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Purification of Electron-Transferring Flavoprotein from *Megasphaera elsdenii* and Binding of Additional FAD with an Unusual Absorption Spectrum

Kyosuke Sato*, Yasuzo Nishina and Kiyoshi Shiga

Department of Molecular Physiology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Kumamoto, 860-8556

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Electron-transferring flavoprotein (ETF), its redox partner flavoproteins, *i.e.*, D-lactate dehydrogenase and butyryl-CoA dehydrogenase, and another well-known flavoprotein, flavodoxin, were purified from the same starting cell paste of an anaerobic bacterium, *Megasphaera elsdenii*. The purified ETF contained one mol FAD/mol ETF as the sole non-protein component and bound almost one mol of additional FAD. This preparation is a better subject for investigations of *M. elsdenii* ETF than the previously isolated ETF, which contains varying amounts of FAD and varying percentages of modified flavins such as 6-OH-FAD and 8-OH-FAD. The additionally bound FAD shows an anomalous absorption spectrum with strong absorption around 400 nm. This spectral change is not due to a chemical modification of the flavin ring because the flavin released by KBr or guanidine hydrochloride is normal FAD. It is also not due to unknown small molecules because the same spectrum appears when ETF is reconstituted from its guanidine-denatured subunits and FAD. A similar anomalous spectrum was observed for AMP-free pig ETF under acidic conditions, suggesting a common flavin environment between pig and *M. elsdenii* ETFs.

Key words: electron-transferring flavoprotein, FAD binding, FAD release, *Megasphaera elsdenii*, unusual absorption spectrum.

Abbreviations: AFR, activity to flavin ratio; BCD, butyryl-CoA dehydrogenase; DLDH, D-lactate dehydrogenase; DTT, dithiothreitol; ETF, electron-transferring flavoprotein; FAD, flavin adenine dinucleotide.

Electron-transferring flavoproteins (ETF) have been isolated and characterized from several species (*1*). They are classified into two groups. One group includes ETFs from mammals (*2*, *3*), the methylotrophic bacterium *Methylophilus methylotrophus* (*4*, *5*), and soil bacterium *Paracoccus denitrificans* (*6*, *7*). These ETFs are heterodimeric, contain one non-covalently bound FAD and one AMP per molecule, and function as electron carriers between other flavoproteins such as acyl-CoA dehydrogenases, sarcosine dehydrogenase, ETF-CoQ oxidoreductase, and trimethylamine dehydrogenase depending on the source species (*1*). The other group includes the ETF from an anaerobic bacterium, *Megasphaera elsdenii* (previously called *Peptostreptococcus elsdenii*). This ETF is also a heterodimer but contains two FAD molecules (*8*). The presence of AMP has not been reported. The function of *M. elsdenii* ETF is to transfer electrons between flavoproteins, D-lactate dehydrogenase (DLDH) (*9*) and butyryl-CoA dehydrogenase (BCD), and additionally to oxidize NADH (*8*). Recently, ETF was isolated from the anaerobic bacterium *Clostridium propionicum* as a tight complex with propionyl-CoA dehydrogenase (*10*). This ETF is probably included in the second group because it oxidizes NADH and contains no AMP, although the FAD content is unclear.

The three-dimensional structures of the human (*11*) and *P. denitrificans* (*12*) ETFs have been published. In both structures, the FAD resides in the peripheral cleft between the two subunits, while the AMP is entirely buried in the smaller subunit. The three-dimensional structure of *M. elsdenii* ETF has not been solved. A comparison of the amino acid sequence of *M. elsdenii ETF* with other ETFs of known three-dimensional structure has previously been described (*12*, *13*). Although the sequence similarity at the FAD- and AMP-binding sites was recognized, it is still unclear whether the AMP in the first group corresponds to one FAD or NAD(H) in the second group.

Our interest is in the function of the AMP contained in the ETF of the first group. We discovered the existence of AMP in the pig ETF (*3*). AMP-free ETF can be prepared by denaturation and renaturation in the absence of AMP. AMP-free ETF and holo-ETF show the same enzymatic activity of electron transfer from acyl-CoA dehydrogenase to an artificial electron acceptor, dichlorophenolindophenol. A function of the AMP that we recognized is its effect on the assembly process of the components of ETF: AMP enhances the rate of formation of the holoprotein (*3*, *14*).

It is expected that another function of the AMP will be recognized by comparison of ETFs in the different groups because the AMP in the first group is probably related evolutionally to one FAD or NAD(H) in the second group. Therefore, we began a study of the *M. elsdenii* ETF. Native (*8*) and recombinant (*13*) *M. elsdenii* ETF have

*To whom correspondence should be addressed. Tel: +81-96-373-5052, Fax: +81-96-373-5052, E-mail: satok@medic.kumamoto-u.ac.jp

been purified, but they involve some complexities. The isolated native ETF contains about 1.4 mol FAD per ETF molecule (*8*, *15*). It also contains 6-OH-FAD and 8-OH-FAD as well as normal FAD. The proportion of these modified FADs are 1–50% of the total flavin (*8*) depending on the preparation. The modified FADs are thought not to exist *in vivo* but to be generated during purification upon being exposed to oxygen (*13*, *15*), although experimental evidence for this has not been published. The recombinant ETF was isolated from extracts of *Escherichia coli* by a different purification procedure (*13*). The purified recombinant ETF contains 1.06 mol FAD per ETF. Modified FADs such as 6-OH-FAD and 8-OH-FAD are not present. The recombinant ETF binds 0.5–0.6 mol of additional FAD per mol of pre-bound FAD, and the FAD-saturated ETF contains 1.6–1.8 mol FAD (*13*). This suggests that the recombinant ETF preparation has lost some FAD-binding ability.

In the past, most research on *M. elsdenii* ETF has used isolated native ETF, which varies from preparation to preparation in FAD content and in the percentage of modified flavins. This makes the interpretation of the experimental results somewhat unclear. In this study, we isolated native ETF by a revised procedure. The ETF obtained contained one mol FAD without modified flavins and retained FAD-binding ability allowing recovery of the almost complete holoprotein with two mol FAD bound. This preparation will be useful for investigating the functions of the two FADs. In addition to ETF, our procedure resulted in the simultaneous isolation of the redox partners of ETF, *i.e.*, DLDH and BCD, and another well-known flavoprotein, flavodoxin. Only three chromatography columns were used to prepare the four flavoproteins, which minimizes time and labor. The simultaneous preparation of ETF, DLDH, and BCD will encourage studies on the interactions between these flavoproteins.

The FAD-saturated ETF shows an anomalous flavin absorption spectrum, showing strong absorption around 400 nm (*8*, *13*). The nature of this unusual spectrum has not been solved. We found in this study that pig ETF shows a similar spectrum under certain conditions. This finding will provide a clue for understanding the unusual flavin spectrum of *M. elsdenii* ETF.

MATERIALS AND METHODS

Reagents—$ZnCl_2$ was obtained from Wako Pure Chemicals Industries. NADH was obtained from Sigma. 2,6-Dichlorophenolindophenol (DCIP) was obtained from Katayama Chemical. All other chemicals were obtained from Nacalai Tesque. FAD was further purified as described (*3*). Ferricenium hexafluorophosphate ($Fc^+PF_6^-$) (*16*) and butyryl-CoA (*17*) were prepared as described previously.

General—A Hitachi U-3210 spectrophotometer and a Shimadzu MultiSpec-1500 photodiode array spectrophotometer were used for spectrophotometric measurements. The N-terminal amino acid sequence was determined using an Applied Biosystems 477A Protein Sequencer. SDS-PAGE was carried out (*18*) using vertical slab gels of 12.5% concentration. Protein bands were stained with Coomassie Brilliant Blue, and molecular weights were estimated using Precision Protein Standards (Bio-Rad).

HPLC Analysis of Guanidine Extracts of Flavoproteins—About 10 μl of about 200 μM flavoprotein solution was mixed with three volumes of aqueous solution of 8 M guanidine hydrochloride and then ultrafiltered using a Microcon-10 centrifugal filter (Millipore). The filtrate was analyzed by C18 reverse phase chromatography. The column used was Cosmosil 3C18 (Nacalai Tesque), and the elution was carried out with a 20-min linear gradient from 10 to 60% methanol in 10 mM potassium phosphate ($K\text{-}P_i$) buffer (pH 7) at a flow rate 0.5 ml/min. Detection was with an MCPD-3600 spectro multi channel photo detector (Otsuka Electronics) with recording of the absorption spectrum (250–800 nm) at each time (2-s intervals).

UV-LS-RI Method—Gel filtration HPLC was performed with a Superdex 200 HR 10/30 column (Amersham Biosciences) at a flow rate of 0.5 ml/min at 20°C with a running buffer of 50 mM $K\text{-}P_i$ (pH 6). The eluate was monitored by the following detectors connected in series: an L-4200 UV-VIS detector (Hitachi), an L-8000 low-angle laser light scattering photometer (Tosoh) with a He-Ne laser (wavelength 632.8 nm) as a light source, and an RI-8011 differential refractometer (Tosoh) with a light-emitting diode (wavelength 660 nm) as a light source. The peak heights of the outputs from the three detectors, denoted as *LS* (light scattering), *UV* (ultraviolet-visible absorbance), and *RI* (refractive index) can be represented by the following equations (*19*, *20*):

$$LS = k\left(\frac{dn}{dc_w}\right)^2 Mc_w \quad (1)$$

$$UV = k'\varepsilon c_m \quad (2)$$

$$RI = k''\left(\frac{dn}{dc_w}\right)c_w \quad (3)$$

where dn/dc_w is specific refractive increment, M is molecular weight, c_w is weight concentration, ε is molar extinction coefficient, and c_m is molar concentration. Above and below, k and K represent instrument constants. Equations 1 and 3 lead to

$$M = K\frac{LS}{(dn/dc_w)RI}.$$

For simple proteins, the value of dn/dc_w is almost constant (*19*, *20*). Therefore the molecular weight of a sample can be determined by comparing *LS*/*RI* values between the sample and standards of known molecular weights. Equations 1, 2, and 3 lead to

$$\varepsilon = K'\frac{LS \times UV}{RI^2}.$$

The ε value of a sample can be determined by comparing $LS \times UV/RI^2$ values between the sample and standards of known ε values.

The standard proteins were bovine serum albumin (M = 66,296, ε_{278} = 43,623 $M^{-1}\cdot cm^{-1}$) and ovalbumin (M = 42,756, ε_{280} = 29,972 $M^{-1}\cdot cm^{-1}$) purchased from Sigma. The M and ε values are from the literature (*21*).

Spectrophotometric Titration of ETF with FAD—The titration was carried out at 25°C using a made-to-order spectrophotometric microcell with a screw cap. A 100 μl sample in the cell was titrated with FAD using a Hamil-

ton gas-tight microsyringe (10 μl) fitted to a Hamilton repeating dispenser. At all time except during the addition of the titrant, the screw cap was kept closed to prevent evaporation of the sample. The absorbance change was completed during the operation of mixing (about 10 s). The absorption spectrum was measured after each addition of titrant. Each spectrum was corrected for dilution to 100 μl, and then the spectra were combined to a matrix form, where columns represent the absorption spectrum at each FAD concentration and rows represent the titration profile at each wavelength. The matrix was analyzed by the matrix least squares method based on singular value decomposition (*22*). This analysis uses titration profiles at all wavelengths to determine parameters such as the dissociation constant and FAD concentration at a 1:1 molar ratio, and simultaneously to determine the spectra of the three components (initial ETF, FAD-saturated ETF, and free FAD). The computer program was written and run on IGOR Pro version 4 for Windows (WaveMetrics).

Enzyme Assay—All assays were carried out at 25°C in 50 mM K-P_i (pH 7). The diaphorase activity of ETF was assayed with 50 μM NADH and 50 μM DCIP. The reduction of DCIP was monitored by the decrease in absorbance at 600 nm. LDH activity was assayed with 0.2 M sodium DL-lactate and 1 mM potassium ferricyanide. The reduction of ferricyanide was monitored by the decrease in absorbance at 420 nm. BCD activity was assayed with 200 μM butyryl-CoA and 200 μM $Fc^+PF_6^-$. The reduction of $Fc^+PF_6^-$ was monitored by the decrease in absorbance at 300 nm. In all assays, one unit/ml was defined as the concentration of enzyme giving an absorbance decrease of 1 min^{-1} with the lightpath 1 cm. The activity to flavin ratio (AFR) was defined as the ratio of units/ml to absorbance at 450 (ETF), 454 (DLDH), and 430 nm (BCD). Concentrations were determined from the molar extinction coefficients: ε_{340} = 6.22 $mM^{-1}\cdot cm^{-1}$ for NADH, ε_{600} = 21.0 $mM^{-1}\cdot cm^{-1}$ for DCIP, ε_{260} = 16.4 $mM^{-1}\cdot cm^{-1}$ for butyryl-CoA (*23*), ε_{617} = 0.41 $mM^{-1}\cdot cm^{-1}$ for $Fc^+PF_6^-$ (*16*).

Bacterial Cell Paste—The anaerobic bacterium *Megasphaera elsdenii* (JCM 1772), obtained from the Japan Collection of Microorganisms (the Institute of Physical and Chemical Research, Saitama), was grown in the medium described by Engel (*24*) at 37°C in 50-l culture. The cell paste (160 g) collected by centrifugation was stored at –30°C until used.

Purification of Flavoproteins—The frozen cell paste was thawed at 4°C for 20 h and then suspended in 50 mM K-P_i (pH 6) with a total volume of 800 ml. Cells in a glass beaker surrounded by an ice-water bath were broken using a Model 250 Sonifier (Branson Ultrasonics) with power output 9 and duty cycle 50% for 7 cycles of 3-min sonication and 2-min rest.

All steps described below were carried out at 4°C. Cell debris and denatured proteins were removed by centrifugation (4 times of 30 min × 10,000 ×*g*), and the cell extract was applied to a DEAE-cellulose (Whatman DE-52) column (diameter × height = 4.4 × 35 cm) equilibrated with 50 mM K-P_i (pH 6). The column was washed with 1,100 ml of 0.1 M K-P_i (pH 6). Of the flavoproteins adsorbed by DE-52, ETF was first eluted with a 2,600 ml linear gradient from 0.1 to 0.3 M K-P_i (pH 6). Diaphorase activity peak was observed with the A_{450} peak. Fractions with a diaphorase activity greater than half the peak activity were pooled (crude ETF). The column was then washed with 2,200 ml of 0.2 M K-P_i (pH 7) containing 1 mM dithiothreitol (DTT). DTT is essential for the recovery of DLDH activity (*25*) but interferes with the diaphorase assay, so it was not present during ETF elution. During washing, a pink rubredoxin band moved faster than the large yellow flavodoxin band, the leading part of which contained greenly fluorescent DLDH; the green BCD band broadened somewhat but stayed near the top of the column. At the end of the washing step, some of the rubredoxin band had eluted. DLDH and flavodoxin were eluted with 1,800 ml of 0.2 M K-P_i (pH 7), 0.1 M KCl and 1 mM DTT. A small DLDH peak was observed at the leading edge of the large flavodoxin peak as a shoulder on the A_{450} profile. Fractions with LDH activities more than 20% of the peak activity were pooled (crude DLDH). The rest of the yellow fractions with an A_{450} more than 20% of the peak value were collected (crude flavodoxin). At this point, the green BCD band had broadened to occupy almost all the column space. BCD was eluted with a 1,100 ml gradient from 0.1 to 1.0 M KCl in 0.2 M K-P_i (pH 7). Fractions with an A_{450} more than 20% of the peak value were pooled (crude BCD).

(A) ETF Purification—The buffer used for ETF purification was 50 mM K-P_i (pH 6) containing 0.3 mM EDTA. Solid ammonium sulfate was added to the crude ETF preparation to 30% saturation, and the solution was applied to a Phenyl Sepharose CL-4B (Amersham Biosciences) column (2.5 × 28 cm) equilibrated with 30% ammonium sulfate in the buffer. The ETF band bound tightly to the top of the column. The column was washed with 600 ml equilibrium buffer. While the ETF band moved very slowly, a broader free FAD band was eluted. ETF was eluted with a linear gradient formed by 500 ml of 30% ammonium sulfate in the buffer and 500 ml of 50% v/v ethylene glycol in the buffer. Fractions with an A_{450} more than 50% of the peak value were pooled. The solvent of the ETF solution was changed to 0.2 M KCl in the buffer by cycles of concentration and dilution using an Amicon Stirred Cell Model 8200 with a YM30 ultrafiltration membrane (Millipore) until more than 99% replacement was achieved. The solution was diluted 5-fold with pure water and applied to a Q-Sepharose (Amersham Biosciences) column (2.2 × 18.5 cm) equilibrated with the buffer. The ETF band bound tightly to the top of the column was developed with 560 ml of 0.1 M KCl in the buffer and then the broadened band was eluted with a 560 ml linear gradient from 0.1 to 0.3 M KCl in the buffer. The leading edge contained protein impurities and the trailing edge contained ETF with bound 6-OH-FAD. The fractions with an A_{450} more than 60% of the peak value were collected, diluted 5-fold with pure water, and reapplied to the same Q-Sepharose column, which was re-activated with 1 M KCl and equilibrated without repacking. ETF was eluted and the fractions were collected in the same way as above. The solvent of the collected ETF was changed to 0.2 M KCl in the buffer with a Stirred Cell, and the ETF was stored frozen at –80°C.

(B) DLDH Purification—Concentrated solutions of DTT and $ZnCl_2$ were added to the crude DLDH preparation to concentrations of 1 and 0.1 mM, respectively. After

1 d, the LDH activity had increased about 2-fold. The addition of 0.1 mM FAD had little effect on the recovery. To this solution, solid ammonium sulfate was added to 40% saturation. After removing small particles by centrifugation, the solution was applied to the Phenyl Sepharose column (the same column used for ETF purification and re-activated without repacking with 30% 2-propanol as described in the instruction manual for Phenyl Sepharose) equilibrated with 40% ammonium sulfate in 50 mM K-P_i (pH 7) and 1 mM DTT. Elution was carried out with a 1,000 ml linear gradient from 40 to 0% ammonium sulfate in 50 mM K-P_i (pH 7) and 1 mM DTT, and an additional 200 ml of final buffer. A large flavodoxin band moved faster than the fluorescent DLDH band, and the two were completely separated. The DLDH fractions with an A_{450} more than 20% of the peak value were collected, concentrated using a Stirred Cell with a YM30 membrane, and stored frozen at –80°C.

(C) Flavodoxin Purification—The crude flavodoxin was further purified by the same chromatography procedure (Phenyl Sepharose) as DLDH but without preincubation with DTT and $ZnCl_2$ and without DTT in the running buffer. Fractions with an A_{450} more than 20% of the peak value were collected. This flavodoxin solution contained some visible rubredoxin on SDS-PAGE. The solvent of the solution was exchanged for 50 mM K-P_i (pH 7) by cycles of concentration-dilution using a Stirred Cell with YM10 membrane until more than 99% replacement was achieved. The sample was applied to the Q-Sepharose column used for ETF purification and re-activated without repacking and equilibrated with 50 mM K-P_i (pH 7). The column was washed with 420 ml of 0.2 M K-P_i (pH 7). The pink rubredoxin band and a small part of the leading edge of the flavodoxin band were eluted. The remaining main part of the flavodoxin was eluted with a 500 ml linear gradient from 0 to 0.5 M KCl in 0.2 M K-P_i (pH 7). Fractions that gave only a flavodoxin band on SDS-PAGE were collected and the precipitate obtained by centrifugation after 90% saturation of ammonium sulfate was stored at 4°C.

(D) BCD Purification—The crude BCD was subjected to ammonium sulfate fractionation (55–75% saturation) and the obtained precipitate was dialyzed against 50 mM K-P_i (pH 7). After removing the cloud of denatured protein by centrifugation, solid ammonium sulfate was added to 20% saturation and the solution was applied to the Phenyl Sepharose column (the same as above) equilibrated with 20% ammonium sulfate in 50 mM K-P_i (pH 7). Elution was carried out with a 580 ml linear gradient from 20 to 0% ammonium sulfate in 50 mM K-P_i (pH 7) and an additional 340 ml of the final buffer. Fractions with an A_{450} more than 20% of the peak value were collected. BCD was stored at 4°C as the precipitate obtained by 75% ammonium sulfate.

Denaturation-Renaturation of ETF—A solution of denatured subunits free from small molecules was prepared by repeated cycles of concentration and dilution using a Centricon-30 (Millipore) with 6 M guanidine hydrochloride in 50 mM K-P_i (pH 6) and 10 mM DTT at 25°C. The concentrated denatured protein (200–400 μM of each subunit) was then quickly diluted to 2 μM in 50 mM K-P_i (pH 6) containing 1 mM DTT, and 10 μM FAD. After 4-h incubation at 25°C, the solution was passed through a Millex-LH filter (0.45-μm pore size, Millipore) and subjected to cycles of ultrafiltration and dilution with 50 mM K-P_i (pH 6) at 4°C to remove free FAD. The recovery of the protein-bound FAD was about 140% of the starting ETF.

Pig ETF—The holoprotein of ETF was purified from pig kidney by the reported method (*2*). AMP-free ETF was prepared by the same denaturation-renaturation method described above for *M. elsdenii* ETF except for the following points: a Centricon-10 was used, the buffer pH was 7.6, the incubation and storage buffer contained 5% v/v glycerol, and the FAD concentration for incubation was 3 μM. This is a revision of our previously reported method (*3*). The recovery of protein-bound FAD was about 60% of the starting holoprotein. The resulting AMP-free ETF showed a single band on isoelectric focusing at the position of pI = 7.1, which is consistent with the previous report (*3*) and distinct from that of the holoprotein (pI = 6.7).

RESULTS AND DISCUSSION

Protein Purification—The present purification procedure provides four flavoproteins from the same starting material. The entire purification was completed in 10 d by one person. The isolated flavoproteins were pure on SDS-PAGE (Fig. 1). The determined protein molecular mass values indicated in the figure are almost the same as the previously reported values: 29.1 and 36.1 kDa (ETF, from amino acid sequence) (*13*), 55 kDa (DLDH, SDS-PAGE) (*25*), 25 kDa (flavodoxin, SDS-PAGE) (*26*), and 41.4 kDa (BCD, from amino acid sequence) (*27*). The yields of ETF, DLDH, and flavodoxin were as much as the previous yields, but that of BCD was significantly lower (Table 1). The reason for this low yield is unknown. Oxidation of the cell extract by stirring in air (*24*) was omitted in the present procedure. This is not the cause of the low yield because the amount of the purified BCD was not increased even when we employed the oxidation step. Omitting the oxidation step resulted in BCD with a lower A_{710}/A_{430} ratio (0.1) than the previous method (0.33–0.46) (*28*). A low A_{710}/A_{430} ratio implies a low content of CoA persulfide bound to the active site (*29*) and has the disadvantage of causing the instability of BCD (*30*). However, we did not employ the oxidation step because it damages DLDH activity. When the oxidation step was included, the AFR of the resulting DLDH was about 80, while the AFR was more than 100 when the step was omitted (Table 1), which is as high as that of the previous preparation (*25*).

The enzyme activity of flavodoxin was not examined. The identity of our preparation of flavodoxin was confirmed by the following evidence. The N-terminal amino acid sequence of the purified enzyme was determined to be MVEIVYWSGTGNTEAMANEI, which is identical to the reported sequence (*31*, *32*). As described above, the molecular mass determined by SDS-PAGE was the same as in the previous report (*26*) (note that the true molecular mass is 14.5 kDa as determined from the amino acid sequence). The cofactor liberated by 6 M guanidine hydrochloride as judged by C18 reverse phase chromatography was flavin mononucleotide (FMN), as reported (*33*).

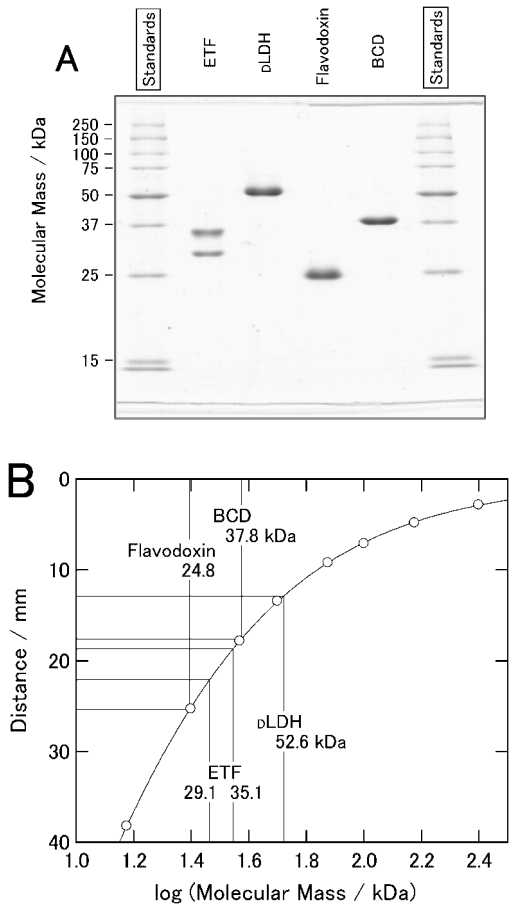

Fig. 1. **SDS-PAGE of the purified flavoproteins.** A: SDS-PAGE gel after staining. Amounts of sample flavoproteins were all 1 μg. B: Molecular mass determination. Circles show the data for standards and the perpendicularly connected lines show the data for the purified flavoproteins.

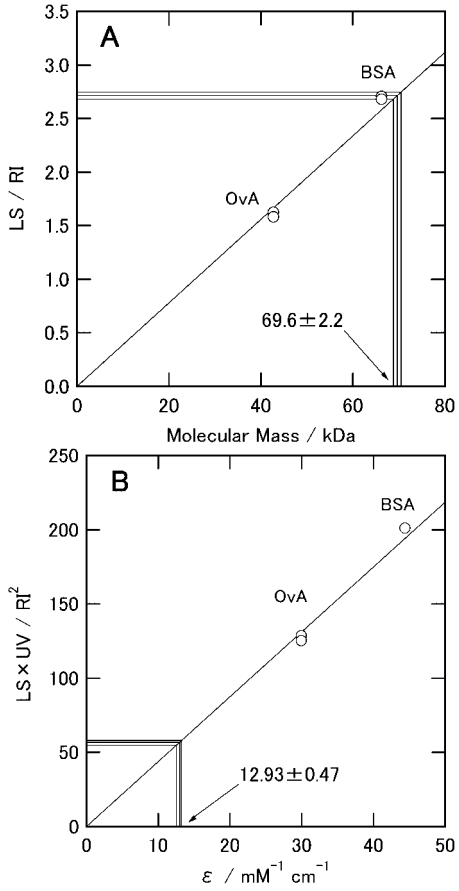

Fig. 2. **Determination of molecular weight (A) and molar extinction coefficient at 450 nm (B) of purified ETF by UV-LS-RI method.** The standards were bovine serum albumin (BSA) and ovalbumin (OvA). Two different amounts of each standard were used (n_{std} = 4). The data for the ETF sample are shown by perpendicularly connected lines. Three different amounts of ETF were analyzed (n_{smp} = 3). Calculation was as follows. The mean value (x_0) was calculated from $x_0 = y_0/r_0$, where y_0 is the mean of the y values of the samples and r_0 is the mean of the y/x value of the standards. The error (Δx) was calculated by $\Delta x = \Delta y/r_0 + y_0\Delta r/r_0^2$, where Δy is the standard deviation of y of the samples divided by $\sqrt{n_{smp}-1}$ and Δr is the standard deviation of y/x of the standards divided by $\sqrt{n_{std}-1}$.

Properties of the Purified ETF—The native molecular mass of the purified ETF was determined to be 69.6 ± 2.2 kDa by the UV-LS-RI method (Fig. 2). The value was independent of ETF concentration (0.8–9 μM) at elution from the column, indicating that ETF does not exist in an equilibrium of multimerization in this concentration range. The value is essentially the same as the sum of the two subunits (36.1 + 29.1 = 65.2 kDa) (*13*), consistent with a heterodimer. It is also similar to the 72–75 kDa value estimated by gel filtration (*8*).

Table 1. **Purification of Enzymes.** The yields are in mg protein per 100 g starting wet cell paste. AFR (activity to flavin ratio) is defined in "MATERIALS AND METHODS."

	Previous[a]	Present	
Enzyme	Yield	Yield	AFR
ETF	27[b]	27	707
DLDH	34	36	103
Flavodoxin	156[bc], 64[bd]	131	n.d.[e]
BCD	200–400[b]	42	560

[a]Obtained from the literature: ETF (*8*), DLDH (*25*), flavodoxin (*33*), and BCD (*28*). [b]Cited in the literature based on dry weight of the starting cells, and thus corrected using the ratio by which 140 g wet cell corresponds to 28 g dry weight (*24*). [c]After the first crystallization, where the sample shows almost the same specific activity as the final one. [d]Final purified enzyme after the fourth crystallization. [e]Not determined.

The bound flavin was solely FAD as judged by liberation by guanidine hydrochloride and C18 reverse phase chromatography (Fig. 3a). Other flavins such as 8-OH-FAD and 6-OH-FAD, which are found in the ETF obtained by the previous method (*8*), were not detected. The ETF fraction removed by the Q-Sepharose steps contained 6-OH-FAD as well as normal FAD (Fig. 3b) as described in "MATERIALS AND METHODS." AMP, which is contained in ETF from other sources, was not detected. An investigation of the presence of AMP in *M. elsdenii* ETF has not been described previously (*8*, *13*).

The purified ETF showed a typical flavin spectrum (Fig. 4a), with absorbance maxima at 272 nm (6.1), 374 nm (0.79), and 450 nm (1.0) (the values in parentheses are relative absorbance values). Two kinds of molar extinction coefficient at 450 nm were determined. First,

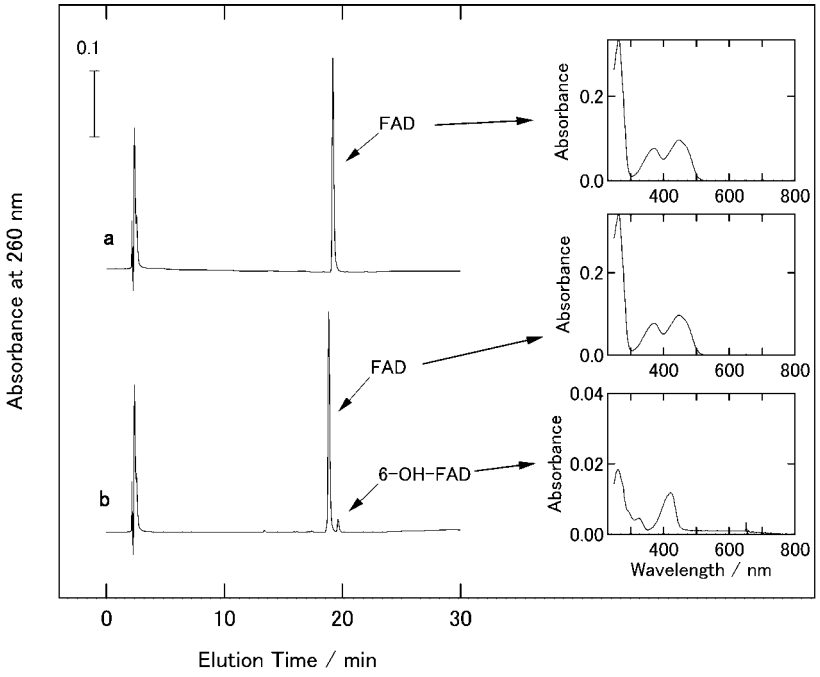

Fig. 3. **C18 reverse phase HPLC of guanidine extract of ETF.** The samples were purified ETF (a) and the fraction at the trailing edge of the ETF peak on the last two Q-Sepharose chromatography steps (b). The noisy peaks at 2.5 min are due to guanidine hydrochloride contained in the samples. The insets show the peak absorption spectra monitored by the photodiode array detector. The main peaks at 19 min were identified as FAD by comparing the position with authentic FAD and the absorption spectrum. 6-OH-FAD was identified by comparing the absorption spectrum with reported spectrum (*39*). The bar at the left indicates the absorbance scale.

the absorption per molar concentration of bound FAD was determined to be ε_{450} = 11.3 $mM^{-1}\cdot cm^{-1}$ by comparing the spectra of native and 6-M guanidine-denatured ETFs and those of FAD under the same native and denaturing conditions at 25°C with the known extinction coefficient of FAD. The spectra of both ETF and FAD under the denaturing conditions were the same, confirming that FAD bound to ETF was completely liberated under these denaturing conditions. The same value has been reported for recombinant ETF (*13*). Second, the absorption per molar concentration of ETF molecule was determined by the UV-LS-RI method to be ε_{450} = 12.93 ± 0.47 $mM^{-1}\cdot cm^{-1}$ (Fig. 2B). From these two ε_{450} values, the FAD content of the present ETF is calculated to be 1.14 ± 0.04 mol FAD/mol of ETF. The results obtained by the UV-LS-RI method could be slightly erroneous and the FAD content should be taken to be 1.0 rather than 1.14, as discussed later.

The purified ETF bound additional FAD as also reported for the previous preparations (*8*, *13*). FAD titration resulted in the binding of 90% more FAD with a dissociation constant of 0.07 μM (inset to Fig. 4). Because the initial ETF contained one mol FAD, FAD-saturated ETF contains 1.9 mol FAD. The absorption spectrum of FAD-saturated ETF (curve b in Fig. 4) shows an anomalously strong absorption around 400 nm. The A_{400}/A_{450} ratio increased considerably from 0.44 (initial form) to 0.88 (FAD-saturated form).

Assuming that the spectrum of the originally bound FAD is not changed by the binding of the second FAD, the spectrum of the second FAD can be illustrated by curve a in Fig. 5, which is the difference spectrum between the FAD-saturated and initial ETFs. This spectrum is very unusual for a flavin spectrum: the absorption band that is normally located around 370 nm is largely shifted rightward to about 400 nm. The extinction coefficients at the peaks are 9.8 $mM^{-1}\cdot cm^{-1}$ at 450 nm and 14.3 $mM^{-1}\cdot cm^{-1}$ at 401 nm. The nature of this unusual spectrum is discussed later. Curve c in Fig. 5 is the difference spectrum of curve a against the spectrum of free FAD

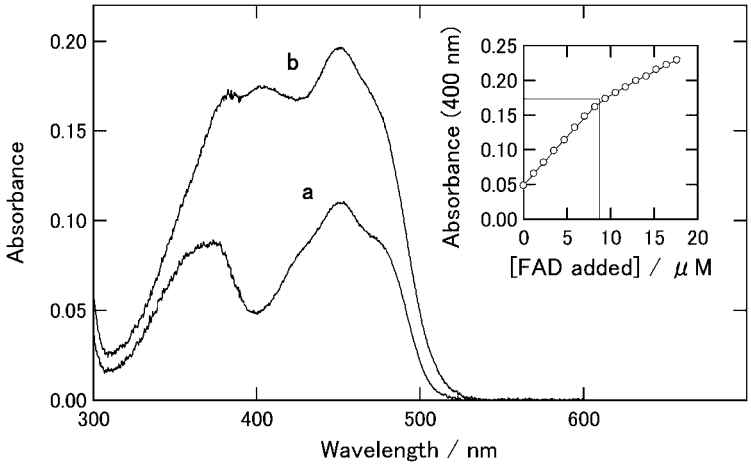

Fig. 4. **Absorption spectrum of ETF.** A 100 μl solution of 9.77 μM purified ETF (a) was titrated with 587 μM FAD (0.2 μl per addition) at 25°C in 50 mM K-P_i, pH 6, containing 6 mM KCl, which was derived from a stock solution of ETF. The spectrum of the FAD-saturated ETF (b) was obtained by analyzing the set of spectra recorded during the titration. The inset shows the titration plot of absorbance at 400 nm and a theoretical curve with a dissociation constant of 0.07 μM and an added FAD concentration at 1:1 molar ratio of 8.76 μM (solid curve). The perpendicularly connected lines indicate the 1:1 point.

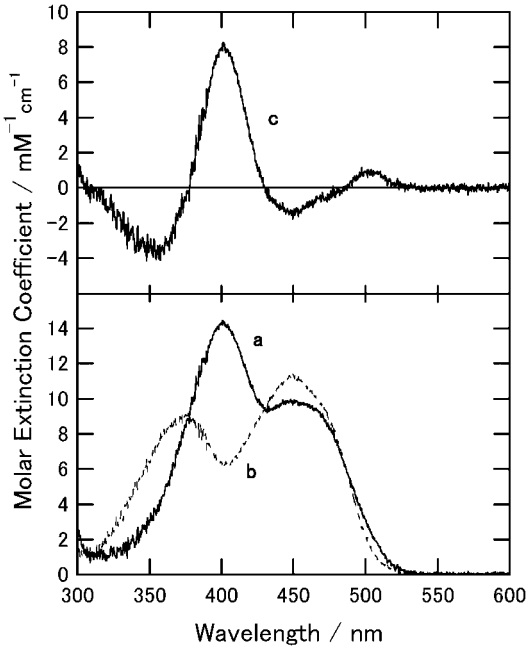

Fig. 5. **Spectral change of FAD upon binding to ETF.** Curve a shows the difference spectrum between a and b in Fig. 4 divided by the added FAD concentration at a 1:1 ratio to convert to the molar extinction coefficient. Curve b shows the spectrum of free FAD and curve c shows the difference spectrum, a – b.

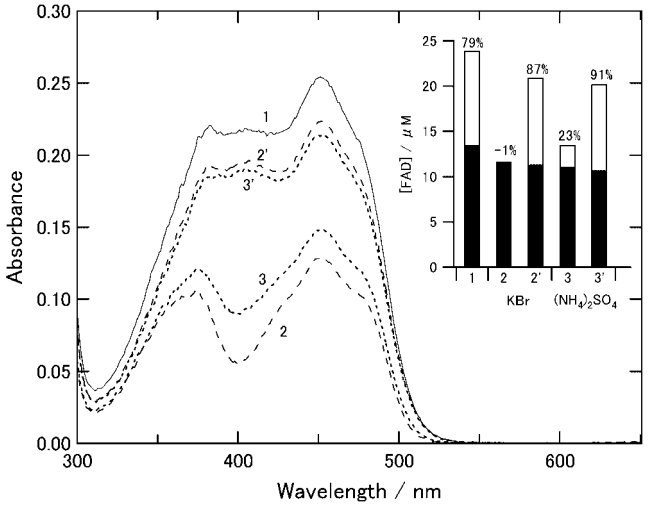

Fig. 6. **Release of the second FAD from ETF by potassium bromide and ammonium sulfate.** Three batches of 200 μl mixtures of 6.6 μM purified ETF and 9.8 μM FAD in buffer (50 mM K-P_i, pH 6) were prepared at 25°C and then concentrated using a Microcon-30 (Millipore) at 4°C to about 2 μl (called sample A). Curve 1: Sample A was washed with buffer. Curve 2: Sample A was washed with 1 M KBr in buffer and then washed with buffer without KBr. Curve 3: Sample A was washed with 1 M $(NH_4)_2SO_4$ in the buffer and then washed with the buffer without $(NH_4)_2SO_4$. Here the meaning of "washing" is two cycles of addition of 200 μl solution and concentration to about 2 μl by using the Microcon-30 at 4°C. All the samples prepared as above were brought to be 100 μl in the buffer and subjected to the measurement of the absorption spectra. Curves 2′ and 3′ show the FAD-saturated forms of ETF of curves 2 and 3, respectively. These two spectra were obtained by FAD titration. The inset shows the concentrations of the first (closed bars) and second FADs (open bars) as estimated by spectral decomposition performed as described in the legend to Fig. 8. The percentages of the second to first FAD concentrations are indicated above the bars.

(curve b). The corresponding spectra of the previous native ETF (*8*) and the recombinant ETF (*13*) have been reported, and they are almost the same as the present result.

The properties of ETF are compared among preparations in Table 2. The FAD content in the purified ETF is dependent on the ammonium sulfate used in the purification (described later). From the results described above, the A_{400}/A_{450} ratio and FAD content (x) can be related by the equation, $A_{400}/A_{450} = \{5.0+14.3(x-1)\}/\{11.3+9.8(x-1)\}$, where $(x-1)$ is the content of the second FAD and the constants are the ε values at 400 and 450 nm of the first and second FADs. The A_{400}/A_{450} ratio and FAD content of the previous preparations are not consistent with this equation. The reason for this inconsistency is unclear, but it could be partly due to the instability of ETF (described below), which had not been recognized.

The FAD-binding ability of the present ETF was somewhat unstable. Immediately after the last Q-Sepharose chromatography step, the peak fraction showed a FAD-binding ability (mol additionally bound FAD/mol initially bound FAD) of 95%, which is the highest value we have observed. The FAD-binding ability of the purified ETF was gradually lost in 50 mM K-P_i (pH 6) at 4°C with a rate constant of 0.13 d^{-1}. When 0.2 M KCl was added to the storage buffer, the rate constant was 0.02 d^{-1}, 6.5 times more stable than without KCl. Therefore, we employed buffer containing 0.2 M KCl for storage. ETF should be stored at –80°C rather than –20°C. We sometimes observed that the FAD-binding ability dropped unexpectedly to less than half after one night of storage at –20°C and thawing in an ice-water bath. The binding ability was preserved by storage at –80°C for more than 2 months. Cycles of freezing at –80°C and thawing in an

Table 2. **Comparison of the properties of different preparations of *M. elsdenii* ETF.**

		Ref . *8*	Ref .*13*	Present
Purified	A_{400}/A_{450}	0.59	0.53[a]	0.44
	FAD content	*ca.* 1.4[b]	1.06	1.0
	% modified FADs	1–50	0	0
FAD-saturated	A_{400}/A_{450}	0.78	0.74[a]	0.88
	FAD content	2.0	1.6–1.8	1.9
K_d (second FAD)		—	*ca.* 1 μM	0.07 μM

[a]Obtained from the figure. [b]Cited elsewhere (*15*).

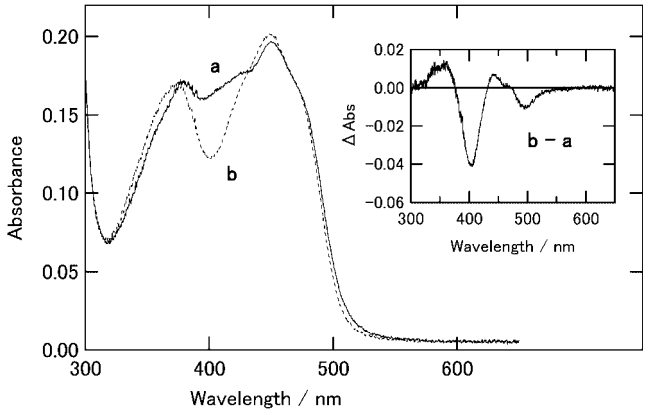

Fig. 7. **Absorption spectra of crude ETF with and without 1 M KBr.** A solution of crude ETF was mixed with the same volume of buffer (50 mM K-P_i, pH 6) (a) or 2 M KBr in the buffer (final 1 M KBr) (b) at 25°C. The inset shows the difference spectrum.

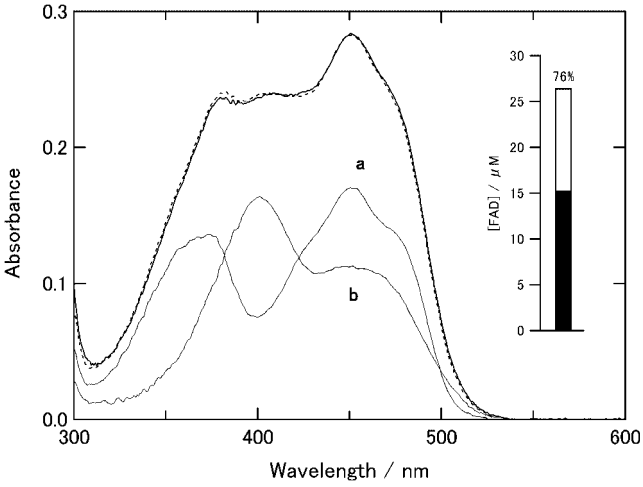

Fig. 8. **Absorption spectrum of reconstituted ETF.** ETF was reconstituted from guanidine-denatured subunits and FAD as described in "MATERIALS AND METHODS." The spectrum of the reconstituted ETF (solid thick curve) was decomposed into the spectra of the first (curve a) and second FADs (curve b). The dotted curve is the sum of curves a and b. The inset shows the concentrations of the first (closed bar) and second FADs (open bar) estimated by decomposition. The percentage of the second to first FAD concentration is indicated above the bar. Decomposition was performed with the formulation, $A_\lambda = c_1B_\lambda + c_2C_\lambda + \varepsilon_\lambda$ (λ = 350–550 nm, 1-nm step), where A_λ is the spectrum of the subject, B_λ and C_λ are the spectra of the first and second FADs (curves a in Figs. 4 and 5, normalized to show extinction coefficients), c_1 and c_2 are the concentrations of the first and second FADs, and ε_λ is the residual. The c_1 and c_2 values that minimize $\sum_\lambda \varepsilon_\lambda^2$ were determined by the linear least squares method.

ice-water bath slightly damage the FAD-binding ability: about 1.7% loss with every cycle.

In summary, we prepared ETF containing one mol FAD and no modified flavins. This ETF binds almost one additional FAD to form an almost complete holoprotein. Proper storage conditions were also found.

Dissociation of the Second FAD from ETF—Previously, FAD was released from ETF by gel filtration with 1.55 M guanidine hydrochloride (*8*), where almost all the bound FAD was removed. Here we report the method for the selective removal of the second FAD.

Potassium bromide (KBr) is widely used to release flavins from flavoproteins. Figure 6 shows experiments that demonstrate that the second FAD of ETF is released by KBr. In all the experiments shown in Fig. 6, the starting material was the purified ETF mixed with excess FAD. First, this ETF-FAD mixture was repeatedly subjected to ultrafiltration and dilution in buffer to remove free FAD (curve 1). The A_{400}/A_{450} ratio was 0.85: a small amount of the second FAD was lost from the saturating complex (ratio of 0.88) due to the slightly weak binding (K_d = 0.07 μM). Second, the ETF-FAD mixture was subjected repeatedly to ultrafiltration and dilution with 1 M KBr and then with buffer (curve 2). The A_{450} value was about half that of curve 1 and the A_{400}/A_{450} ratio of 0.44 was the same as that of the purified ETF, indicating the almost complete removal of the second FAD by KBr treatment. This ETF sample bound 85% more FAD by titration with FAD, and the FAD-saturated form (curve 2′) shows an A_{400}/A_{450} ratio of 0.87, which is almost the same as that of the FAD-saturated form of purified ETF (0.88), indicating that most of the FAD-binding ability was not injured by KBr treatment.

The shape of the spectrum of the purified ETF (curve a in Fig. 4) was unchanged by KBr treatment (data not shown), indicating that the purified ETF does not contain a second FAD. This is why we indicated above that the FAD content of the purified ETF is 1.0 rather than 1.14. The content of the first and second FADs can be estimated by fitting the spectra with a linear combination of the spectra of the first and second FADs. The fittings of the spectra in Fig. 6 were all fairly good (not shown but like Fig. 8) and the estimated contents of the two FADs are shown in the inset. Bars 2 and 2′ clearly show that KBr treatment completely removed the second FAD and caused little damage to the FAD-binding ability.

The second FAD was also released by ammonium sulfate (curve 3 in Fig. 6), but 23% of the second FAD was retained. ETF and FAD are probably in an equilibrium of association and dissociation in 1 M ammonium sulfate and thus the removal of the second FAD was not completed by only two cycles of dilution-concentration (see the legend to Fig. 6). FAD titration of this ETF resulted in the FAD-saturated form (curve 3′), which contained as much of the second FAD (91% of the first FAD) as the FAD-saturated form of the purified ETF: ammonium sulfate treatment also caused little damage to the FAD-binding ability.

The Phenyl Sepharose step in the present ETF purification procedure involves 30% (1.2 M) ammonium sulfate in the running buffer. The second FAD, which had been bound to the crude ETF, was released during this step (see "MATERIALS AND METHODS"). Figure 7 shows the spectra of the crude ETF in the absence and presence of 1 M KBr (curves a and b, respectively). The difference spectrum (inset to Fig. 7) is very similar to the reverse of curve c in Fig. 5, indicating that about 5 μM of the second FAD was released by 1 M KBr. The dissociation constant of the second FAD in 1 M KBr was estimated to be 30 μM by FAD titration of purified ETF in 1 M KBr (data not shown). FAD titration of the crude ETF in the absence of KBr indicated that the 2.4 μM second FAD-binding site is vacant in crude ETF (data not shown). These data lead to

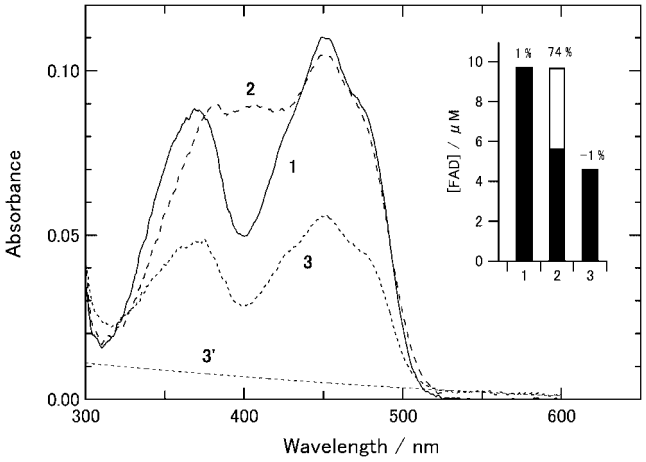

Fig. 9. **Spectral change of ETF by prolonged reduction and reoxidation.** The purified ETF (9.8 μM in 100 μl) in 50 mM K-P_i (pH 6) and 6 mM KCl at 25°C (curve 1) was made anaerobic as described previously (*17*). The ETF was reduced with sodium dithionite (about 20 μM) for 2 h and reoxidized in air (curve 2). This ETF solution was then washed with 1 M KBr and then with buffer using a Microcon-30 and brought to 100 μl (curve 3). The inset shows the concentration of the first (closed bars) and second FADs (open bars) estimated by the decompositions of the spectra. In the decomposition of curve 3, additional terms of a quadratic function of wavelength were included to correct for the baseline rise. The obtained baseline is shown by curve 3′.

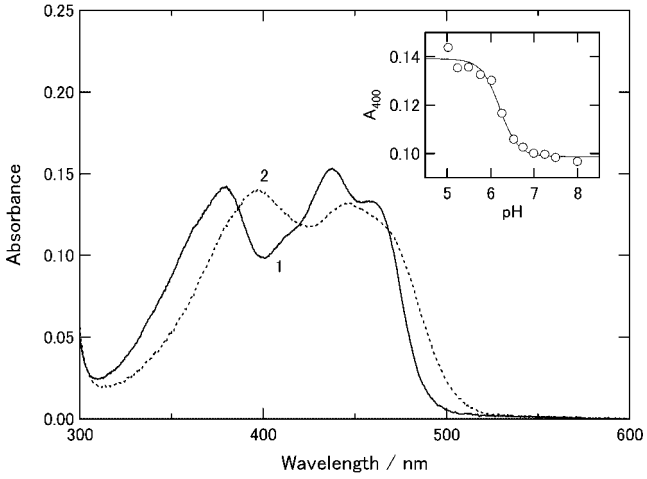

Fig. 10. **Spectral change of AMP-free pig ETF depending on pH.** The absorption spectra of 11.7 μM AMP-free pig ETF was measured in 50 mM potassium citrate buffer containing 5% v/v glycerol at various pH values at 20°C. A set of spectra was combined to a matrix form and analyzed by the matrix least squares method based on singular value decomposition as in the case of FAD titration of *M. elsdenii* ETF, but the reaction mechanism was formulated as $\log([B]/[A]) = n(pH - pK)$ where [B]/[A] is the molar concentration ratio of the alkaline to acidic forms. Curves 1 and 2 show the spectra of the alkaline and acidic forms obtained by the analysis. The inset shows the absorbance at 400 nm at various pH values and the theoretical curve obtained with the parameters $pK = 6.21$ and $n = 2.06$.

the estimation that the crude ETF contains a total 8.6 μM second FAD-binding site, 6.2 μM of which is occupied by FAD and 2.4 μM is vacant: the second FAD-binding site is about 70%-saturated in the crude ETF. The saturation of the second FAD-binding site *in vivo* is presently unknown.

The purification of the recombinant ETF also includes Phenyl Sepharose chromatography with 1 M ammonium sulfate (*13*). This is why the FAD content of the recombinant ETF is nearly one. The previous purification method of native ETF uses reverse ammonium sulfate chromatography (*8*), which involves 60–50% (2.4–2 M) ammonium sulfate and DEAE-cellulose instead of Phenyl Sepharose. The release of the second FAD during this chromatography could be incomplete, resulting in the FAD content of about 1.4.

In the case of ETFs from pig (*34*), human (*35*), and *P. denitrificans* (*7*), the bound FAD is released but the bound AMP is not released by KBr. In this point, the second FAD of *M. elsdenii* ETF resembles FADs of mammalian and other bacterial ETFs, and the first FAD resembles the AMP of other ETFs.

Absorption Spectrum of the Second FAD—Both the guanidine extract (containing both the first and second FAD) and 1-M KBr extract (containing only the second FAD) of FAD-saturated ETF showed a single FAD peak on C18 chromatography (data not shown). This indicates that the unusual spectrum of the second FAD (curve a in Fig. 5) is due to the flavin environment in the protein not to some chemical modification of the flavin ring.

Previously, the apoprotein of ETF was prepared by using 1.55 M guanidine hydrochloride (*8*), and ETF was reconstituted by mixing the apoprotein and FAD. In that case, the concentration of guanidine hydrochloride was relatively low so that the protein may not have unfolded completely. In this study, we prepared completely unfolded subunits by using 6 M guanidine hydrochloride and ETF was reconstituted by dilution of the guanidine-denatured subunits with a solution of FAD. The absorption spectrum of the reconstituted ETF was decomposed into the spectra of the first and second FADs (Fig. 8): the unusual spectrum (curve b) was also observed after reconstitution. This result excludes the possibility that some small molecule contained in ETF contributes to the unusual spectrum, because small molecules were removed during the preparation of guanidine-denatured subunits by cycles of ultrafiltration. The buffer ingredients, *i.e.*, phosphate and potassium ions, do not contribute to the unusual spectrum because a similar spectrum was observed after reconstitution in histidine-HCl buffer (not shown).

A similar anomalous spectrum was observed after prolonged reduction of the purified ETF followed by reoxidation (Fig. 9) as reported previously (*8*). The mechanism of this spectral change had not been resolved. The spectrum after reoxidation was dependent on the time during which the ETF was in the reduced form: the A_{400}/A_{450} ratio after reoxidation was 0.58 (10-min reduction with NADH), 0.70 (30 min), 0.81 (1 h), 0.83 (1.5 h), and 0.84 (2 h), that is, the half completion time was about 0.4 h. Curve 2 in Fig. 9 is the spectrum of reoxidized ETF after a 2-h reduction, which was again decomposed into the spectra of the first and second FADs. The total FAD concentration (9.6 μM) was unchanged before and after reduction-reoxidation, but about a half of the initial FAD was changed to the second FAD (inset to Fig. 9). These

observations can be explained as follows. The oxidized form of the first FAD is bound tightly to the apoprotein while the reduced form of the first FAD dissociates slowly from the protein. The released FAD rebinds to the second FAD-binding site of another ETF molecule with the first FAD bound, resulting in similar amounts of holoprotein (with two FAD bound) and apoprotein (without FAD). This mechanism is consistent with the fact that the standard redox potential of the first FAD (–200 mV *vs.* standard hydrogen electrode) is more negative than that of free FAD (–160 mV) at pH 6 (*36*), because a more negative standard redox potential of protein-bound FAD than free FAD implies that the reduced FAD binds to the protein more loosely than the oxidized FAD does. Our preliminary experiment showed a much more positive standard redox potential of the second FAD than free FAD (*37*), and thus tighter binding of reduced FAD than oxidized FAD to the second FAD-binding site, which is also consistent with the mechanism. The second FAD produced by reduction-reoxidation was released by KBr treatment (curve 3 in Fig. 9). Curve 3 contains a contribution of the baseline absorbance (curve 3′) probably due to denaturation of the FAD-unbound apoprotein during KBr treatment. The spectrum of the purified ETF mixed with equimolar FAD, which showed an A_{400}/A_{450} ratio of 0.85, was not changed by reduction-reoxidation as reported previously (*8*): this is also consistent with the mechanism. A similar rearrangement of FAD was also observed after treatments that cause partial protein denaturation such as heating of the purified ETF, insufficiently cautious evacuation for anaerobic experiments, and repeated cycles of freezing (–20°C) and thawing: the A_{400}/A_{450} ratio was raised in all the cases.

An unusual flavin absorption spectrum similar to that of the second FAD of *M. elsdenii* ETF was observed for AMP-free pig ETF. At alkaline pH, both the holoprotein of pig ETF (containing AMP and FAD) and AMP-free pig ETF (containing only FAD) show normal absorption spectra (Fig. 10, curve 1) as reported previously (*3*). Whereas the spectrum of the holoprotein was almost unchanged in the pH range 5–9 (data not shown), the spectrum of AMP-free pig ETF was altered by acidification (inset to Fig. 10) and the effect reversed by re-alkalization. The acidic form (Fig. 10, curve 2) showed a spectrum similar to the unusual spectrum observed for *M. elsdenii* ETF (Fig. 5, curve a). The parameters of the pH profile formulation, $\log([B]/[A]) = n(\mathrm{pH} - \mathrm{p}K)$, where [B]/[A] is the molar concentration ratio of the basic and acidic forms of AMP-free ETF, varied depending on the buffer and buffer concentration: pK = 5.3–6.3 and n = 1.3–2.2. This result suggests that two residues are responsible to the spectral change of AMP-free pig ETF, and that the pK of each residue is dependent on the solvent conditions. Such residues with a pK of about 5.5 include histidine, glutamate, and aspartate. Although the spectrum of the second FAD of *M. elsdenii* ETF, obtained by FAD titration, was almost unchanged in the pH range of 6–8.5 (data not shown), there should be some similarity in the flavin environment between *M. elsdenii* and pig ETFs: again the second FAD of *M. elsdenii* ETF shows a resemblance to the FAD of the other ETFs. It is unlikely that the unusual spectrum is due to the protonation of the flavin ring itself, because the spectrum of free FAD in 10 N HCl is a different one, showing a single absorption band in the visible region with a peak at 400 nm.

A similar unusual flavin spectrum has also been reported for the riboflavin bound to 6,7-dimethyl-8-ribityllumazine synthase from *Schizosaccharomyces pombe* (*38*). The crystallographic structures of these three flavoproteins, none of which have been resolved, would provide important information to clarify the nature of the unusual spectrum.

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