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MECHANISM OF ENZYME ACTION

II. CHARACTERIZATION OF THE PURPLE INTERMEDIATE IN THE ANAEROBIC REACTION OF D-AMINO-ACID OXIDASE WITH D-ALANINE

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SUMMARY

1. The purple intermediate in the anaerobic reduction of D-amino-acid oxidase (D-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) with D-alanine had a broad absorption band in the vicinity of 550 mμ, an extrinsic negative Cotton effect in optical rotatory dispersion having a trough at 430 mμ, an inflexion point close to 400 mμ and a peak at 380 mμ, but no significant electron spin resonance signal.

2. After the preparation had been aged in the dark at 5°, a new absorption peak appeared at 492 mμ, accompanying the gradual disappearance of the broad absorption band peaking at 550 mμ and the appearance of an electron spin resonance signal. The increase in $A_{492\ m\mu}/A_{550\ m\mu}$ was proportional to the decrease in the amplitude of the trough at 430 mμ, and the increase in the concentration of unpaired electrons.

3. The purple intermediate changed into the benzoate complex of the oxidized form when the preparation was treated with an excess of triturated sodium benzoate under anaerobic conditions, indicating that this intermediate is a molecular complex between the enzyme and substrate that can be replaced by benzoate. However, in the aged preparation only part of the total enzyme changed into the benzoate complex, and no significant change was found in the concentration of unpaired electrons before and after the addition of benzoate; this indicates the presence of two species in the aged material, the molecular complex and the semiquinoid enzyme which is derived from the former.

4. The conversion from the diamagnetic purple intermediate into the paramagnetic species occurred without light, although light accelerated this conversion. This phenomenon is probably explained in terms of a charge separation process of shared electrons between the enzyme and the substrate moiety.

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

5. The reflectance spectrum of the suspension of the "Michaelis complex"* crystals, freshly prepared according to a previous report, was essentially identical with the absorption spectrum of the purple intermediate. This observation indicates that the "Michaelis complex" crystals obtained in the previous work correspond to this species.

INTRODUCTION

To obtain practical information on the mechanism of enzymic catalysis, it is important to clarify the physical and chemical properties of the reaction intermediates, such as the Michaelis complex. To put this idea into practice, extensive efforts have been made by many workers to demonstrate the intermediate complex by a variety of methods. However, the isolation of such a complex in a stable crystalline form was not reported until the isolation by crystallization of a purple intermediate complex of D-amino-acid oxidase (D-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) was described by YAGI AND OZAWA[3] who reduced the enzyme with excess D-alanine under anaerobic conditions. Their crystals contained equimolar amounts of the coenzyme and substrate moieties, and showed a weak electron spin resonance (ESR) signal. They termed the crystalline material a "Michaelis complex". Soon afterwards, they obtained larger but somewhat differently colored, pinkish-purple crystals by modifying the experimental conditions, namely by storing at 5° in the dark for more than a week and in the presence of a large excess of pyruvate[4]. The absorption spectrum of a single crystal and of its mother liquor both showed a peak at 492 mμ; spectra were similar to that of the semiquinoid enzyme produced by half-reduction with dithionite[5]. However, they were fairly different from the spectrum reported by KUBO, WATARI AND SHIGA[6] who observed a broad absorption band in the vicinity of 550 mμ but no peak at 492 mμ when this enzyme reacted with the same substrate in the presence of excess pyruvate. A weak ESR signal was observed in their preparation. A similar absorption spectrum was also reported by NAKAMURA, NAKAMURA AND OGURA[5] and by MASSEY AND GIBSON[7], who, however, found no significant ESR signal in their samples.

Later, YAGI AND OKAMURA[8] studied in detail the changes in the absorption and the ESR spectra during the preparation of the pinkish-purple crystals[4], and found that at the initial stage the solution showed an absorption spectrum similar to that observed by KUBO, WATARI AND SHIGA[6] and other workers[5,7]. This showed no significant ESR signal, whereas after a 4-day storage in the dark at 5°, a peak appeared at 492 mμ and a weak ESR signal was detected. MASSEY AND GIBSON reported that a similar conversion was accelerated by irradiation with visible light[7]. YAGI AND OKAMURA[8] tentatively named these two species type 550 and type 492, respectively.

The present paper chiefly deals with the characterization of the purple inter-

* If any of the series of the enzyme–substrate complexes appearing in the enzymatic catalysis were to be called a Michaelis complex[1], the crystalline complex obtained in the previous work could be termed a Michaelis complex, because it is composed of the enzyme and the substrate moiety that is readily converted into the product. However, when the term "Michaelis complex" is specially reserved for the intermediate that controls the reaction rate[2], the question whether our crystalline complex should be included in this category awaits further investigation.

mediate (type 550), as well as type 492, to clarify the nature of the crystalline enzyme–substrate complex reported previously[3]. Some parts of this investigation have been reported preliminarily[8,9].

MATERIALS AND METHODS

Crystalline holoenzyme of hog kidney D-amino-acid oxidase was prepared according to the method described by YAGI *et al.*[10]. This enzyme preparation possessed 1 mole of flavin per 49 400 g, and showed a molecular activity of 435 (moles substrate/mole flavin/min) at 20° when it was measured by the routine assay system[10,11].

D-Alanine, sodium benzoate, and other chemicals were of reagent grade.

Crystalline enzyme–substrate complex ("Michaelis complex") was prepared as reported previously[3].

Measurement of the enzymic activity was performed by routine polarographic procedure using a Beckman oxygen sensor[10,11]. The protein content of the enzyme solution was determined by the biuret method[12]. The flavin content of the enzyme sample was estimated by direct spectrophotometry after the enzyme protein had been removed by the addition of trichloroacetic acid (5% final concentration)[10].

The absorption spectrum was recorded by a Beckman DK-2A spectrophotometer. Optical rotatory dispersion (ORD) was measured by using a JASCO-ORD/UV recorder at room temperature. In anaerobic experiments, a Thunberg-type cuvette was used throughout. The reflectance spectrum of the crystalline suspension was measured by using a Shimadzu multipurpose self-recording spectrophotometer MPS type 50.

Sedimentation studies were performed by using a Spinco Model E instrument with temperature control unit. To minimize the effect of air the cell was flushed with argon before use. Measurements were made at 5° and at 56 100 rev./min. Photographs were taken at 16-min intervals. For the calculation of the $s_{20,w}$ of this purple intermediate, the viscosity and the density of the solvent used were assumed to be close to the values of 0.0167 M sodium pyrophosphate buffer, pH 8.3, containing $5.0 \cdot 10^{-2}$ M D-alanine, $1.0 \cdot 10^{-1}$ M pyruvate, and $5.0 \cdot 10^{-2}$ M ammonium sulfate.

ESR measurements were made at room temperature with a JES 3B X-band ESR spectrometer from the Japan Electron Optics Laboratory Co. which was combined with a 300-mm electromagnet and a proton resonance fluxmeter. Solutions to be tested were introduced into a quartz tube, having a capillary of 1.0 mm diameter, by weak suction from its lower end, which was then sealed by a plastic cap. The capillary part of this cell was inserted into the usual cell of 5.0 mm diameter with the aid of lines marked in the cell, and was firmly fixed with rubber tubing so that it was situated in a reproducible position when placed in the magnetic field. This made it possible to estimate the concentration of free radicals in the test solutions with reliable reproducibility. For the measurement of the crystalline sample, crystals were collected into a capillary of a specially designed centrifuge tube (*cf.* ref. 3) with the aid of weak gravity (*e.g.* 1000 × *g*). Before the estimation of the free radical content, the saturation curve of the sample was made by plotting the relative signal intensity *vs.* attenuation (—db) of the power of klystron (9V-5A). The concentration of unpaired electrons in the test sample, expressed as percentage of the total FAD present, was calculated by the usual double graphical integration of the derivative

spectrum, using purified diphenylpicrylhydrazyl of known concentration (in benzene) as standard. The g value was determined by measuring the field strengths at the center of ESR spectra of the sample and of diphenylpicrylhydrazyl. All the measurements were made in the one above-mentioned capillary cell in order to avoid any experimental error emanating from cell differences.

Preparation of the purple complex (type 550). The purple complex, designated type 550, was prepared by mixing enzyme of known concentration with a given amount of D-alanine in the presence of lithium pyruvate ($1.0 \cdot 10^{-1}$ M) and ammonium sulfate ($5.0 \cdot 10^{-2}$ M), under anaerobic conditions. In a typical experiment, the enzyme ($1.54 \cdot 10^{-4}$ M FAD) was reduced by $5.0 \cdot 10^{-2}$ M D-alanine. The resulting absorption spectrum is shown in Fig. 2 (Curve II). This complex, in accordance with the results of other workers[5,7], gave no significant ESR signal (Fig. 3, Curve I).

This type of complex could also be prepared as described by MASSEY AND GIBSON[7], from the enzyme fully reduced with D-alanine, by mixing the latter with excess pyruvate and ammonium sulfate under anaerobic conditions.

Preparation of the semiquinoid enzyme. The preparation of the semiquinoid enzyme was performed according to essentially the same procedure as used by NAKAMURA, NAKAMURA AND OGURA[5]. In some experiments, the semiquinoid enzyme was prepared by illuminating the holoenzyme under anaerobic conditions, according to HWANG *et al.*[13]. A typical absorption spectrum is shown in Fig. 2 (Curve III).

RESULTS

Some physicochemical properties of the purple intermediate (type 550). The purple intermediate prepared in the present study sedimented as a symmetrical 5.1 S boundary* in the ultracentrifuge at 5° (Fig. 1). No change in the absorption spectrum was observed before or after the ultracentrifuge experiment. This value is con-

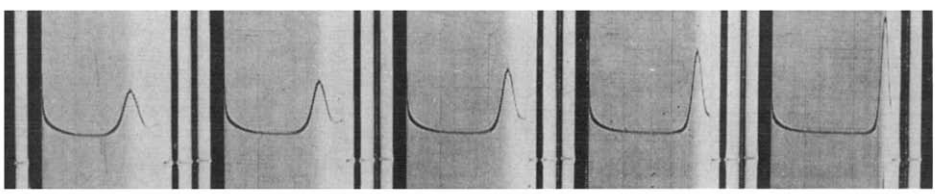

Fig. 1. Ultracentrifuge patterns of the purple intermediate of D-amino-acid oxidase. Measurements were made at 5° and at 56 100 rev./min. Photographs were taken at 16-min intervals. Protein concentration, 11 mg/ml.

siderably smaller than that of the holoenzyme (6.6 S) measured at 5°. The reason for this difference remains to be elucidated.

Besides the differences in the absorption and ESR spectra (Figs. 2 and 3), the purple intermediate (type 550) is clearly distinguished from the semiquinoid enzyme by comparison of their ORD. As indicated in Fig. 4, type 550 showed a clear trough at 430 mμ, an inflexion point close to 400 mμ, and a peak at 380 mμ (Curve I), whereas

* The larger value was quoted as an unpublished result in a previous paper (ref. 14). However, the larger value could be attributed to the prolonged dialysis performed in the previous experiments.

the semiquinoid enzyme gave a simple dispersion pattern (Curve IV) in the 300–600 mμ region. The appearance of this negative Cotton effect indicates the occurrence of an asymmetric character at a position involved in or close to a chromophore that absorbs light at 400 mμ. This chromophore is involved in the isoalloxazine nucleus, because neither protein nor substrate moiety absorbs light at this wavelength. Therefore, this extrinsic Cotton effect should be attributed to the interaction between the isoalloxazine nucleus of FAD and the apoenzyme, or to that between the isoalloxazine moiety of the enzyme and the substrate. Since this Cotton effect was not observed in the holoenzyme[15], it is ascribed to the combination between the

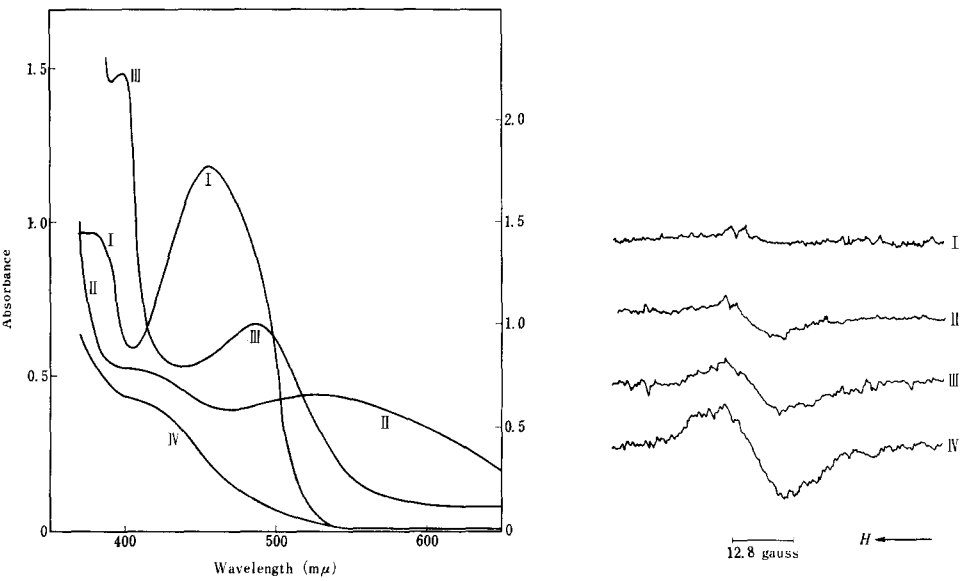

Fig. 2. The absorption spectra of the holoenzyme, the purple intermediate, and the semiquinoid form of D-amino-acid oxidase. (I) The holoenzyme ($1.54 \cdot 10^{-4}$ M in respect to FAD). (II) The purple intermediate. I was mixed with $5.0 \cdot 10^{-2}$ M D-alanine in the presence of $1.0 \cdot 10^{-1}$ M pyruvate and $5.0 \cdot 10^{-2}$ M ammonium sulfate. (III) The semiquinoid enzyme. I was half-reduced with sodium dithionite according to NAKAMURA[5]. (IV) Fully reduced enzyme. I was reduced with excess D-alanine ($3.3 \cdot 10^{-2}$ M). All operations were performed at room temperature under anaerobic conditions. The left scale of the ordinate is for Curves II, III and IV, and the right scale for Curve I.

Fig. 3. Electron spin resonance absorption spectra of the purple (type 550) and the aged (type 492) intermediates. (I) The type 550 solution ($3.72 \cdot 10^{-4}$ M in respect to FAD). (II) I was aged for 5 days in the dark at 5°. (III) I was aged for 10 days in the dark at 5°. (IV) The enzyme half-reduced with sodium dithionite. All measurements were made at room temperature. Field modulation, 100 kcycles 5 gauss; gain 80 db; response 1 sec, magnetic field scanning speed, 20 gauss/min; chart speed, 3 cm/min.

flavin moiety of the enzyme and the substrate. The positive Cotton effect having an inflection close to 380 mμ appeared when the enzyme formed a complex with benzoate[15]. This signifies an interaction between enzyme and benzoate.

Conversion of type 550 to type 492. As reported preliminarily by YAGI AND OKAMURA[8], type 550 complex gradually changed its light absorption when it was stored in the dark at 5° for several days. A typical change in the absorption spectrum

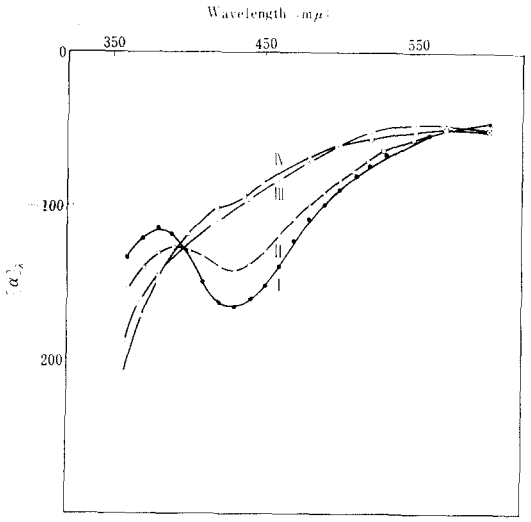

Fig. 4. Optical rotatory dispersion of the purple intermediate and its related states. (I) The purple intermediate. (II) I was aged for 10 days in the dark at 5°. (III) I was illuminated with a Mazda daylight lamp (30 W; 20 cm distant) for 24 h at 5°. (IV) The enzyme half-reduced with sodium dithionite. Measurements were made at room temperature.

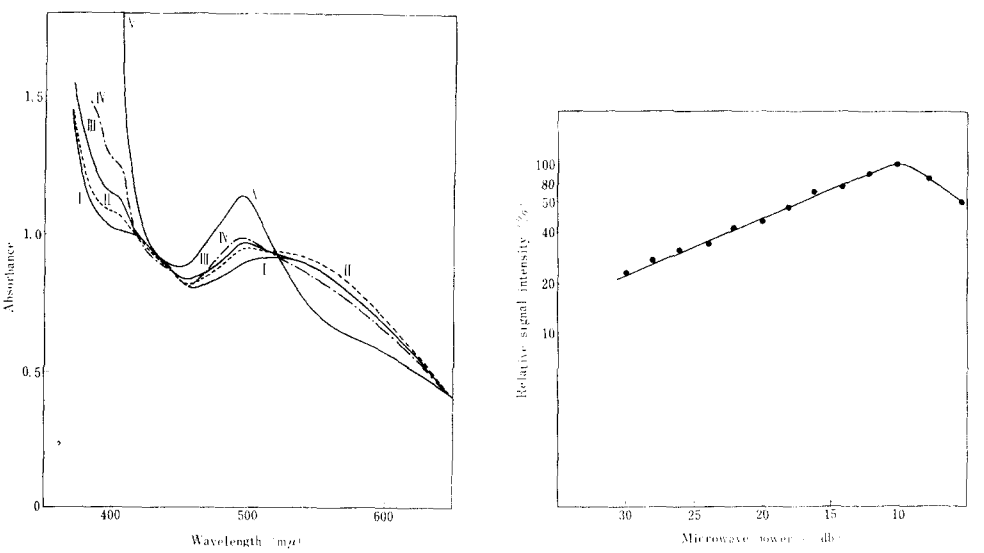

Fig. 5. Change in the absorption spectrum of the type 550 complex by its aging in the dark at 5°. (I) The type 550 complex ($3.12 \cdot 10^{-4}$ M in respect to FAD). (II) After 3 days. (III) After 5 days. (IV) After 10 days. (V) After 1 month.

Fig. 6. Electron spin resonance saturation curve of the aged intermediate (type 492). The relative signal intensity was plotted against attenuation (—db) of the power of klystron. The saturation experiment was made at room temperature.

of type 550 during aging in the dark is shown by Curves I–V in Fig. 5. This conversion is characterized by the appearance of a new peak at 492 mμ, which may derive from the semiquinoid form of this enzyme (see Fig. 2, Curve III); this change in fact ran parallel with an increase in intensity of the ESR signal (Fig. 3, Curves I–III). A significant decrease in the amplitude of the trough at 430 mμ was also found in ORD (Fig. 4, Curve II).

To clarify the mechanism of the conversion of the type 550 complex during storage in the dark, periodical changes in the increase of the unpaired electrons and in the decrease of the amplitude of the trough at 430 mμ were investigated. For the estimation of the concentration of unpaired electrons of the aged sample, saturation curves were previously prepared at each step of aging to minimize the experimental

TABLE I

CHANGES IN THE ABSORPTION SPECTRUM, OPTICAL ROTATORY DISPERSION, AND IN THE FREE RADICAL CONCENTRATION OF THE PURPLE INTERMEDIATE (TYPE 550) DURING STORAGE IN THE DARK

Time of aging (days)	$[\alpha]_{430}$	$\frac{A_{492\ m\mu}}{A_{550\ m\mu}}$	*Content of semiquinoid enzyme (%)*	
			*From free radical concentration**	*From trough at 430 mμ***
0	−167°	1.02	0	0
3	−159°	1.24	6.5	9
10	−147°	1.48	17.8	28
30	−132°	1.74	35.0	52
Enzyme half-reduced with dithionite	−96°	4.29	71.2	100

* The free radical concentration was calculated by the double graphical integration method using purified diphenylpicrylhydrazyl as standard.

** Assuming $[\alpha]_{430} = -167°$ for 100% type 550; $[\alpha]_{430} = -96°$ for 0% type 550 and a linear interpolation.

error as much as possible. A typical saturation curve of the 10-day-old sample is shown in Fig. 6.

As indicated in Table I, the increase in the concentration of unpaired electrons was proportional to the increase in the ratio between the absorbance at 492 mμ and that at 550 mμ ($A_{492\ m\mu}/A_{550\ m\mu}$), and to the decrease in the amplitude of the trough at 430 mμ in ORD, suggesting a stoichiometric relation between the decrease of a compound having the negative Cotton effect and the increase of another compound having unpaired electrons. That is to say, the appearance of unpaired electrons in the flavin nucleus signifies the disappearance of the asymmetric character in the flavin chromophore. This conversion can be attributed to the change in the electronic state of the complex. Therefore, it seems reasonable to consider that this conversion is a process of separating the paired electrons that contribute to the binding between the flavin moiety of this oxidase and the substrate moiety in the type 550 complex.

This conversion progresses without light, which may be extremely important in reactions of biological systems that include a charge transfer process.

MASSEY AND GIBSON[7] reported that a similar conversion was observed when the purple complex was illuminated. In order to investigate this photo-induced conversion in more detail, the following experiments were performed.

The type 550 complex ($7.3 \cdot 10^{-5}$ M FAD) was exposed to a Mazda 30-W daylight lamp at a distance of 20 cm at 5° for a desired length of time, and changes in the absorption spectrum, in ORD, and in the concentration of unpaired electrons were investigated in parallel. This photo-induced conversion was found to be essentially identical with the conversion observed without light, although the former was much faster than the latter, and was accompanied by a considerable decomposition of FAD. Table II indicates the periodical change in the absorption spectrum ($A_{492\ m\mu}/A_{550\ m\mu}$), in the amplitude of the trough at 430 mμ, and in the concentration of unpaired electrons. These results undoubtedly indicate the formation of the semiquinoid enzyme, probably through the charge separation of the purple intermediate. However, evidence for the presence of a substrate radical has not yet been obtained. Perhaps it may be decomposed further because of its instability. If this assumption be true, all the unpaired electrons in the medium should be attributed to the semiquinoid enzyme; in support of this view, from experimental results it was calculated that the concentration of unpaired electrons was 67% of the total FAD remaining in the solution, after the purple complex was illuminated for 48 h.

TABLE II

CHANGES IN THE ABSORPTION SPECTRUM, OPTICAL ROTATORY DISPERSION, AND IN THE FREE RADICAL CONCENTRATION OF THE PURPLE INTERMEDIATE (TYPE 550) BY ILLUMINATION

The purple intermediate was illuminated with a Mazda 30-W daylight lamp at a distance of 20 cm, at 5°.

Time of illumination (h)	$[\alpha]_{430}$	$A_{492\ m\mu}/A_{550\ m\mu}$	*Loss of FAD** (%)	*Content of semiquinoid enzyme (%)*	
				*From free radical concentration***	*From trough at 430 mμ****
0	−167°	1.02	0	0	0
24	−134°	1.39	20.9	35	49.3
48	−113°	1.83	34.0	67	76.1

* Approximately the same moles of the enzyme protein were denatured and sedimented at the bottom of the cell. This sediment did not disturb the measurements.

** The free radical concentration was calculated by the double graphical integration method using purified diphenylpicrylhydrazyl as standard.

*** Assuming $[\alpha]_{430} = -167°$ for 100% type 550; $[\alpha]_{430} = -96°$ for 0% type 550 and a linear interpolation. (See Table I.)

However, the spectroscopic nature of this species possessing an unpaired electron was not always identical with that of the semiquinoid enzyme prepared according to NAKAMURA, NAKAMURA AND OGURA[5] or HWANG *et al.*[13]. As indicated in Fig. 9, Curve I, the minute absorption peak at 400 mμ is quite noticeable in the latter,

but not in the former (Fig. 9, Curve III). This minute peak was also scarcely observed in the aged purple intermediate (type 492, see Fig. 7, Curve III). The disappearance of this peak might be due to the presence of D-alanine in the medium. In fact, this peak merges into the stronger absorption at shorter wavelength, when the semiquinoid enzyme prepared according to NAKAMURA[5] or HWANG[13] is mixed with D-alanine*. This spectral change may be the sign of a combination between the semiquinoid enzyme and this amino acid. However, no change occurs in free radical concentration before or after the addition of the amino acid.

The enzymic activities of these species were examined by the routine procedure to check the loss of the enzyme by denaturation during the conversions. To recover the oxidized form of the enzyme, the type 550 complex and the other species mentioned above were dialyzed in a cold room maintained at 5° against 0.0167 M sodium pyrophosphate buffer, pH 8.3, containing 0.1% of sodium benzoate. From the

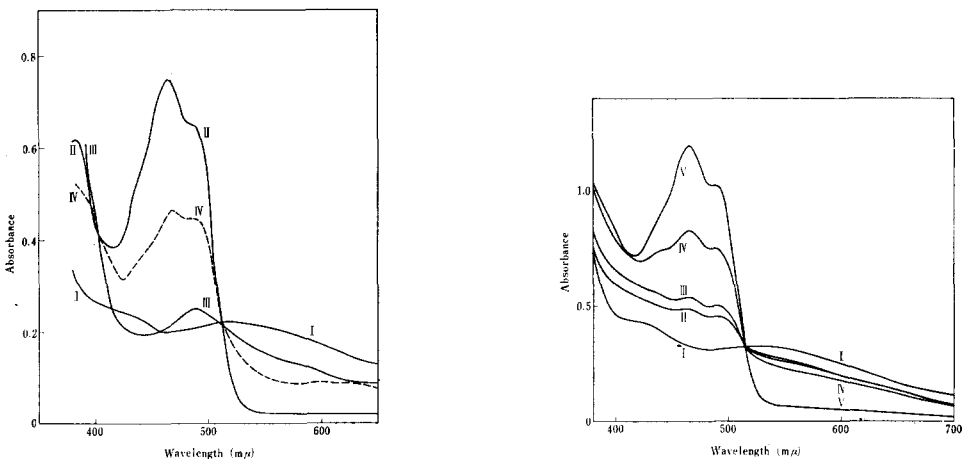

Fig. 7. Reaction of the type 550 complex and its related states with sodium benzoate under anaerobic conditions. (I) The type 550 complex ($6.41 \cdot 10^{-5}$ M in respect to FAD). (II) I was mixed with triturated sodium benzoate (final concentration, $1.0 \cdot 10^{-1}$ M). (III) I was aged for 1 month. (IV) III was mixed with triturated sodium benzoate (final concentration, $1.0 \cdot 10^{-1}$ M). All operations were performed at room temperature.

Fig. 8. Change in the absorption spectrum of the purple intermediate upon mixing with known concentrations of sodium benzoate. (I) The purple intermediate ($1.06 \cdot 10^{-4}$ M in respect to FAD). The amounts of sodium benzoate added to this solution were, in final concentrations, (II) $5.0 \cdot 10^{-3}$ M; (III) $1.0 \cdot 10^{-2}$ M; (IV) $5.0 \cdot 10^{-2}$ M; and (V) $1.0 \cdot 10^{-1}$ M. All operations were made under anaerobic conditions.

type 550 complex, the oxidized form of the enzyme–benzoate complex possessing full activity in the routine assay system was recovered quantitatively. The type 492 solution obtained after a 2-week storage was also converted quantitatively into the active oxidized form, indicating that the conversion from type 550 to type 492 was not caused by denaturation of the protein moiety. However, when the type 550 complex was converted into the semiquinoid enzyme by illumination, the recovery

* K. YAGI, N. SUGIURA, K. OKAMURA AND A. KOTAKI, unpublished results. Details will be published in a future paper.

of the oxidized form was fairly low. This may be attributed to the photodecomposition of FAD and denaturation of the enzyme in the presence of pyruvate[16]. The amount of the active enzyme recovered was almost identical with that of FAD remaining in the semiquinoid derivative produced by illumination. The configuration essential for the enzymic catalysis was therefore maintained in this semiquinoid enzyme.

This semiquinoid enzyme could not be reduced further with D-alanine under anaerobic conditions, whereas the type 550 complex could be reduced further by increasing the amount of substrate or reducing the concentration of pyruvate in the medium*.

Reactivity of type 550 and other species with benzoate. To obtain further information on the conversion of the type 550 complex during storage in the dark, interactions of both the type 550 and the type 492 solutions with benzoate were investigated under anaerobic conditions. Thus, an aqueous benzoate solution was placed in a side-arm of a Thunberg-type cell, the sample to be tested being placed in the main chamber. The cell was then evacuated and filled with argon gas alternately until the solution in the side-arm was dried. During the evacuation, the side-arm was warmed with hot water. When the type 550 complex (Fig. 7, Curve I) ($6.41 \cdot 10^{-5}$ M FAD) was mixed with sodium benzoate (final concentration, $1 \cdot 10^{-1}$ M) in this way, the purple color of the solution gradually turned to yellow. As indicated in Fig. 7, Curve II, the resulting absorption spectrum was identical with that of the oxidized enzyme complexed with benzoate. Since this reaction was performed under anaerobic conditions, it seems difficult to suppose that the enzyme–benzoate complex is formed after the enzyme is oxidized by molecular oxygen. Hence, it may be that this apparent change in the electronic state of the flavin moiety is attributed to the expulsion of the substrate from its binding site of the enzyme by benzoate.

This conversion may be in an equilibrium process; the spectra of the purple intermediate and the benzoate complex are isosbestic at 515 mμ, as shown in Fig. 8. This fact indicates that this purple intermediate is a complex between the enzyme and substrate which is exchangeable for benzoate.

Benzoate was added to the aged intermediate solution (Fig. 7, Curve III) under conditions in which the purple intermediate is completely converted into the benzoate complex. However, formation of the oxidized state was only partial, and the resulting solution showed a dark yellow color (Fig. 7, Curve IV). ESR measurements showed that the values of the concentration of unpaired electrons before and after the addition of benzoate were 35 and 32%, respectively, indicating no significant difference.

After the addition of benzoate to the aged intermediate solution, weak absorption bands in the longer wavelength region were observed. These bands were the same as those appearing when the semiquinoid enzyme, obtained by dithionite reduction, was mixed with benzoate (Fig. 9, Curve II). Therefore, these bands could be ascribed to the semiquinoid enzyme–benzoate complex, which has a unique blue color. No significant difference was found in the concentration of unpaired electrons before and after the addition of benzoate. In one experiment, the values of the concentration of unpaired electrons were 71 and 70%, respectively, before and after the addition of benzoate. On aeration, however, the blue color disappeared, and the

* Details will be reported in a future paper of this series by K. YAGI, K. OKAMURA, N. SUGIURA AND A. KOTAKI.

absorption spectrum of fully oxidized enzyme–benzoate complex appeared. This result indicates the oxidation of the semiquinoid enzyme–benzoate complex. All these characteristics may be intrinsic for the semiquinoid form of this oxidase.

The derivative produced by illuminating the type 550 complex showed a similar behavior to that of the semiquinoid enzyme obtained by half-reduction with dithionite when mixed with benzoate (Fig. 9, Curve IV). The oxidized form was not observed, thus indicating the disappearance of the type 550 complex. No significant difference was observed in the concentration of unpaired electrons before and after the addition of benzoate.

With the enzyme fully reduced with D-alanine or dithionite, no significant spectral change was observed on the addition of benzoate under anaerobic conditions.

Addition of trichloroacetic acid. Although it was found that the "Michaelis com-

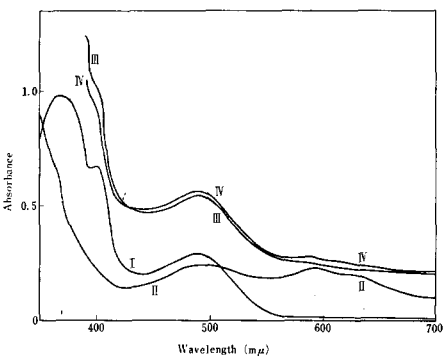

Fig. 9. Reaction of the semiquinoid enzyme with sodium benzoate under anaerobic conditions. (I) The enzyme half-reduced with sodium dithionite. This was also obtained by the method of HWANG[18]. (II) I was mixed with triturated sodium benzoate (final concentration, $1.0 \cdot 10^{-1}$ M). (III) The semiquinoid enzyme induced from the type 550 complex by illumination. The type 550 complex ($1.46 \cdot 10^{-4}$ M in respect to FAD) was illuminated for 24 h at 5°. (IV) III was mixed with triturated sodium benzoate (final concentration, $1.0 \cdot 10^{-1}$ M).

plex" crystal produced a carmine red color on decomposition by trichloroacetic acid under anaerobic conditions and that the color could be attributed to rhodoflavin[17], this phenomenon was checked again on both the purple (ref. 3) and the aged crystal (ref. 4).

After the crystals were washed under anaerobic conditions as reported previously (see ref. 17), trichloroacetic acid was added and agitated anaerobically. The carmine color was observed. However, neither the enzyme fully reduced with D-alanine nor the fully oxidized enzyme gave such a color. From these results, it is considered that the purple crystal may produce semiquinoid rhodoflavin on decomposition by trichloroacetic acid.

Nature of the "Michaelis complex" crystal. To examine the spectroscopic nature of the "Michaelis complex" crystal (ref. 3), it may be necessary to avoid strong light for the above-mentioned reasons. The microspectrophotometer that had been employed for the measurement of the absorption spectrum of the aged crystal (ref. 4) may not be suitable here, because a strong beam is focused on the crystal for a long time. In the present study, therefore, the spectroscopic nature of the crystalline purple complex was characterized by measuring its reflectance spectrum.

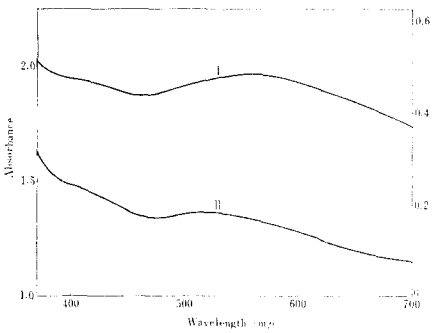

Fig. 10. Reflectance spectrum of the "Michaelis complex" crystals[3]. (I) Reflectance spectrum of the crystal[3]. (II) Absorption spectrum of the washings (see text). The left scale of the ordinate is for Curve I, and the right scale for Curve II.

The crystalline complex was therefore collected by centrifugation and washed with chilled, oxygen-free 0.1 M pyrophosphate buffer, pH 8.3, containing $5 \cdot 10^{-2}$ M D-alanine, $1 \cdot 10^{-1}$ M lithium pyruvate, and $5 \cdot 10^{-2}$ M ammonium sulfate. This washing was repeated twice. The reflectance spectrum of the crystalline suspension is shown in Fig. 10 (Curve I). The absorption spectrum of the final washing is also indicated in the same figure (Curve II). These spectra are similar to the spectrum of type 550.

No significant ESR signal was found in the crystal; less than 0.2% FAD was found to have unpaired electrons.

The results indicate that freshly prepared crystal is composed of the type 550 complex.

DISCUSSION

The purple complex examined in the present study was characterized by a broad absorption band in the vicinity of 550 mμ (type 550) in accordance with the observations of other workers[5-7]. The absorption spectrum of this species is obviously different from both the oxidized and the fully-reduced enzyme[18]. Therefore, the redox state of the coenzyme moiety should be intermediate between the oxidized and the fully-reduced states. Moreover, it is known that the fully-reduced enzyme could be converted into the purple complex by mixing it with the products, pyruvate and ammonium ion, and that the purple complex could be converted into the oxidized enzyme–benzoate complex by the anaerobic addition of benzoate. These facts indicate that the purple complex is an intermediate in the reaction sequence of the anaerobic reduction of this enzyme with D-alanine. This may be simply expressed as

$$E + SH_2 \rightleftharpoons \text{purple intermediate} \leftrightharpoons EH_2 - S$$
$$E + B \rightleftharpoons E - B$$

where E represents the enzyme, SH_2 the substrate, and B the benzoate.

The purple crystals reported in the previous paper, in which they were termed a "Michaelis complex"[3], are identical with this purple intermediate. Since the crystals contain equimolar amounts of coenzyme and substrate, and the present purple intermediate solution showed an interaction between coenzyme and substrate

in ORD measurements, this intermediate is assumed to be a complex between enzyme and substrate. In view of these considerations, the broad absorption band in the vicinity of 550 mμ, which is found in neither enzyme nor substrate, may be attributed to a charge transfer complexing.

The purple complex is apparently distinct from the "semiquinoid enzyme" as judged by their absorption spectra; the latter has an absorption peak at 492 mμ but no broad absorption band at around 550 mμ. However, the indisputable fact is that the type 550 complex gradually changes in the dark into a paramagnetic species, and that light accelerates this change. It was demonstrated by the appearance of an absorption peak at 492 mμ and of an ESR signal, and by the disappearance of the negative Cotton effect. The converted product from type 550 complex was well identified as a semiquinoid enzyme.

Since the semiquinoid enzyme could not be reduced further by D-alanine under anaerobic conditions and it was not oxidized by the anaerobic addition of benzoate, but was transformed to the semiquinoid–benzoate complex, it may not be an intermediate in the physiological sequence of the enzymic reaction under anaerobic conditions.

In spite of this possibility, the mechanism of the conversion of the purple intermediate to the semiquinoid enzyme must be considered. As noted above, this conversion means a change from the diamagnetic into the paramagnetic state accompanied by the disappearance of the interaction between coenzyme and substrate. Such a conversion would be explained in general by the complete transfer of one electron from the electron donor (D) to the acceptor (A). That is, it would be an electron transfer process between the flavin moiety of the enzyme and the substrate moiety. The reaction may be expressed as:

$$A + D \rightleftharpoons A \cdots D \rightleftharpoons A^- - D^+ \rightleftharpoons A^- + D^+$$

The acceptor anion A^- may correspond to the semiquinoid enzyme, and D^+ to the substrate radical that is probably decomposed rapidly because it is unstable.

If the ionization potential of the donor is low enough, and the electron affinity of the acceptor is high enough, this electron transfer would be one process in which diamagnetic donor–acceptor complex may be the intermediate. Ionizations of this kind have been demonstrated in non-enzymic systems by the works of KAINER AND ÜBERLE[19], EASTMAN, ENGELSMAN AND CALVIN[20], KUBOYAMA[21], FOSTER AND THOMSON[22], STAMIRES AND TURKEVICH[23], and others.

In view of this type of dissociation, it would be possible to conclude that the purple intermediate is an 'inner complex' described by MULLIKEN[24]. If such an explanation be valid, the appearance of a diffuse absorption band centered at 550 mμ would be comprehensible in terms of quantum mechanics.

The nature of the large crystal reported previously[4] probably corresponds to that of type 492. The absorption spectrum of the single crystal measured by a microspectrophotometer seems to be identical with that of the photo-induced semiquinoid enzyme. This may be feasible, because a high intensity of light and a long time were required for the measurement.

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REFERENCES

1 J. B. NEILANDS AND P. K. STUMPF, *Outline of Enzyme Chemistry*, Wiley, New York, 1958, p. 96.
2 B. CHANCE, in E. C. SLATER, *Flavins and Flavoproteins* (BBA Library, Vol. 8), Elsevier, Amsterdam, 1966, p. 223.
3 K. YAGI AND T. OZAWA, *Biochim. Biophys. Acta*, 81 (1964) 29.
4 K. YAGI AND T. OZAWA, *Nature*, 203 (1964) 864.
5 T. NAKAMURA, S. NAKAMURA AND Y. OGURA, *J. Biochem.*, 54 (1963) 512.
6 H. KUBO, H. WATARI AND T. SHIGA, *Bull. Soc. Chim. Biol.*, 91 (1959) 981.
7 V. MASSEY AND Q. H. GIBSON, *Federation Proc.*, 23 (1964) 18.
8 K. YAGI AND K. OKAMURA, *J. Biochem.*, 58 (1965) 417.
9 K. YAGI, K. OKAMURA, M. NAOI AND A. KOTAKI, *J. Biochem.*, 59 (1966) 527.
10 K. YAGI, M. NAOI, M. HARADA, K. OKAMURA, H. HIDAKA, T. OZAWA AND A. KOTAKI, *J. Biochem.*, 61 (1967) 580.
11 K. YAGI, M. HARADA AND A. KOTAKI, *Biochim. Biophys. Acta*, 122 (1966) 182.
12 A. G. GONALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
13 K. J. HWANG, K. ASHIDA, M. SHIN, T. SHIGA AND H. WATARI, *Symp. Enzyme Chem. Japan*, 18 (1966) 10.
14 K. YAGI, T. OZAWA AND T. OOI, *Biochim. Biophys. Acta*, 77 (1963) 21.
15 A. KOTAKI, M. NAOI AND K. YAGI, *J. Biochem.*, 59 (1966) 625.
16 K. YAGI AND K. NATSUME, *J. Biochem.*, 55 (1964) 529.
17 K. YAGI, J. OKUDA AND K. OKAMURA, *J. Biochem.*, 58 (1965) 300.
18 K. YAGI AND K. OKAMURA, *Biochem. Biophys. Res. Commun.*, 21 (1965) 399.
19 H. KAINER AND A. ÜBERLE, *Chem. Ber.*, 88 (1955) 1149.
20 J. W. EASTMAN, G. ENGELSMAN AND M. CALVIN, *J. Am. Chem. Soc.*, 84 (1962) 1339.
21 A. KUBOYAMA, *Electronic Spectra* (Jikken-Kagaku-Koza, Vol. 11 ,of new series), Maruzen Co., Tokyo, 1965, p. 495.
22 R. FOSTER AND T. J. THOMSON, *Trans. Faraday Soc.*, 58 (1962) 860.
23 D. N. STAMIRES AND J. TURKEVICH, *J. Am. Chem. Soc.*, 85 (1963) 2257.
24 R. S. MULLIKEN, *J. Phys. Chem.*, 36 (1952) 801.