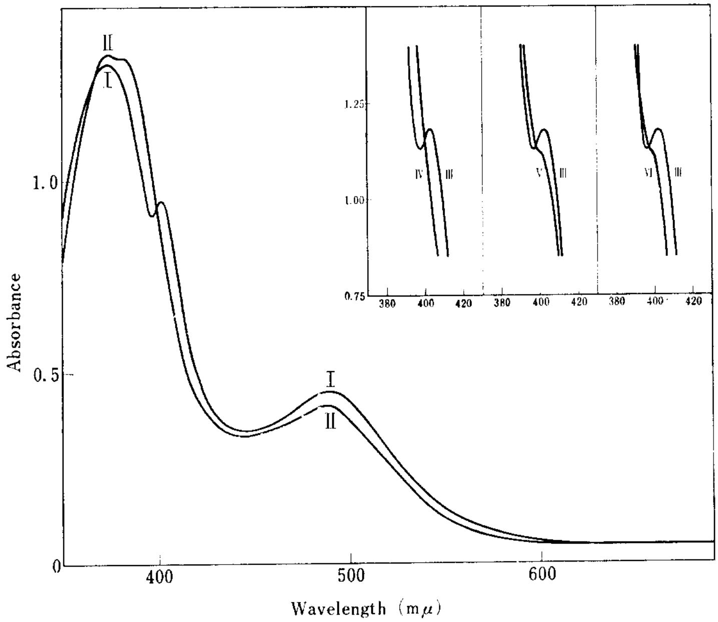

Fig. 4. Spectroscopic demonstration of the interactions between the semiquinoid enzyme and the substrate or the products. I, the semiquinoid enzyme prepared by photo-reducing the oxidized form of the enzyme ($1.04 \cdot 10^{-4}$ M, in respect to FAD) according to Hwang *et al.*[6]; II, I was mixed with $5 \cdot 10^{-2}$ M D-alanine; III, the semiquinoid enzyme prepared by titrating the oxidized form of the enzyme ($1.34 \cdot 10^{-4}$ M, in respect to FAD) with 1 M sodium dithionite according to Nakamura, Nakamura and Ogura[5]; IV, III was mixed with $5 \cdot 10^{-2}$ M D-alanine; V, III was mixed with $1 \cdot 10^{-1}$ M lithium pyruvate + $5 \cdot 10^{-2}$ M $(NH_4)_2SO_4$; VI, III was mixed with $5 \cdot 10^{-2}$ M D-alanine + $1 \cdot 10^{-1}$ M lithium pyruvate + $5 \cdot 10^{-2}$ M $(NH_4)_2SO_4$. All operations were carried out at room temperature under anaerobic conditions.

V, and the absorption spectrum of the mother liquor by Curve VI, in Fig. 1. These spectra are identical. However, it is noted that the free semiquinoid enzyme showed a sharp absorption peak at 400 mμ, whereas it was indistinct both in the spectrum of the aged intermediate[2] and that of the mother liquor of the present crystalline preparation. To explain such a difference, the free semiquinoid enzyme was mixed with D-alanine and/or the products (pyruvate + ammonia). As a result, it was found that the absorption peak at 400 mμ merges into the strong absorption band in the shorter wavelengths, when it was mixed with D-alanine (Fig. 4, Curves II, IV). On the other hand, when it was mixed with the products, the peak did not move, but changed to a shoulder (Fig. 4, Curve V). When it was mixed with both D-alanine and the products, the shoulder became smaller (Fig. 4, Curve VI) as seen in the absorption spectrum of the aged intermediate (type 492)[2] or that of the present preparation (see Fig. 1). These facts indicate that both D-alanine and the products interact with the semiquinoid enzyme. Probably in type 492 and in the present preparation, the semiquinoid enzyme exists as its complex with co-existing substrate or products.

The validity of this assumption was further examined by the selective elimination of pyruvate present in the type 492 solution with H_2O_2 under anaerobic conditions. By this treatment, the shoulder at 400 mμ clearly diminished, showing that all the semiquinoid enzyme in the medium now forms a complex with the substrate (Fig. 5). Decrease in absorbance in the longer wavelengths may be ascribed to the conversion of the purple intermediate to the fully-reduced enzyme probably due to the elimination of pyruvate which shifts the equilibrium.

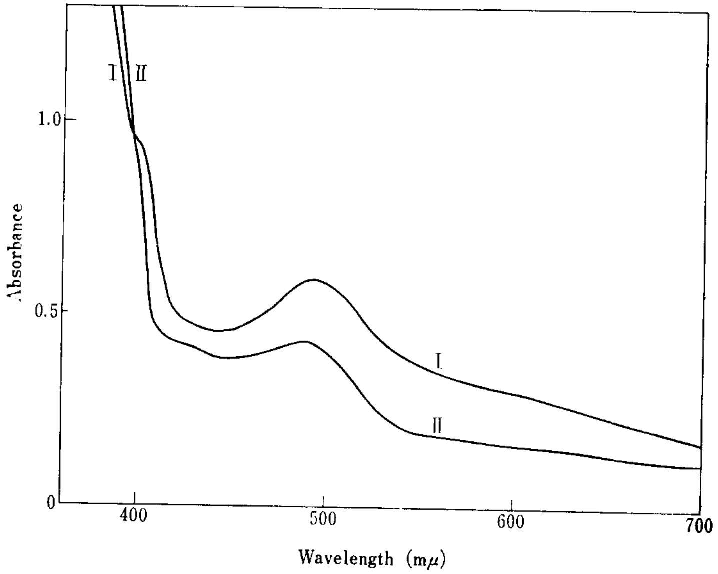

Fig. 5. Changes in the absorption spectrum of the aged intermediate (type 492)[2] when mixed with H_2O_2. I, the aged intermediate ($1.56 \cdot 10^{-4}$ M, in respect to FAD); II, the aged intermediate solution (4 ml) was mixed with 0.3 ml of 3% H_2O_2. The spectrum was recorded 2 min after the mixing. All operations were carried out at room temperature under anaerobic conditions.

Another support for this interpretation was obtained by analyzing the constituents of the present crystalline preparation. Thus, the crystals were analyzed according to the procedure reported previously[1]. As a result, it was found that, after the third washing, [pyruvate + alanine]/[FAD] ratio of the crystals was almost unity. Even after sixth washing, both [pyruvate]/[FAD] and [alanine]/[FAD] ratio of the crystals were found to be more than 0.2. These results probably support the abovementioned view.

Reactivity of the semiquinoid enzyme

Although the semiquinoid enzyme was found to combine with either D-alanine or the products as mentioned above, the substrate failed to reduce it further, and the products failed to oxidize it. However, the semiquinoid enzyme was found to be reactive with molecular oxygen. When the mother liquor was mixed with air, the purple intermediate reappeared. This indicates that it is oxidized rapidly to form a purple complex with the substrate present in the medium. This preparation was also able to reduce methylene blue as reported by HWANG *et al.*[6].

These results indicate that the semiquinoid enzyme is a good electron donor. However, its fairly low electron affinity should be noted. As reported in Fig. 6A, the

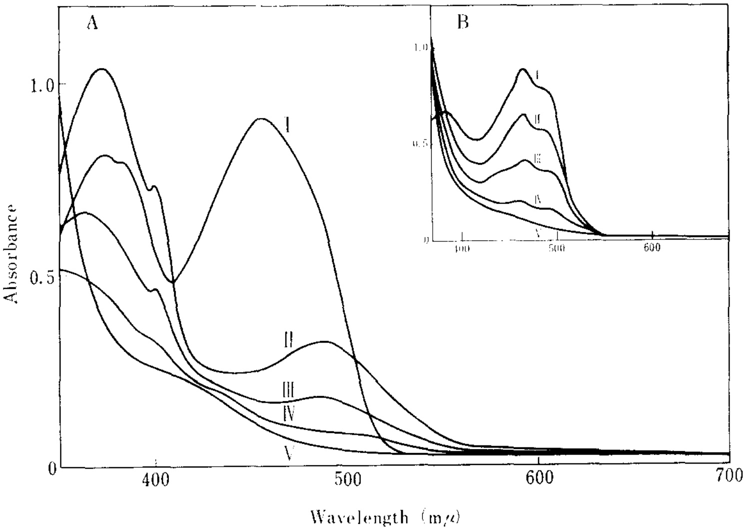

Fig. 6. A. Reduction of the oxidized form of D-amino-acid oxidase with excess sodium dithionite. I, absorption spectrum of the oxidized enzyme ($8.0 \cdot 10^{-5}$ M, in respect to FAD); II, I was mixed with 18 μmoles of sodium dithionite. After repeated evacuation and flushing with argon gas, an aqueous solution of sodium dithionite, in the side chamber of a Thunberg-type cuvette, was introduced into the main chamber which contained 4 ml of the enzyme solution, and the resulting absorption spectrum was recorded immediately after the mixing. III, 10 min after II; IV, 20 min after II; V, 30 min after II. All operations were carried out at room temperature. B. Reduction of the benzoate complex of D-amino-acid oxidase with excess sodium dithionite. I, absorption spectrum of the benzoate complex ($8.0 \cdot 10^{-5}$ M, in respect to FAD). The solution contained $1 \cdot 10^{-2}$ M sodium benzoate .II, I was mixed with 18 μmoles of sodium dithionite. After repeated evacuation and flushing with argon gas, an aqueous solution of sodium dithionite, in the side chamber of a Thunberg-type cuvette, was introduced into the main chamber which contained 4 ml of the enzyme solution, and the resulting absorption spectrum was recorded 10 min after the mixing. III, 30 min after the mixing; IV, 60 min after the mixing; V, 90 min after the mixing. All operations were carried out at room temperature.

oxidized form of the enzyme was rapidly half-reduced with excess dithionite, while the semiquinoid enzyme thus formed was considerably resistant to further reduction with this reagent. In contrast with these results, the enzyme–benzoate complex was not reduced to a semiquinoid state, but was converted slowly but directly into the fully-reduced state, when it was mixed with dithionite under the same conditions, as indicated in Fig. 6B. In addition, the complex was found to be almost insensitive to photoreduction, suggesting that it is a poor one-electron acceptor, despite its potential two-electron-accepting nature.

The free semiquinoid enzyme formed a blue colored substance without any change in its paramagnetic nature, when mixed with sodium benzoate[2,9]. The absorption spectrum of the mixture is shown by Curve II in Fig. 7, which appears to be identical with those of the 'blue radical' of some flavoproteins reported by MASSEY AND PALMER[10]. As shown in Table I, the red radical (according to their nomenclature[10]) of D-amino-acid oxidase was easily converted into a blue-colored substance only by mixing at pH 8.1 with benzoate or its derivatives under anaerobic conditions. This mixing was not accompanied by a significant change in pH. In addition, conversion of the red radical to the blue substance was found to be only partial (Curve IV in Fig. 7) when the red radical (Curve I) was mixed with benzoate in the presence of D-alanine. This fact indicates a competition between benzoate and D-alanine in forming complexes with the semiquinoid enzyme. Pyruvate behaved in the same way as D-alanine.

The results listed in Table I indicate that the semiquinoid enzyme forms the blue complexes when mixed with meta- or para-substituted benzoates, whereas it

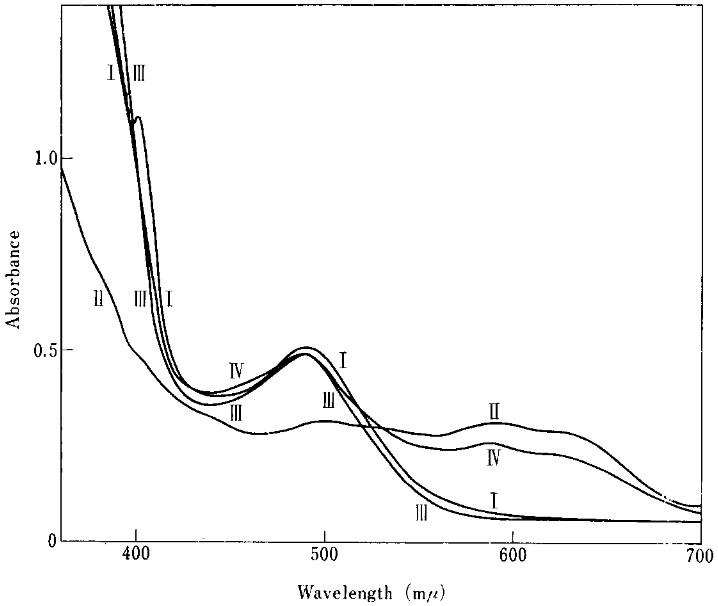

Fig. 7. Effect of D-alanine on the complex formation of the semiquinoid enzyme with benzoate. I, the semiquinoid enzyme ($1.15 \cdot 10^{-4}$ M, in respect to FAD) prepared according to the method of NAKAMURA *et al.*[5]; II, I was mixed with $1 \cdot 10^{-1}$ M sodium benzoate; III, I was mixed with $5 \cdot 10^{-2}$ M D-alanine; IV, I was first mixed with $5 \cdot 10^{-2}$ M D-alanine and then mixed with $1 \cdot 10^{-1}$ M sodium benzoate. All operations were carried out at room temperature under anaerobic conditions.

TABLE I

CHANGE IN THE COLOR OF THE SEMIQUINOID ENZYME UPON ADDITION OF BENZOATE OR ITS DERIVATIVES

The semiquinoid enzyme was prepared by half-reduction of the enzyme ($1.19 \cdot 10^{-4}$ M, in respect to FAD) with sodium dithionite. Final concn. of added benzoate or its derivatives is 0.1 M.

Addition	*Formation of blue complex*
Benzoate	+
Benzoate*	+
o-Aminobenzoate	—
p-Aminobenzoate	±
m-Aminobenzoate	+
o-Toluate	—
p-Toluate	+
m-Toluate	+

* The semiquinoid enzyme used here was prepared by illuminating the oxidized enzyme ($1.19 \cdot 10^{-4}$ M, in respect to FAD) with 1-kW tungsten lamp for 40 min at 5°, under anaerobic conditions.

cannot when mixed with ortho-substituted derivatives. This might be ascribed to the so-called ortho-effect.

Effect of light on the production of the semiquinoid enzyme

As already reported[2], light accelerates the spontaneous charge separation of the purple complex. On the other hand, HWANG *et al.*[6] and MASSEY AND PALMER[10] reported

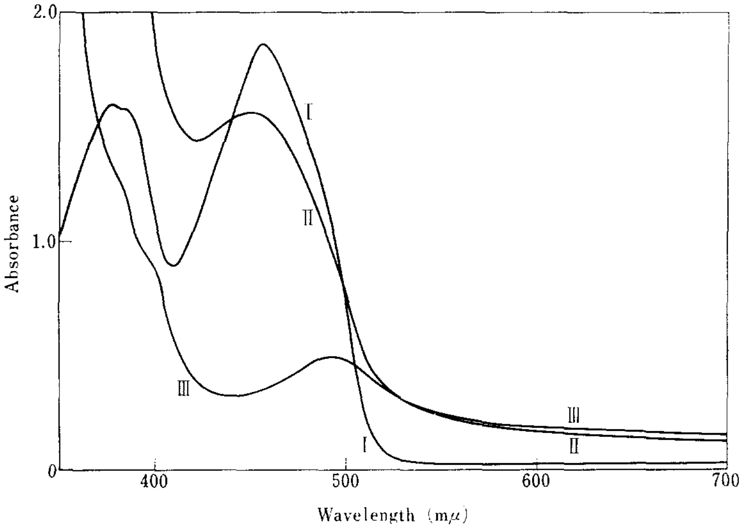

Fig. 8. Illumination of the oxidized form of D-amino-acid oxidase under the conditions where the semiquinoid enzyme is produced from the purple complex. I, absorption spectrum of the oxidized enzyme ($1.65 \cdot 10^{-4}$ M, in respect to FAD); II, I was illuminated for 48 h by use of a Mazda daylight lamp (30 W) at a distance of 20 cm at 5°; III, the semiquinoid enzyme produced from the purple complex ($1.65 \cdot 10^{-4}$ M, in respect to FAD) by illumination under the same conditions. All operations were carried out under anaerobic conditions.

that the semiquinoid form of D-amino-acid oxidase could be produced simply by irradiating the oxidized form of the enzyme with strong light under anaerobic conditions. Hence, another interpretation can be made as to the mechanism of the conversion of the purple intermediate by illumination; it could be ascribed to a half-reduction of free enzyme co-existing with the purple complex in the medium[6]. However, as indicated in Fig. 8, the semiquinoid enzyme was not produced from the oxidized form when illuminated with a weak light (30 W), under which the purple complex can readily convert to the semiquinoid enzyme (Curve III). No significant ESR signal was found in the solution of Curve II. The half-reduction of the free oxidized enzyme in fact required a strong light (*e.g.*, from a 1.0-kW lamp). These results may rule out the possibility mentioned above and strongly support our previous interpretation[2].

DISCUSSION

As reported previously[2], the aged intermediate solution contains a semiquinoid enzyme which is considered to be formed from the purple intermediate through the spontaneous charge transfer from the substrate to the enzyme. This was confirmed in this study by crystallizing the semiquinoid enzyme from the illuminated intermediate solution. Since the illumination accelerates this charge separation, the crystalline sample thus obtained is considered to be essentially identical with the semiquinoid enzyme in the aged intermediate. However, its absorption spectrum and reactivity with benzoate are obviously different from those of free semiquinoid enzyme[2]. The present results have indicated that such differences should be ascribed to the presence of the substrate and the products in the media of illuminated or aged intermediate, in which the semiquinoid enzyme forms catalytically meaningless complexes with the substrate and the products.

The present result has revealed that the enzyme is a potential one-electron acceptor when it is reduced with dithionite[5] or strong light[6], while it is a poor one-electron acceptor, but a potential two-electron acceptor, when it forms a complex with benzoate. It may be valuable to recall that benzoate combines with the enzyme at the substrate sites[11,12] and that the complex is considered to be an enzyme–substrate complex model[13]. It would thus be natural to consider that the enzyme becomes a potential two-electron acceptor, when it is complexed with the substrate or some carboxylic acids.

The complex formation of the semiquinoid form of D-amino-acid oxidase with some carboxylic acids was shown in this study. In particular, benzoate and its derivatives form blue-colored complexes. On the other hand, MASSEY AND PALMER[10] found that, when glucose oxidase was illuminated at higher pH (*e.g.*, pH 10.5), the resulting spectral change was practically identical with that of D-amino-acid oxidase, whereas at lower pH (*e.g.*, pH 5.98) a blue radical was formed. This they ascribed to the deprotonation of the semiquinone to anion radical at high pH media. They found an inflexion point at about pH 7.5 when the glucose oxidase semiquinone was titrated. However, they could not find any formation of blue substance when D-amino-acid oxidase was illuminated at pH 6.0. Their findings are interesting, but our results would not fit their interpretation. The change in color from red to blue observed by us was not accompanied by a significant change in pH of the medium. Accordingly, it may be difficult to explain the change in color from blue to red of glucose oxidase radical only by the ionization of its semiquinoid form to radical anion. Another explanation might be possible; the change in color mainly depends on the change in the environment surrounding the isoalloxazine nucleus. Such a difference in environment would be afforded by the protein moiety, the conformation of which appears to be sensitive to change in pH. Probably at a low pH, the glucose oxidase semiquinone is present in a form which is identical with that of the benzoate complex of the D-amino-acid oxidase semiquinone.

In conclusion, the semiquinoid form of the enzyme is not involved in the regular anaerobic reaction of this enzyme; its formation is only due to the unnatural reduction of the enzyme with chemical reagents or illumination. Otherwise, the semiquinoid enzyme is formed through the spontaneous charge separation process of the purple intermediate. This charge separation, confirmed further in the present study, supports the view that the purple intermediate previously crystallized in our laboratory is a kind of 'inner complex'[14] in the category of charge–transfer complex.

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