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Apoenzyme from *Cryptococcus humicolus* UJ1 D-aspartate oxidase

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Abstract

The apoenzyme of D-aspartate oxidase from *Cryptococcus humicolus* UJ1 was obtained by dialyzing the holoenzyme against 3 M KBr in 250 mM potassium phosphate (pH 7.0), 0.3 mM EDTA and 5 mM 2-mercaptoethanol, followed by gel filtration on Superdex 200 to separate from the remaining holoenzyme. Apo-D-aspartate oxidase is entirely present as a monomeric protein of 40 kDa, while the reconstituted holoenzyme is a tetramer of 160 kDa. The equilibrium binding of FAD to apoprotein was measured from the quenching of flavin fluorescence: a very small value of K_d (8.2×10^{-12} M) was calculated. The kinetics of formation of the apoprotein–FAD complex was studied by the quenching of protein and flavin fluorescence and by activity measurements. The reaction apparently proceeded in two stages, a rapid first phase, followed by a slower secondary phase. The rapid phase was observed by the change in protein fluorescence and an initial rapid phase of decrease in FAD fluorescence, representing at least initial binding of FAD to the monomeric apoprotein. The slower phase correlated with a secondary phase of FAD fluorescence quenching and the appearance of catalytic activity.

Keywords: D-Aspartate oxidase; Apoprotein; Apoenzyme; Flavoprotein; (Yeast (*C. humicolus*))

1. Introduction

D-Aspartate oxidase and D-glutamate oxidase are FAD enzymes that oxidatively deaminate acidic D-amino acids as shown in Fig. 1. The enzymes are similar to D-amino acid oxidase in the type of reaction they catalyze but differ in substrate specificity. They have been purified from bovine kidney [1], octopus hepatopancreas [2], the yeast *Cryptococcus humicolus* UJ1 [3] and the yeast *Candida boidinii* 2201 [4]. In addition, D-aspartate oxidase has been recently cloned from bovine kidney [5] and human brain [6]. Although the enzymes have been characterized fairly well, information on the apoprotein is very limited. It has been prepared only from the bovine D-aspartate oxidase [1], while the apoform of D-amino acid oxidase has been prepared from several sources and characterized well [7,8]. We have found that the D-aspartate

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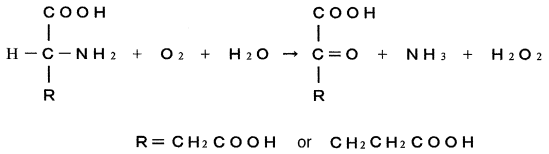

Fig. 1. Oxidative deamination of acidic D-amino acids.

oxidase from *C. humicolus* is different from the bovine enzyme in many aspects, especially in the quarternary structure; the former is a homotetramer of 160 kDa [3], whereas the latter is a monomer of 39 kDa [1]. This difference tempted us to study the apoprotein of the yeast enzyme. The present paper describes its preparation and properties.

2. Materials and methods

The yeast *C. humicolus* UJ1 was grown in a medium containing 10 g D-aspartate, 10 g glucose, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.0 g corn steep liquor powder in a total volume of 1 l at pH 7.0. D-Aspartate oxidase holoenyzme was prepared from yeast cells by the reported procedure [3] with the following minor modifications: the final two stages of purification procedure employed FPLC on SP-Sepharose and FPLC on Superdex 200 in this order, in place of twice repeated FPLC on Superdex 200 originally reported; a larger scale of purification procedure than that originally described [3] was adopted. The enzyme was eluted from the SP-Sepharose column with a linear gradient from 0 to 500 mM NaCl in 20 mM MES buffer (pH 6.5) containing 2 mM EDTA. These modifications produced purified preparations with a specific activity of about 150 units/mg protein, twice as that reported [3]. Enzyme activity was measured colorimetrically with 2,4-dinitrophenylhydrazine as described [9], by 10-min incubation at 37°C of the assay mixture containing 20 mM D-aspartate, 20 μM FAD and 0.025 mg/ml bovine serum albumin in 50 mM potassium phosphate buffer (pH 7.5). FAD was omitted from the assay mixture when holoenzyme activity was measured. Concentrations of holo- and apo-enzyme were expressed in terms of the concentration of monomer subunit (40 kDa) [3] calculated from the protein concentrations determined by the method of Lowry et al. [10].

FAD (from Sigma) was purified by chromatography on J'sphere ODS-M80 column (4.6 × 250 mm) using a Shimadzu HPLC apparatus according to the method of Light et al. [11]; purified FAD was used for all fluorescence and spectroscopy experiments. An absorption coefficient of 11,300 M^{-1} cm^{-1} at 450 nm was used for free FAD [8].

Absorption spectra were measured with a Shimadzu UV-2500PC UV and visual recording spectrophotometer. Fluorescence measurements were performed with a Shimadzu RF-5300PC spectrofluorometer equipped with Taitec EL-80 coolnit bath for temperature control. Calculation of the dissociation constant of the apoprotein–FAD complex was carried out on assumption of a simple 1:1 equilibrium, according to the method of Stinson and Holbrook [12], employing Eq. (1):

$$1/(1-a) = 1/K_{EL}([L_o]/a - [E_o]), \qquad (1)$$

where a = fractional saturation of the total concentration of binding sites, K_{EL} = dissociation constant, $[L_o]$ = total ligand concentration and $[E_o]$ = total enzyme concentration.

3. Results

3.1. Preparation of apoprotein

Homogeneous D-aspartate oxidase (70–150 μg/ml, 1.8–3.8 μM) was dialyzed for 3 days at 4°C in the dark against four changes of a 100 volume of 250 mM potassium phosphate buffer, pH 7.0 containing 3 M KBr, 0.3 mM EDTA and 5 mM 2-mercaptoethanol, until the catalytic activity decreased to 5–10% of its initial value. Dialysis was then continued for 6 h against three changes of the same buffer without KBr.

The dialyzate was applied to FPLC gel filtration on a column of Superdex 200 equilibrated with the same buffer without KBr. As shown in Fig. 2, two protein peaks of 160 and 40 kDa, respectively, were separated. The former fraction was fully active without addition of FAD, indicating that it was the remaining holoenzyme, while the latter fraction absolutely required FAD for activity, showing its identity as apoprotein with a monomer structure. This pattern was the case in the range of 5–240 μg/ml protein concentrations, and no occurrence of any protein of 80 kDa was observed. The remaining holoenzyme was about 10% of the original preparation that underwent the dialysis, and the yield of apoprotein was about 60%. The low yield was probably due to denaturation of apoprotein since a small amount of precipitated protein was always observed at the end of dialysis. Addition of glycerol to the dialyzing buffer did not improve the yield. Presence of malonate, a competitive inhibitor of the enzyme, did not protect the apoprotein from denaturation. Rather, malonate appeared to prevent the dissociation of holoenzyme. Use of a lower concentration (2 M) of KBr markedly slowed down the production of the apoenzyme; holoenyzme activity did not decrease to 5% of the original even after 17 days of dialysis under the conditions. More prolonged dialysis using 3 M KBr enabled us to remove holoenyzme completely, but this method was not adopted because it lowered the yield of apoprotein markedly.

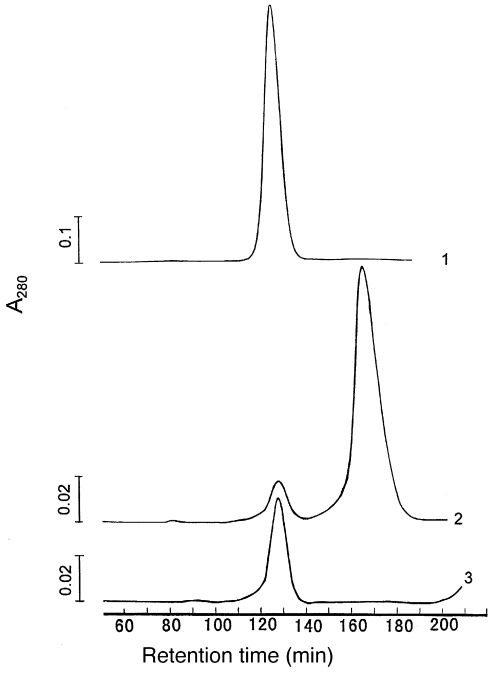

Fig. 2. Gel-filtration profile for D-aspartate oxidase native holoenzyme, apoprotein and reconstituted holoenzyme. (1) Native holoenzyme (2.1 mg); (2) a mixture of apoprotein and residual holoenzyme (total protein, 400 μg) obtained after the treatment of native holoenzyme with 3 M KBr for 3 days; and (3) reconstituted holoenzyme (ca. 200 μg) obtained by incubation of apoprotein with 100 μM FAD for 36 h at 4°C, were applied to FPLC gel filtration on a column (1.6×60 cm) of Superdex 200. (1) and (3) were eluted with 20 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA, and (2) was eluted with 250 mM potassium phosphate buffer (pH 7.0) containing 0.3 mM EDTA and 5 mM 2-mercaptoethanol. The protein peaks at around 128 and 167 min were estimated to have molecular masses of 160 and 40 kDa, respectively.

Table 1
Kinetic parameters of the native and reconstituted holoenzymes of *C. humicolus* D-aspartate oxidase
Measurements were performed in the same way as the measurement of enzyme activity described in Section 2, except that D-aspartate was used at concentrations of 0.67–20 mM.

	K_m (mM)	V_{max} (μmol/min·mg)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Native holoenzyme	3.61	191.6	127.7	35,370
Reconstituted holoenzyme	3.66	201.1	134.1	36,640

When the apoprotein, thus, obtained was incubated with excess FAD and underwent the same gel filtration as described above, the chromatogram showed only one protein peak of 160 kDa, demonstrating the reconstitution of holoenzyme having a homotetramer structure (Fig. 2). The reconstituted holoenzyme gave identical K_m and V_{max} values as those for the native enzyme (Table 1). On SDS-polyaclamide gel electrophresis, both of the apoprotein and the reconstituted holoenyzme gave a single band of 40 kDa as previously reported for the native enzyme [3].

3.2. Stability of the apoprotein

The apoprotein in 250 mM potassium phosphate buffer, pH 7.0 containing 5 mM 2-mercaptoethanol and 0.3 mM EDTA was stable at 4°C at least for 1 month. It was also stable at 16°C for 24 h. At temperatures higher than 30°C, it became markedly unstable and the activity decreased to 56% and 0% of the original by 10-min preincubation at 37°C and 45°C, respectively. This was in contrast to the finding that the holoenzyme kept its activity higher than 90% after incubation at 45°C. Presence of 2-mercaptoethanol was essential for stabilization during dialysis for apoprotein formation as indicated by observed rapid protein precipitation, when the thiol was omitted from the dialysis buffer.

3.3. Spectrophotometric and fluorometric properties of the apoprotein

The apoprotein showed no absorption band at around 450 nm which was shown by the holoenzyme containing FAD. In this respect, the spectrum of the reconstituted holoenzyme was essentially similar to that of the native holoenzyme (Fig. 3). However, when compared in detail, some differences were shown between the spectra in the shape of absorption bands at around 370 and 480 nm and in molar extinction coefficients at around 270 and 450 nm.

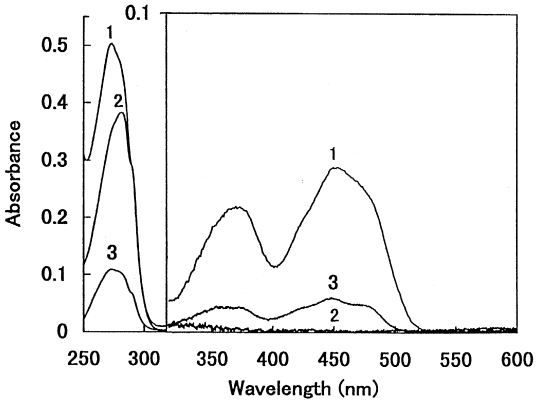

Fig. 3. Absorption spectra of D-aspartate oxidase native holoenzyme, apoprotein and reconstituted holoenzyme. (1) 4.4 μM native holoenzyme in 20 mM potassium phosphate (pH 7.0) containing 2 mM EDTA. (2) 4.8 μM apoprotein in 250 mM potassium phosphate (pH 7.0) containing 5 mM 2-mercaptoethanol and 0.3 mM EDTA. (3) 1.5 μM reconstituted holoenzyme in 20 mM potassium phosphate (pH 7.0) containing 2 mM EDTA, which was prepared by incubation of apoprotein with 100 μM FAD for 36 h at 4°C followed by gel filtration on Superdex 200 column.

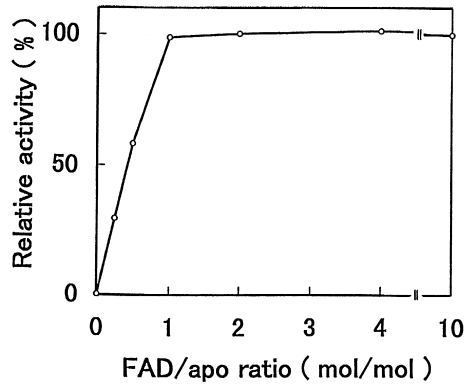

Fig. 4. Activity titration curve of D-aspartate oxidase apoprotein with FAD. Incubation mixtures contained 300 μl 3.5 μM apoprotein and 50 μl FAD solution at different concentrations to give the indicated molar ratios, in 250 mM potassium phosphate (pH 7.0) containing 5 mM 2-mercaptoethanol and 0.3 mM EDTA. After incubation at 16°C for 60 min, 10-μl aliquots were withdrawn from each incubation mixture and assayed for holoenzyme activity.

When excited at 285 nm, the apoprotein showed a protein fluorescence spectrum having a peak at 338 nm with a markedly increased intensity (3.5 times) in comparison to that of the holoenzyme with a peak at 335 nm. When excited at 450 nm, the apoprotein showed no flavin fluorescence, while the native holoenzyme and the reconstituted holoenzyme showed a flavin fluorescence with a peak at 522 nm whose intensity was about 10% of that of free FAD. Fluorescence titration shown in Fig. 5 also indicates that the flavin fluorescence for the reconstituted holoenzyme was about 10% in comparison to that of free FAD.

3.4. Binding of FAD to the apoprotein and reactivation

Activity titration of apoprotein with increasing amounts of FAD gave the curve shown in

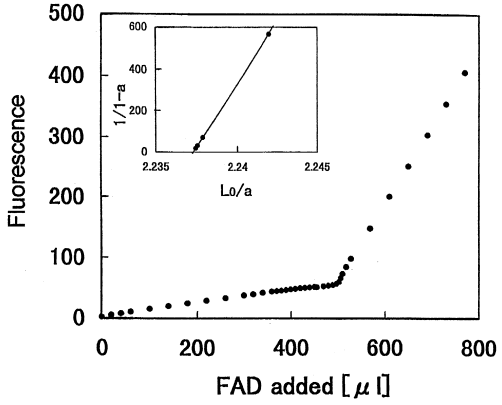

Fig. 5. Equilibrium binding of apoprotein with FAD measured by flavin fluorescence and determination of the dissociation constant for the binding. A 2-ml solution of 1.62 μM apoprotein in 250 mM potassium phosphate (pH 7.0) containing 5 mM 2-mercaptoethanol and 0.3 mM EDTA was titrated by the addition of small aliquots 9.12 μM FAD under magnetic stirring at 16°C, and the fluorescence at 522 nm (slit, 10 nm) was recorded by excitation at 450 nm (slit, 5 nm) 20 min after each addition to allow the system to reach equilibrium. Points were shown after correction for dilution occurring upon each addition. The experimental points, which deviated from the two linear parts of the curve, were used to calculate the dissociation constant as shown in the inset based on the relationship described in Section 2, using the actual values of $[L_o]$ and $[E_o]$, corrected for dilution.

Fig. 4, indicating that maximal activity was attained at 1 mol FAD/mol apoprotein, when the acitivity was measured after preincubation for 60 min before addition of the substrate. Full activity recovery required at least 30-min preincubation as shown in Fig. 8.

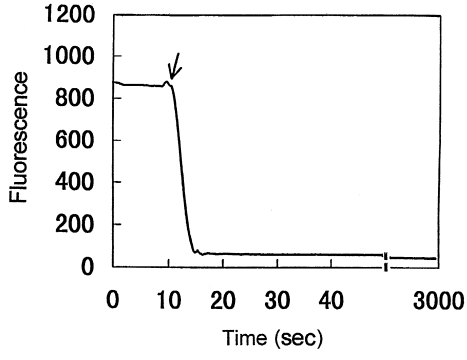

Fig. 6. Quenching of apoprotein fluorescence following FAD addition. Changes in fluorescence at 337 nm (slit, 3 nm) by excitation at 285 nm (slit, 1.5 nm) were followed after mixing 3 μM apoprotein with 60 μM FAD in 250 mM potassium phosphate (pH 7.0) containing 5 mM 2-mercaptoethanol and 0.3 mM EDTA, in a total volume of 2 ml, at 16°C.

The equilibrium binding of FAD to apoprotein was measured by the quenching of FAD fluorescence as shown in Fig. 5. A small number of points that deviated from the two linear parts of the curve were used to calculate the dissociation constant as shown in the inset, and a K_d of 8.2×10^{-12} M was obtained. The amount of FAD which underwent quenching is consistent with a 1:1 molar ratio of binding as observed in the activity titration experiments (Fig. 4).

The kinetics of the formation of the apoprotein–FAD complex were studied by following the quenching of protein fluorescence and flavin fluorescence, and the appearance of catalytic activity. As shown in Fig. 6, protein fluorescence quenching was very rapid and hard to

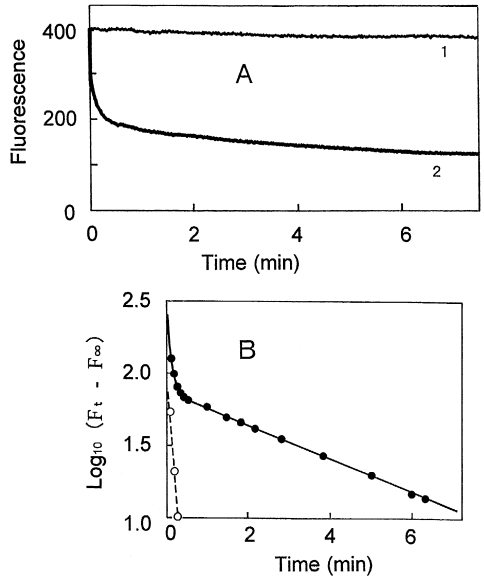

Fig. 7. Quenching of flavin fluorescence following apoprotein addition. (A) 0.5 μM FAD was incubated at 16°C by itself (1) or with 1.9 μM apoprotein (2) in 250 mM potassium phosphate (pH 7.0) containing 5 mM 2-mercaptoethanol and 0.3 mM EDTA, in a total volume of 2 ml, and changes in fluorescence at 525 nm (slit, 10 nm) by excitation at 450 nm (slit, 5 nm) were followed. (B) First-order analysis of the results shown by (2) in (A) with correction for the decrease shown by (1). Fluorescence level observed after 20 to 60 min was employed as the final fluorescence level reached (F_∞) and was subtracted from fluorescence level at indicated times (F_t). (●), primary plot; (○), results obtained for the initial rapid phase after subtracting the contribution of the slow phase.

analyze kinetically. But, it may be seen that a half maximal change occurred in a few seconds after addition of FAD. Similar results were obtained with 0.02 μM apoprotein and 20 μM FAD.

Quenching of FAD fluorescence was rapid initially and continued slowly as shown in Fig. 7A. Fig. 7B indicates that the slow secondary phase subjected to the first order kinetics with a rate constant of 0.27 min^{-1}. When the contribution of this slow phase was subtracted from the data for the initial rapid phase, the differences replotted were apparently in accord with the first order kinetics with a rate constant of 10.6 min^{-1}. It is calculated with this rate constant that a half of the initial rapid quenching is brought about in about 4 s. This suggests a possibility that the rapid phase reflects a same process as shown by the quenching of protein fluorescence.

Fig. 8 shows that the activity was considerably high even at 1 min after mixing of FAD with the apoprotein, and, thereafter, it increased slowly to the full activity. No reliable data were obtained by incubation for periods shorter than 1 min. The high activity at 1 min seems reasonable, since the incubation for activity measurement allowed a further formation of holoenzyme without doubt. On the other hand, the later changes are considered to reflect the effect of preincubation examined. A first order plot of these changes gave the results shown in the inset of Fig. 8, allowing the determination of a rate constant of 0.13 min^{-1}. When similar experiments were performed with 0.02, 0.2 and 2 μM apoprotein at a constant FAD concentration of 20 μM, the rate constant appeared independent of apoprotein concentrations, being 0.16 ± 0.10 min^{-1}.

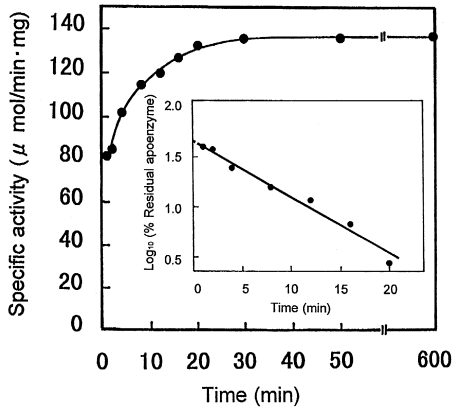

Fig. 8. Time course of enzyme activity recovery on addition of FAD to apoprotein. 3 μM apoprotein was mixed with 3 μM FAD in 250 mM potassium phosphate (pH 7.0) containing 5 mM 2-mercaptoethanol and 0.3 mM EDTA, in a total volume of 0.445 ml at 16°C. 10-μl aliquots were withdrawn from the mixture at the indicated times and assayed for holoenzyme activity in a total volume of 1 ml by 10-min incubation at 37°C. The inset shows the first order plot of the results.

4. Discussion

In the present work, apoprotein of *C. humicolus* D-aspartate oxidase was successfully prepared essentially according to the method of Massey and Curti [7], which depends on the ability of Br^- to weaken the binding of flavin to apoprotein [13]. In this respect, the present method was the same as that used for removal of FAD from bovine kidney D-aspartate oxidase [1], but differed in that 2-mercaptoethanol and high concentrations of phosphate ions were also required. Phosphate ions were effective to stabilize apoprotein as pointed out by Casalin et al. [8], who prepared apoprotein of D-amino acid oxidase of *Rhodotorula gracilis*. Requirement for 2-mercaptoethanol was obvious since its omission induced protein precipitation, whereas no such requirement has been reported for bovine D-aspartate oxidase. This difference may be related to the finding that the present enzyme, a tetrameric protein, dissociates into apoprotein monomers when FAD is removed, while the bovine enzyme is a monomer both in its holo-and apo-form. It is probable that SH groups become exposed to the environment by the dissociation into monomers and oxidized.

A K_d of 8.2×10^{-12} M was calculated for the dissociation of FAD from D-aspartate oxidase from the binding experiments based on the

quenching of FAD fluorescence. Although this value may be vulnerable to error due to the small number of data used for calculation, it seems to be accurate enough to roughly characterize the enzyme. This value is markedly lower than that determined for the bovine D-aspartate oxidase (5.0×10^{-8} M) [1], *R. gracilis* D-amino acid oxidase (2.0×10^{-8} M) [8] and pig D-amino acid oxidase (2.2×10^{-7}M) [14]. Considering that the dissociation constant for most flavoproteins is of the order of 10^{-7} to 10^{-10} M [15], the binding in the present enzyme may be classified among the tightest ones. This tight binding as well as instability of the apoprotein in the absence of high concentrations of phosphate ions and SH protecting agents may suggest that the apoenzyme is not present in the yeast cells under physiological conditions.

Kinetic studies of the formation of holoenyzme from FAD and apoprotein have shown that it is at least a two-stage process.

The first stage was shown by the very rapid quenching of protein fluorescence and the rapid phase of FAD fluorescence quenching. Probably, this stage reflects at least the binding of FAD to monomeric apoprotein. The second stage was shown by the secondary slow phase of quenching of FAD fluorescence and the activity appearance, both of which subjected to the first-order kinetics with comparable rate constants. In addition, this phase of activity appearance seemed to be independent of protein concentrations. These observations suggest that this stage is associated with some intramolecular changes which are essential for activity. The changes could be in the conformation of the protein at the active site so that the bound FAD might be held in a correct manner as required for the catalytic activity, thus converting a holoenzyme precursor to the active holoenzyme.

Since apoprotein is monomeric, while holoenzyme is tetrameric, tetramerization must occur between initial binding of FAD to monomeric apoprotein and final formation of active holoenzyme. Since the activity appearance was not dependent on apoprotein concentrations, tetramerization step is unlikely to be included in the second stage. On the other hand, it remains possible that tetramerization occurs very rapidly following the formation of monomeric apoprotein–FAD complex and cannot be observed separately from the first stage in the present experiments. Further work is required to confirm this possibility.

For the present, we might assume that the first stage includes the following process to produce inactive holoenzyme precursor: $4(E + FAD) \rightleftharpoons 4(E \cdot FAD) \rightleftharpoons (E \cdot FAD)_4$, and the second stage produces the holoenzyme: $(E \cdot FAD)_4 \rightleftharpoons$ Holoenzyme

References

[1] A. Negri, V. Massey, C.H. Williams Jr., J. Biol. Chem. 262 (1987) 10026.
[2] A. D'Aniello, E. Rocca, Comp. Biochem. Physiol. B 41 (1972) 625.
[3] R. Yamada, H. Ujiie, Y. Kera, T. Nakase, K. Kitagawa, T. Imasaka, K. Arimoto, M. Takahashi, Y. Matsumura, Biochim. Biophys. Acta 1294 (1996) 153.
[4] S. Fukunaga, S. Yuno, M. Takahashi, S. Taguchi, Y. Kera, S. Odani, R. Yamada, J. Ferment. Bioeng. 85 (1998) 579.
[5] T. Simonic, S. Duga, A. Negri, G. Tedeschi, M. Malcovati, M.L. Tenchini, S. Ronchi, Biochem. J. 322 (1997) 729.
[6] C. Setoyama, R. Miura, J. Biochem. 121 (1997) 798.
[7] V. Massey, B. Curti, J. Biol. Chem. 241 (1966) 3417.
[8] P. Casalin, L. Pollegioni, B. Curti, M. Pilone Simonetta, Eur. J. Biochem. 197 (1991) 513.
[9] R. Yamada, H. Nagasaki, Y. Wakabayashi, A. Iwashima, Biochim. Biophys. Acta 965 (1988) 202.
[10] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[11] D.R. Light, C. Walsh, M.A. Marletta, Anal. Biochem. 109 (1980) 87.
[12] R.A. Stinson, J.J. Holbrook, Biochem. J. 131 (1973) 719.
[13] E. Walaas, O. Walaas, Acta Chem. Scand. 10 (1956) 122.
[14] V. Massey, H. Ganther, Biochemistry 4 (1965) 1161.
[15] M. Husain, V. Massey, in: S. Fleischer, L. Packer (Eds.), Methods in Enzymology Vol. 53 Academic Press, New York, 1978, p. 429.