COMPLEX FORMATION OF APO-ENZYME, COENZYME AND SUBSTRATE OF D-AMINO ACID OXIDASE

II. SPECTROPHOTOMETRIC ANALYSIS USING A SUBSTRATE-SUBSTITUTE

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SUMMARY

From the changes of the absorption spectra of FAD, FMN, riboflavin 5'-monosulfate, and adenosine 5'-monosulfate, observed upon adding the apo-enzyme of D-amino acid oxidase and/or benzoate as a substrate-substitute, it was concluded that the protein, FAD and benzoate form a stable complex.

INTRODUCTION

In the previous paper[1], it was shown by kinetic analysis in which FMS and AMS were used as "indicators", that the coenzyme, FAD, combines with the protein part of D-amino acid oxidase by both its FMN and AMP moieties. It may be expected that the spectrophotometric analysis of the complex of apo-enzyme, FAD and substrate will provide valuable information concerning the nature of these bindings as free FAD has a specific absorption spectrum, which will be modified upon combining with apo-enzyme and substrate. However, in the presence of substrate, enzyme-bound FAD will be reduced so that changes of the absorption spectrum of oxidized FAD cannot be observed. For eliminating this difficulty a "substrate-substitute" must be used, *viz.* a compound combining in the same way as substrate with the apo-enzyme without being dehydrogenated, *e.g.* benzoate[2,3]. Therefore the change of the spectrum of FAD in the presence of apo-enzyme and benzoate was studied.

FMS and AMS, the "indicators" studied in the previous paper[1], were used for the analysis of the change in absorption spectrum of FAD upon formation of the complex.

A preliminary note has been published already[4].

MATERIALS

D-*Amino acid oxidase apo-enzyme*

The first step of the procedure for preparation was essentially similar to that used by KUBO *et al.*[5], the second one to that of MASSEY AND PALMER[6] with some modi-

Abbreviations: FMS, riboflavin 5'-monosulfate; AMS, adenosine 5'-monosulfate.

fications[7], and the third one to that of NEGELEIN AND BRÖMEL[8]. The details of the procedure are described below.

Extraction and heat treatment:

1 kg of hog kidney was homogenized with 4 l of pyrophosphate buffer (*M*/60, pH 8.3, containing 0.1% benzoic acid) in a Waring blendor. The homogenate was kept at 38° for 40 min, then cooled below 20°. The pH was adjusted to 5.2 by the addition of 1 *N* acetic acid with stirring. Then, the solution was kept at 40° for 5 min. The precipitate was removed by centrifugation (10000 × *g*, 20 min). $(NH_4)_2SO_4$ (250 g/l) was added to the supernatant, and the solution left over night at 0°. The precipitate formed was sedimented by centrifugation and dissolved in 400 ml of pyrophosphate buffer (*M*/60, pH 8.3, containing 0.1% benzoic acid). Then the pH was adjusted to 5.1 by adding 1 *N* acetic acid. The solution was kept at 52° for 7 min, any precipitate formed being centrifuged off. $(NH_4)_2SO_4$ (250 g/l) was added to the supernatant. After standing at 0° for 30 min, the precipitate was spun down, and dissolved in 135 ml of pyrophosphate buffer (*M*/60, pH 8.3, containing 0.1% benzoic acid). By adding 1 *N* acetic acid the pH was adjusted to 5.1, and the solution heated at 60° for 3 min. The denatured protein was centrifuged off. To the supernatant $(NH_4)_2SO_4$ (114 g/l of the supernatant) was added. After standing at 0° for 30 min the reddish-yellow precipitate formed was spun down and dissolved in 10 ml of pyrophosphate buffer (*M*/60, pH 8.3, containing 0.1% benzoic acid) (Solution A).

Calcium phosphate gel treatment:

The preparation thus obtained was further purified in two ways. In the way similar to that used by MASSEY AND PALMER[6], a column (2.0 cm in diameter) of calcium phosphate gel–cellulose powder (1:30, w/w) suspended in distilled water (final concentration of gel, 1%) was equilibrated with potassium phosphate buffer (0.05 *M*, pH 5.3). Solution A was brought on the top and eluted with potassium phosphate buffer (0.05 *M*, pH 6.3). Reddish brown impurities remained at the top as sharp bands, while the enzyme moved as a clear yellow band. The yellow eluate is called Solution B.

In the second method[7] the mixture of calcium phosphate gel and cellulose powder, as mentioned above, was added to Solution A and stirred at pH 7.1. The pH was then brought to 5.1 by adding 1 *N* acetic acid. The reddish brown precipitate was centrifuged off. Repeating this procedure twice, the clear yellow supernatant was obtained (Solution B).

Separation of FAD:

Solution B obtained in one way or another was diluted with pyrophosphate buffer (*M*/60, pH 8.3) to a final protein concentration of 1%. $(NH_4)_2SO_4$ was added to 0.2 saturation. The solution was cooled below 5°, then 1 *N* HCl was added to bring the pH to 2.8 with continuous stirring. After standing at 0° for 30 min, the precipitate formed was spun down and dissolved. Repeating this procedure twice, apo-enzyme quite free from FAD was obtained (Solution C).

Flavins

FAD was prepared by the method of YAGI *et al.*[9]. The purity of the sample was over 92%. The sample contained no other flavins, nucleic acids and metals.

Riboflavin was purified from a commercial sample by powdered cellulose column chromatography.

Indicators

FMS and AMS were synthesized by the method of TAKAHASHI, YAGI AND EGAMI[10] and by that of EGAMI AND TAKAHASHI[11], respectively.

Substrate-substitute

The sodium benzoate used was a high grade commercial sample.

RESULTS

The absorption spectrum of FAD in pyrophosphate buffer (*M*/60, pH 8.3) is given by Curve I in Fig. 1 and Fig. 2. The peaks are situated at 263 mμ, 375 mμ and 450 mμ. The molecular absorption coefficients calculated for these wave lengths were identical with those reported by WHITBY[12].

When FAD was mixed with the apo-enzyme in a molar ratio 1:2, the peak at 263 mμ was shifted to 268 mμ and the absorption intensity was strengthened as shown by Curve II in Fig. 1*.

When FAD was mixed with an equimolar amount of the apo-enzyme in the pyrophosphate buffer, the absorption intensity in visual wave length region was slightly lowered and the peak at 450 mμ was shifted to 453 mμ as shown by Curve II in Fig. 2.

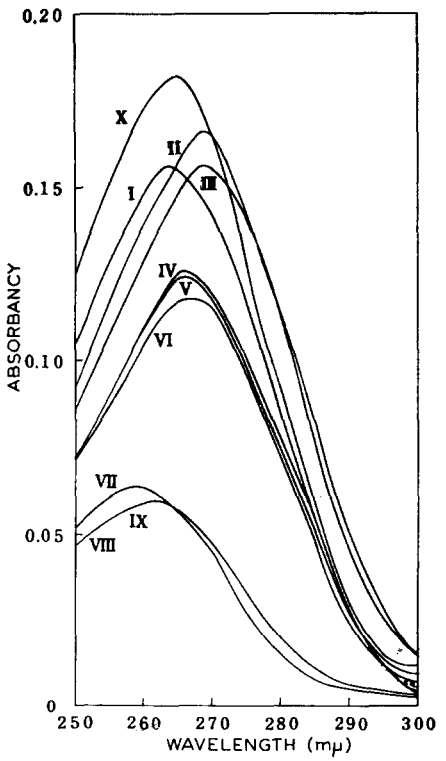

Fig. 1. Absorption spectra of FAD and the indicators (FMS and AMS) in the presence of the apo-enzyme of D-amino acid oxidase and benzoate in pyrophosphate buffer (*M*/60, pH 8.3). I, FAD ($4.0 \cdot 10^{-6}$ *M*); II, FAD + apo-enzyme ($8.0 \cdot 10^{-6}$ *M*); III, FAD + apo-enzyme + benzoate ($1.0 \cdot 10^{-4}$ *M*); IV, FMS ($4.0 \cdot 10^{-6}$ *M*); V, FMS + apo-enzyme ($8.0 \cdot 10^{-6}$ *M*); VI, FMS + apo-enzyme + benzoate ($1.0 \cdot 10^{-4}$ *M*); VII, AMS ($4.0 \cdot 10^{-6}$ *M*); VIII, AMS + apo-enzyme ($8.0 \cdot 10^{-6}$ *M*); IX, AMS + apo-enzyme + benzoate ($1.0 \cdot 10^{-4}$ *M*); X, sum of spectra of FMN ($4.0 \cdot 10^{-6}$ *M*) and AMP ($4.0 \cdot 10^{-6}$ *M*).

* The work described in the preliminary note[4] was carried out with less purified enzyme. The figure in this note should be corrected for Curve II.

When both the apo-enzyme and benzoate were added to FAD solution, an ultraviolet spectrum of FAD was found as shown by Curve III in Fig. 1. Its peak was also situated at 268 mμ as in the case of Curve II in Fig. 1, but the absorbancy was lowered as compared with Curve II.

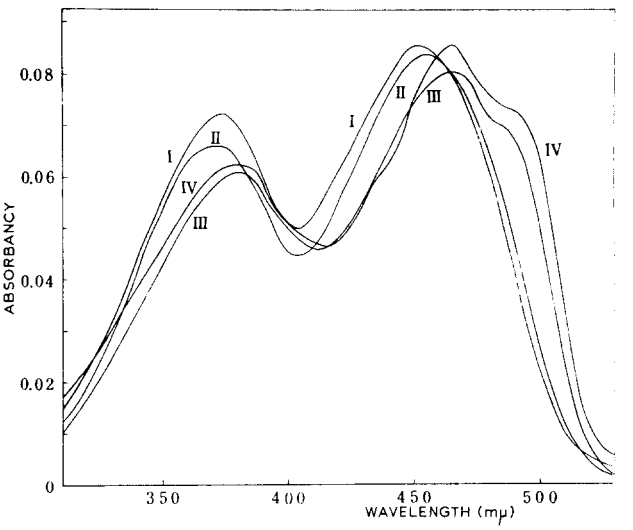

Fig. 2. Absorption spectra of FAD in the presence of the apo-enzyme of D-amino acid oxidase and benzoate in pyrophosphate buffer (M/60, pH 8.3). I, FAD ($7.6 \cdot 10^{-6}$ M); II, FAD + apo-enzyme ($7.6 \cdot 10^{-6}$ M); III, FAD + apo-enzyme + benzoate ($1.0 \cdot 10^{-4}$ M); IV, FAD + apo-enzyme ($1.5 \cdot 10^{-5}$ M) + benzoate ($1.0 \cdot 10^{-4}$ M).

When FAD was mixed with an equimolar amount of the apo-enzyme and an excess of benzoate, the spectrum of FAD in the visual wave length region was changed as follows: the peaks at 375 mμ and 450 mμ were shifted to 380 mμ and 465 mμ, respectively, and a marked shoulder appeared at 490 mμ as shown by Curve III in Fig. 2.

By mixing FAD and the apo-enzyme in the molar ratio 1:2 with an excess of

TABLE I

RATIO OF ABSORBANCIES OF FAD IN ITS MIXTURE WITH BOTH THE APO-ENZYME AND BENZOATE

I, FAD ($7.6 \cdot 10^{-6}$ M) in pyrophosphate buffer (M/60, pH 8.3); II, I + apo-enzyme ($7.6 \cdot 10^{-6}$ M); III, II + benzoate ($1.0 \cdot 10^{-4}$ M); IV, I + apo-enzyme ($1.5 \cdot 10^{-5}$ M) and benzoate ($1.0 \cdot 10^{-4}$ M).

Ratio A/B*	*I*	*II*	*III*	*IV*
380/465	0.910	0.785	0.760	0.735
380/490	1.715	1.325	0.905	0.875
465/490	1.890	1.700	1.180	1.170

* Ratio of absorbancy at A mμ to absorbancy at B mμ was listed.

benzoate, the spectrum of FAD was further modified as shown by Curve IV in Fig. 2: the absorbancy at 465 mμ was increased as compared with Curve III and a shoulder appeared at 440 mμ, while the shoulder at 490 mμ was further strengthened. The spectral changes in the visual wave length region are demonstrated by the change of the ratios of the absorbancies as listed in Table I.

The ultraviolet spectrum of FMS is shown by Curve IV in Fig. 1. It was slightly affected by the addition of the apo-enzyme as shown by Curve V in Fig. 1: the peak at 266 mμ is not shifted, while the absorbancy was only a little lowered. Addition of an excess benzoate to this mixture caused a more pronounced decrease, as shown by Curve VI in Fig. 1.

The peak in the ultraviolet spectrum of AMS had its maximum at 258 mμ as shown by Curve VII in Fig. 1. This was shifted to 263 mμ and the absorbancy decreased by the addition of the apo-enzyme as shown by Curve VIII in Fig. 1. In contrast with the case of FMS, the addition of benzoate to the mixture of AMS and the apo-enzyme did not cause any further change of the spectrum as shown by the coincidence of Curves IX and VIII in Fig. 1.

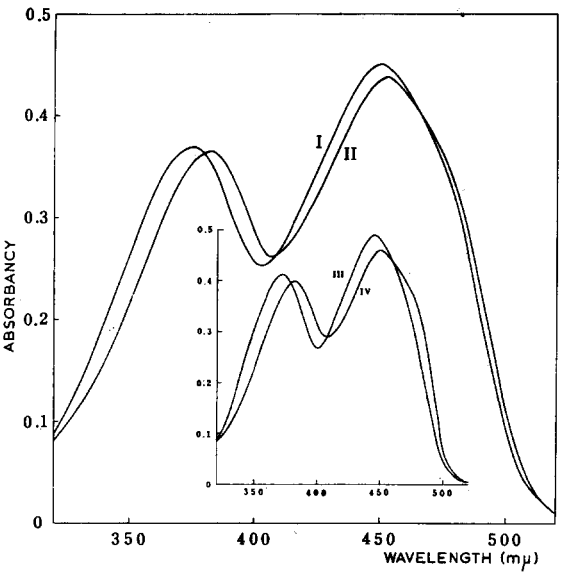

Fig. 3. Absorption spectra of FAD and riboflavin in the presence of excess benzoate. I, FAD ($4.0 \cdot 10^{-5}$ *M*) in pyrophosphate buffer (*M*/60, pH 8.3); II, FAD + benzoate (1.0 *M*); III, riboflavin (4.0^{-5} *M*) in pyrophosphate buffer (*M*/60, pH 8.3); IV, riboflavin + benzoate (1.0 *M*).

By mixing FAD with an excess of benzoate (final concentration, 1.0 *M*) in pyrophosphate buffer (*M*/60, pH 8.3), the absorption intensity of FAD in the visual wave length region was slightly lowered and the peaks at 375 mμ and 450 mμ were shifted to 382 mμ and 453 mμ, respectively, as shown by Curve II in Fig. 3.

By mixing riboflavin with an excess benzoate, a similar change was observed in the spectrum of riboflavin: the absorbancy was slightly lowered and the peaks at 373 mμ and 445 mμ were shifted to 382 mμ and 450 mμ, respectively, as shown by Curve IV in Fig. 3.

DISCUSSION

The absorption of FAD in the ultraviolet ray region is first considered. The shift of the absorption peak of FAD from 263 mμ to 268 mμ by the addition of the apo-enzyme must be caused by the reconstitution of the holo-enzyme.

The combination of FMS and the apo-enzyme causes no shift of the absorption peak of FMS, while the combination of AMS and the apo-enzyme shifts the absorption peak of AMS from 258 mμ to 263 mμ. These findings suggest that the shift of the absorption peak of FAD upon adding apo-enzyme is caused by the combination of the AMP part of FAD with the apo-enzyme (see Bond I in Fig. 4). The addition of benzoate to FMS + apo-enzyme caused a marked decrease of absorbancy of FMS

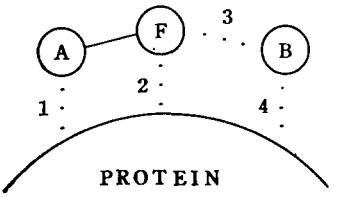

Fig. 4. The scheme of the bindings between apo-enzyme, FAD and benzoate. The adenine nucleus of FAD is designated by A, the isoalloxazine nucleus of FAD by F, benzoate by B, and the apo-enzyme by PROTEIN.

in the ultraviolet region. On the contrary, benzoate did not affect the ultraviolet spectrum of AMS combined with the apo-enzyme (see Curve IX in Fig. 1). As the ultraviolet spectrum of the FAD–apo-enzyme complex is also affected by benzoate, it may be concluded that FAD is also bound to the protein by the isoalloxazine moiety of the FAD (see also Fig. 4).

According to WHITBY[12] the binding between the 3-NH group of the isoalloxazine and the adenine decreases the absorbancy of FAD as compared with the spectrum obtained by summation of the spectra of FMN and AMP (see Curve X in Fig. 1). The increase of the absorbancy of FAD at 268 mμ upon adding apo-enzyme therefore suggests that the intramolecular hydrogen bond in FAD is broken, the more so since the apo-enzyme causes a decrease of the absorbancies of FMS and AMS.

Now, considering the spectral changes of FAD in the visual wave length region, it may be seen that the presence of the apo-enzyme causes a slight effect (Curve II in Fig. 2). The peak at 450 mμ is shifted to 453 mμ, as was also observed in the case of FAD–phenol complex[3]. This may be interpreted as indicating that the isoalloxazine nucleus of FAD combines with a tyrosyl residue of the protein[3]. This binding is shown by Bond 2 in Fig. 4.

From the fact that the 375 mμ peak of FAD is shifted to about 380 mμ upon adding apo-enzyme and/or benzoate and the 375 mμ peak of riboflavin is similarly shifted in the presence of benzoate, while phenol or apo-enzyme fails to affect the 375 mμ peak, it is concluded that 375 → 382 shift must be attributed to binding between the isoalloxazine moiety of FAD and benzoate in the FAD–apo-enzyme–benzoate mixture. This binding is shown by Bond 3 in Fig. 4.

On the other hand, the occurrence of a binding also between benzoate and the apo-enzyme (Bond 4 in Fig. 4) is suggested by the results obtained by kinetic analysis of the enzyme action[3].

It should be noted that the spectral change of FAD observed in FAD–apo-enzyme–benzoate mixture is larger than the sum of the changes observed in the FAD–apo-enzyme mixture and in the FAD–benzoate mixture (compare Curve IV in Fig. 2 with Curve II in Fig. 2 and Curve II in Fig. 3). A marked shoulder at 490 mμ is considered to be characteristic for this artificial complex composed of the apo-enzyme, coenzyme and substrate–substitute. These facts suggest that a tight binding is brought about by the reaction of apo-enzyme, the isoalloxazine moiety of FAD and benzoate. This is supported by still other evidence: the yellow fluorescence of FAD, specific for the isoalloxazine nucleus, is somewhat quenched by the apo-enzyme as reported by WALAAS[13] and the authors[14], but the quenching is much more pronounced by the further addition of benzoate[14].

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