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L-Kynurenine 3-Monooxygenase from Mitochondrial Outer Membrane of Pig Liver: Purification, Some Properties, and Monoclonal Antibodies Directed to the Enzyme[1]

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We have purified L-kynurenine 3-monooxygenase from pig liver mitochondria using a procedure involving seven steps composed of (1) preparation of mitochondrial outer membrane, (2) preparation of the zwitterionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (Chaps)-insoluble outer membrane material, (3) extraction of the enzyme with β-octylglucoside, (4) ammonium sulfate fractionation, (5) DEAE-Sepharose CL-6B chromatography, (6) Matrex gel orange A affinity chromatography, and (7) high-performance liquid chromatography (HPLC) gel filtration. The final preparation had an about 160-fold purified enzyme activity with a yield of 0.8%. The apparent molecular mass of the aggregated form of the native enzyme was determined to be close to 300 kDa by HPLC gel filtration in the presence of 0.005% Triton X-100. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed a main protein band with an apparent molecular mass of about 49 kDa. The enzyme was found to be about 86% pure by the criterion of SDS-PAGE. The dissociated form of the enzyme contains 1 mol of non-covalently bound FAD/mol of protein monomer. The UV/visible spectrum had absorption peaks at 275, 384, and 450 nm, typical of a simple flavoprotein. Five inhibitory monoclonal antibodies against the enzyme were obtained. They could stain moderately a single protein band (49 kDa) in a Western blot.

Key words: kynurenine 3-monooxygenase [EC 1.14.13.9], mitochondrial outer membrane, monoclonal antibody, pig liver, UV/visible absorption spectrum.

Kynurenine was identified as a product of tryptophan metabolism in animal cells by Kotake and his colleagues (*1, 2*) and 3-hydroxykynurenine was isolated as crystals from pupa of the blowfly (*Calliphora erythrocephala*) by Butenandt *et al.* (*3*). Later, the occurrence of 3-hydroxykynurenine as a tryptophan metabolite was demonstrated in animals (*4*). Advanced studies revealed the participation of kynurenine 3-monooxygenase [Fpk, EC 1.14.13.9] in brain function through neurotoxic quinolinic acid formation (*5-8*). Elucidation of the structure-function relationships of the enzyme will be essential to understand its regulation in the brain.

Fpk, which converts kynurenine to 3-hydroxykynurenine was first identified in Brown's laboratory (*9*). One year later, it was found to be a NADPH-dependent monooxygenase (*10*) and FAD-requiring enzyme (*11*) located in the mitochondrial outer membrane (*12*). So far, the purification of the mammalian enzyme from rat liver mitochondria has been reported from three different laboratories (*13-18*), but purification has not been achieved from any other species. Because of the difficulty in purifying this enzyme, especially in separating it completely from hemoprotein contaminants, it has not been possible to establish the typical absorption spectrum of this flavoprotein so far (*15-17*). We have tried for several years to purify the enzyme from rat and pig liver mitochondria (*19-21*) in order to obtain a visible spectrum having no Soret band of hemoprotein.

In the present communication we report the purification procedure and some properties of Fpk from pig liver mitochondrial outer membrane; our findings are very different from those for the previously reported rat liver enzyme (*16-18*). A reasonable UV/visible spectrum and monoclonal antibodies to the enzyme, which inhibit the Fpk activity and recognize the SDS-denatured Fpk protein, have been obtained for the first time.

[1] Dedicated to Emeritus Prof. Toshio Yamano of Osaka University Medical School, on the occasion of his 75th birthday.

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Abbreviations: BSA, bovine serum albumin; Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; HPLC-ECD, high-performance liquid chromatography with electrochemical detector; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-2[2-ethanesulfonic acid]; Ig, immunoglobulin; KIU, kallikrein inhibitor unit; Fpk, kynurenine 3-monooxygenase; mAb, monoclonal antibody; MAO, monoamine oxidase; MES, 2-(*N*-morpholino)ethanesulfonic acid monohydrate; OGS, β-octylglucoside; OM, outer membrane; PBS, phosphate-buffered saline; PCA, perchloric acid; PMSF, phenylmethylsulfonyl fluoride; POD, peroxidase; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, tris-buffered saline.

EXPERIMENTAL PROCEDURES

Materials—Protamine sulfate (Grade X from salmon), Chaps, OGS, FAD disodium salt, and protein A agarose beads were purchased from Sigma (St. Louis, MO, USA). DEAE-Sepharose CL-6B and a molecular weight gel filtration calibration kit were from Pharmacia (Uppsala, Sweden). SDS-PAGE molecular weight standards (high range) and Affi-gel 501 were from Bio-Rad (USA). Dye Matrex Screening Kit, Matrex gel orange A, and Centriplus-100 were from Amicon (USA). TSK-gel G3000 SW and G4000 SW columns (7.5 mm×60 cm) were from Tosoh (Tokyo). L-Kynurenine sulfate and a Cosmosil 5C 18-AR column (4.6 mm×15 cm) were from Nacalai Tesque (Kyoto). NADPH was from Oriental Yeast (Tokyo), PMSF from Wako Pure Chemical (Osaka), Trasylol VLE (aprotinin, 10,000 KIU/ml) from Bayer UK (Haywards Heath, W. Sussex, UK), MES from Dojindo (Kumamoto), Surfact-Amps™ X-100 (10% solution of Triton X-100) from Pierce (Ilinois, USA), rabbit anti-mouse IgG from MBL (Nagoya), and IgG sorb from Funakoshi (Tokyo).

Preparation of Mitochondria—Pig liver (1.2-1.6 kg) was brought to the laboratory on cracked ice from a slaughterhouse. Mitochondria were prepared by a modification of the procedure of Beattie (*22*) as described in detail (*23*), except for the use of buffered 0.25 M sucrose (1 mM Tris-HCl buffer, pH 7.5). Mitochondria were washed five times by gradually diminishing the volume and the centrifugal force with buffered sucrose to remove microsomal contamination as described previously (*23*). The five-times-washed mitochondria were stored at −70°C until use.

Enzyme Assays—Fpk activity was measured spectrophotometrically at room temperature in terms of the disappearance of NADPH absorption at 340 nm (*24*) with a millimolar extinction coefficient of 6.22 $mM^{-1}\cdot cm^{-1}$ in a Shimadzu UV 3000 dual-wavelength/double-beam spectrophotometer. The reaction mixture contained, in a final volume of 1.5 ml, 0.1 M Tris-acetate buffer (pH 8.0), 0.01 M KCl, 40 μM FAD, 0.1 mM L-kynurenine, appropriate amounts of enzyme (in the case of outer membrane, 0.25-0.5 mg of protein), and 147 μM NADPH. The reaction mixture was put into a cuvette of 1 cm light path. Before the addition of L-kynurenine, the endogenous rate of the absorbance change at 340 nm was recorded for about 5 min and after the addition of L-kynurenine, the rate of the absorbance change was measured for about 6 min, the endogenous rate being subtracted. MAO activity was measured fluorometrically using kynuramine as the substrate (*25*) in a Hitachi S-4000 spectrophotofluorometer.

Electrophoresis—SDS-PAGE was performed by the method of Laemmli (*26*) in 7.5% gels. The gels were stained with 0.04% Coomassie Blue R-250, photocopied with a Fujifilm Pictrostat 330, and scanned in a Shimadzu dual-wavelength flying-spot scanner CS-9000.

Optical Spectra—The optical spectra were measured in a Beckman DU-64 spectrophotometer equipped with a 50 μl carat cell at room temperature.

FAD Content—Flavin content was determined by HPLC using a Gilson system composed of a 305 pump, 805 manometric module and 121 filter fluorometer detector equipped with an excitation filter of 450 nm and an emission filter of 510-650 nm. A Nacalai Cosmosil column (5C18-AR, 4.6×150 mm) was used (*27*). Integration and data analysis were performed on an SIC Chromatocorder 11 (Tokyo). Standard FAD obtained from Sigma were purified by HPLC (*27*). The concentration of FAD was determined spectrophotometrically using an absorption coefficient of 11.3 $mM^{-1}\cdot cm^{-1}$ at 450 nm (*28*). Non-covalently bound flavin was extracted from a purified active enzyme preparation by incubation at 85°C for 20 min; after centrifugation at 16,500×g for 15 min, the supernatant was passed through a Millipore filter LCR4-LG (0.22 μm, ϕ4 mm), diluted 4 times with elution buffer and an aliquot (50 μl) was injected into the HPLC column. Otherwise, FAD in the enzyme preparation was determined spectrophotometrically in terms of the decrease in absorbance at 450 nm after reduction with sodium dithionite. The difference between the molecular absorption coefficient of the oxidized and reduced form of FAD was assumed to be 10.3 $mM^{-1}\cdot cm^{-1}$ (*29*).

HPLC Gel Filtration—HPLC gel filtration was performed at room temperature on a TSK-G 3000 SW column and a TSK-G4000 SW column attached to a Gilson 715 HPLC system equipped with an autosampler (Gilson, Model 202). The two columns, each connected to its own pump, ran independently of each other. At the outlet of the first column, we set up the first UV detector (LINEAR UVIS 200) with a recorder (Hitachi, 056) and at the outlet of the detector we placed a high-pressure switching valve (Rheodyne, 7010), which was connected to the inlet of the second column. Thus, unwanted peaks from the first column could be drained and the wanted peak could be introduced into the second column based on monitoring of the first UV detector or recorder. After the outlet of the second column we set up another UV detector (Gilson 115 UV detector) and a VIS detector (Gilson 112 UV/VIS detector), both of which were connected to a two-pen recorder (LC-Master Model 2002). The first and the second UV detectors were set at 280 nm and a filter cartridge of 450 nm was inserted in the UV/VIS detector, whose light source was a phosphor-coated Hg lamp, so that we could detect simultaneously UV absorption and the yellow color of the enzyme.

Protein Content—Protein was measured by the method of Lowry *et al.* (*30*) or Bradford (*31*) using DC protein microassay kits (Bio-Rad) or the Bio-Rad protein assay kit, respectively, with BSA as the standard.

Immunization—Four BALB/c mice were used. Two of them were immunized with three intraperitoneal injections of 50-80 μg of antigen (enzyme preparation after step 6) in 0.1 ml of sterile PBS emulsified in an equal volume of complete (first injection, day 1) or incomplete (second injection, day 15 or third injection, day 24 or 30) Freund's adjuvant. Two other mice were injected as above and further injections were given on day 35 or 40.

Cell Culture Conditions and Fusion of Cells—Mouse plasmocytoma cell line P3-X63-Ag8-U1 (P3U1) was grown in Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum and glutamine (300 mg/liter)+pyruvic acid (110 mg/liter). Spleen cells (1×10^8) were fused to 1.8×10^7 P3U1 cells with the addition of a mixture of 50% polyethylene glycol 1500 in 75 mM HEPES buffer, pH 8.0. The fused cells were seeded into the wells of tissue culture plates and hybridomas were selected by the method of Littlefield (*32*) in a medium containing hypoxanthine, aminopterine, and thymidine.

Hybridomas which produced an antibody (detected by ELISA) were cloned twice by limiting dilution and expanded by tissue culture.

Screening of Cell Culture Supernatant by Inhibition of the Fpk Activity—Each cell culture supernatant (100 μl) was preincubated with 0.6 μg of the enzyme in the presence or absence of the secondary reagents, *i.e.* 15 μl of protein A-agarose beads (*33*) or 40 μl of 1% (v/v) IgG sorb prebound with rabbit anti-mouse IgG (*34, 35*) in the final volume of 200 μl at 37°C for 30 min. After centrifugation at 10,000 × *g* for 10 min, an aliquot of 150 μl of the supernatant was added to 160 μl of mixed solution composed of 400 μl of 0.5 M Tris-acetate buffer, pH 8.0, 400 μl of 0.3 M KCl, 400 μl of FAD (1 mg/ml), and H_2O to make a final volume of 564 μl. Then 30 μl of 30 mM NADPH and 6 μl of 30 mM L-kynurenine were added to start the reaction. After incubation at 37°C for 10 min, the reation was stopped by the addition of 500 μl of ethanol, 50 μl of PCA and 50 μl of 0.2 M EDTA and the amount of 3-hydroxykynurenine was measured by HPLC-ECD (*21*).

Typing of mAbs—This was carried out with a mouse mAb isotyping kit (Pharmingen) by ELISA.

Immunoblot Detection of Fpk with mAbs—SDS-PAGE was carried out on a 10% gel with a 3% stacking gel. After electrophoresis, proteins were horizontally transferred onto PVDF membrane according to the manual of Bio-Rad. Part of the membrane was stained with Coomassie blue and the other part of the membrane was incubated with TBS containing 2% BSA overnight to block non-specific binding. Then it was incubated with hybridoma tissue culture supernatants for 2 h and, after washing, with peroxidase-conjugated goat anti-mouse IgG (H+L) diluted 1:2,000 for another 1 h. It was rinsed with TBS containing 0.1% Tween 20, and developed with a POD immunostaining set (Nitroblue tetrazolium/NADH/H_2O_2) for 10 min.

RESULTS

Purification of Fpk—Step 1. Preparation of crude mitochondrial outer membranes: Mitochondria were thawed and crude mitochondrial outer membranes were isolated by the hypotonic swelling method (*36, 37*), except for the use of 10 mM Tris-HCl buffer, pH 8.0 containing 50 μM FAD. Outer membranes were stored at −70°C until use.

Step 2. Preparation of Chaps-insoluble outer membrane material: We applied the extraction method for rat liver microsomal NADPH-cytochrome P-450 reductase from the pellet of Chaps-protamine sulfate treated microsomes (*38*) to extract Fpk from mitochondrial outer membranes. Outer membranes (about 920 mg protein) suspended in buffer A (0.2 M Tris-acetate buffer, pH 8.0 containing 1 mM DTT/EDTA, 10% glycerol, 50 μM FAD, and 10 KIU Trasylol/ml) at a protein concentration of 10 mg/ml were treated with 10% Chaps in combination with 1.5% protamine sulfate (prepared just before use) at a final concentration of 0.5 and 0.07%, respectively, as described previously (*19–21*). After centrifugation at 170,000 × *g* for 60 min, the Chaps-insoluble pellet was suspended in buffer A by homogenization at a protein concentration of 10 mg/ml.

Step 3. Extraction of Fpk with OGS: To the above-

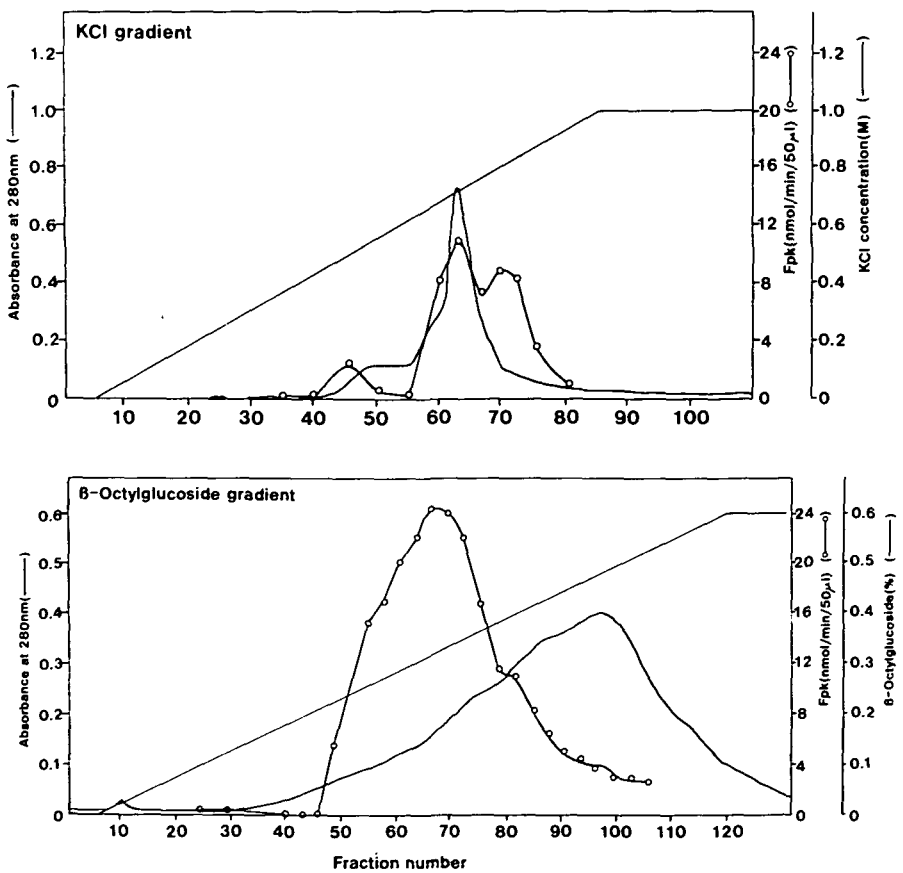

Fig. 1. **Elution pattern of Fpk from DEAE-Sepharose CL-6B column.** The 40–80% floating precipitate with ammonium sulfate was dialyzed against buffer B and centrifuged. The supernatant (41 ml, 127 mg of protein) was loaded onto the column (1.5 × 17 cm). After washing of the column with 60 ml of buffer B′ (buffer B containing 0.1% Chaps, 0.05% OGS, and 10 KIU Trasylol but no FAD), the enzyme was eluted by linearly increasing the concentration of KCl from 0 to 1.0 M in buffer B′, and then with a linear gradient, 0.05 to 0.6%, of OGS in buffer B′ containing 1.0 M KCl. The flow rate was 0.5 ml/min. Note that the active peak obtained with the OGS gradient is higher than that obtained with the KCl gradient and elutes before the protein peak.

described suspension, 20% OGS was added dropwise to make a final concentration of 2% with stirring magnetic stirrer at 4°C for 30 min. After centrifugation as above, the supernatant containing about 70–80% of the total activity

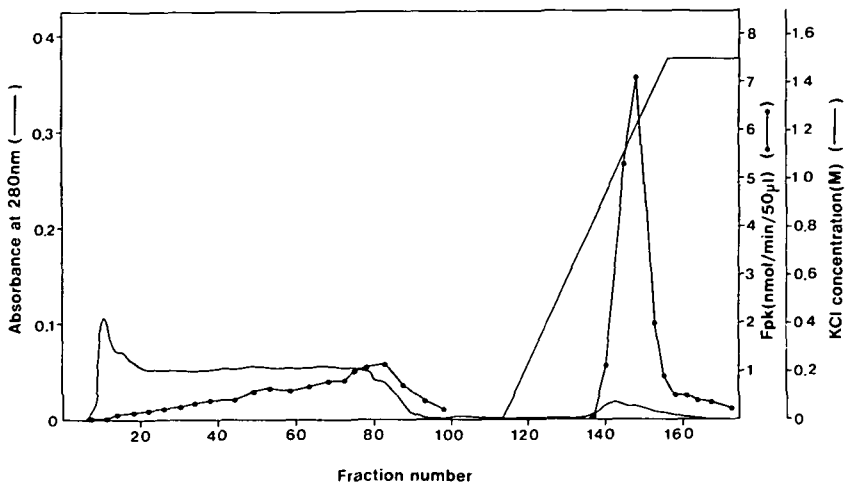

Fig. 2. **Affinity chromatography of the Step 5 preparation on Matrex gel orange A.** Step 5 preparation (81 ml, 38 mg of protein) was loaded onto the affinity column (1.5×12 cm). After washing of the column with equilibration buffer C (50 mM Tris-acetate buffer, pH 8.0, containing 10% glycerol, 1 mM DTT, 0.2 mM EDTA, 50 mM KCl, and 0.1% Triton X-100), the enzyme was eluted with 40 ml of a linear gradient from 0.05 to 1.5 M KCl in buffer C. After completion of the gradient, flow of 1.5 M KCl buffer (30 ml) was continued. The flow rate was 0.15 ml/min.

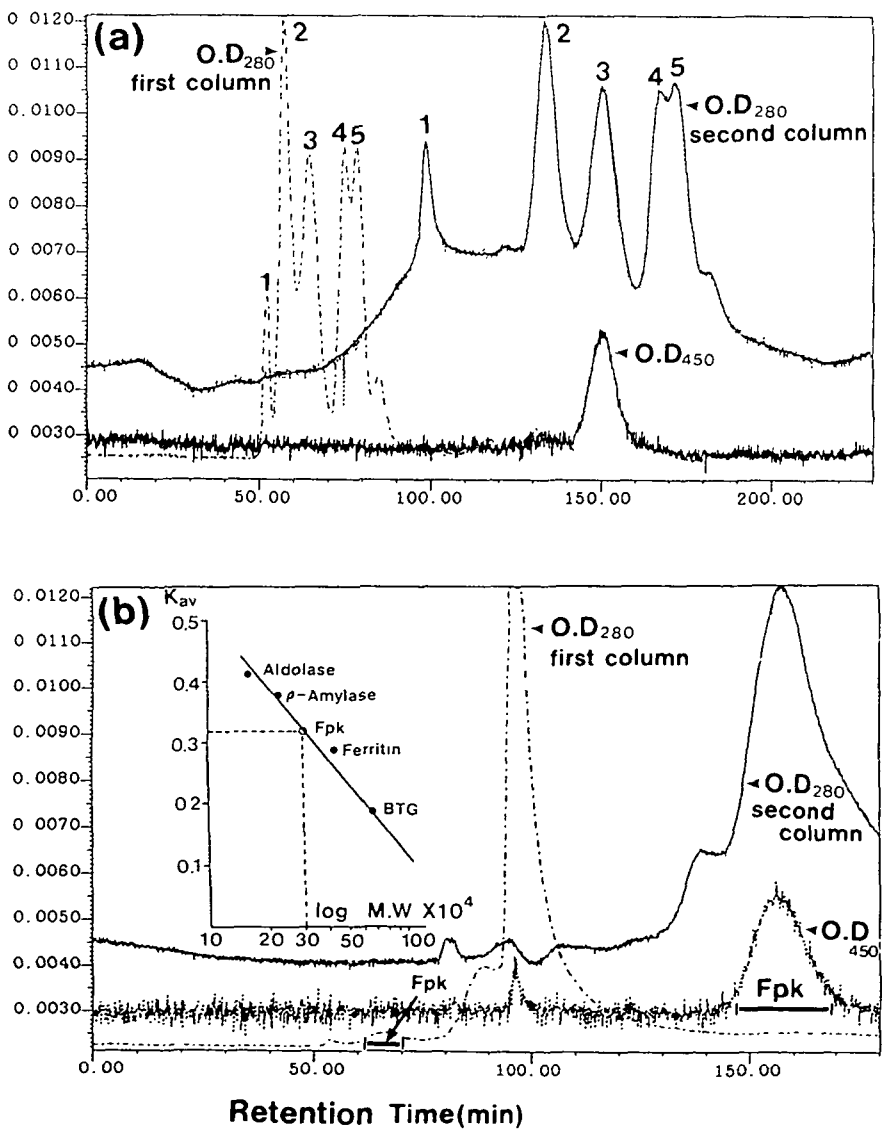

Fig. 3. **Elution profile of Fpk, DNA, and standard proteins from HPLC gel filtration.** (a) DNA and standard proteins. DNA was used in place of blue dextran. The numbered peaks correspond to: 1, DNA (1 μg); 2, bovine thyroglobulin, BTG (50 μg); 3, ferritin (5 μg); 4, β-amylase (25.5 μg); 5, aldolase (50 μg); Injection volume was 100 μl. (b) Fpk sample. The active fraction (about 0.4 ml, 1.3 mg of protein) obtained from the Matrex gel orange A affinity column was filtered through a Millex-GV filter and loaded onto the column (TSK-G3000 SW and TSK-G4000 SW, both 7.5 mm×60 cm). The sample was loaded in aliquots of 60–90 μl. The flow rate was 0.2 ml/min. The broken, dotted, and solid lines indicate readings at 280 nm with the first detector, and at 450 and 280 nm with the second detectors. The ordinate shows optical density at 450 nm. Note that the large peak at about 100 min from the first column was due to micelles of Triton X-100 and was eliminated through the drain. Therefore, it can not be seen in the elution profile from the second column. The inset in panel (b) shows the determination of molecular weight of Fpk by comparison with the standards.

recovered from the Chaps-insoluble pellet was fractionated with ammonium sulfate.

Step 4. Ammonium sulfate fractionation: Solid ammonium sulfate was added to the supernatant. The floating pellet recovered between 40-80% saturation of ammonium sulfate was collected by centrifugation at 70,000×*g* for 30 min. It showed about 40% of the outer membrane activity and was dissolved in buffer A by gentle mixing without homogenization, then dialyzed against buffer B (10 mM Tris-acetate buffer, pH 8.0, containing 10% glycerol, 0.5 mM EDTA, 0.2 mM DTT, and 50 μM FAD) at 4°C for 16 h.

Step 5. DEAE-Sepharose CL-6B column chromatography: After centrifugation at 17,000×*g* for 30 min, the supernatant was applied to a DEAE-Sepharose CL-6B column (1.5×17 cm) attached to an Econo System (Bio-Rad). The column was preequilibrated with buffer B′ (buffer B containing 0.1% Chaps, 0.05% OGS, and 10 KIU Trasylol/ml, but no FAD) and after washing of the column with 60 ml of buffer B′, the enzyme was eluted with a 60 ml linear gradient from 0 to 1.0 M KCl in buffer B′. The flow of 1.0 M KCl buffer (30 ml) was continued, and then a 90 ml linear gradient of 0.05 to 0.6% OGS in 1.0 M KCl buffer was performed and the flow of 0.6% OGS buffer (30 ml) was continued. The active OGS fractions having higher activity than the KCl fractions appeared a little before the protein peak and were pooled and stored at −70°C until use. The elution pattern is shown in Fig. 1. These steps (from Step 2 to Step 5) were repeated two or three times.

Step 6. Matrex gel orange A affinity column chromatography: The FAD-containing monooxygenase has recently been purified from porcine liver microsomes by using Matrex gel red A (*39*). We determined by a preliminary experiment with Dye Matrex Screening Kit that Matrex gel orange A was the most suitable gel for the purification of Fpk. The stored sample (about 80 ml) was thawed, supplemented with Triton X-100 to a final concentration of 0.1%, and dialyzed at 4°C for 16 h against buffer C (50 mM Tris-acetate buffer, pH 8.0 containing 10% glycerol, 1 mM DTT, 0.2 mM EDTA, 50 mM KCl, and 0.1% Triton X-100). The dialysate was applied to an orange A column (1.5×12 cm) attached to an Econo System. The column was pre-equilibrated with buffer C, then washed with 40 ml of buffer C, and the enzyme was eluted with a 40 ml linear gradient from 0.05 to 1.5 M KCl in buffer C. The flow of 1.5 M KCl buffer (30 ml) was continued. As shown in Fig. 2, the enzyme activity was eluted near 1.2 M KCl. The active fractions were pooled and concentrated on an Amicon Centriplus-100 to a final volume of about 400 μl.

Step 7. HPLC gel filtration: The concentrated enzyme was filtered through a Millex GV filter (0.22 μm, Millipore) and applied to two gel filtration columns attached to a Gilson 714 HPLC system, whose construction was described in "EXPERIMENTAL PROCEDURES." The columns were equilibrated with buffer D (0.1 M Tris-acetate buffer, pH 7.7, containing 0.005% Triton X-100, 0.2% Chaps, 10% glycerol, 0.5 mM DTT, 0.2 mM EDTA, and 0.5 M NaCl). The flow rate of each column was 0.2 ml/min. The elution pattern from Tosoh TSK-columns is shown in Fig. 3. Panel a shows the pattern obtained with molecular weight standards, while panel b shows the result obtained with our Fpk sample. The broken line in panel b monitored at 280 nm shows the elution pattern from the first column where all components in the sample, including a large amount of Triton X-100, were separated according to their molecular weight. We loaded the small peak eluted from the first column which appeared in the elution pattern at 60 to 70

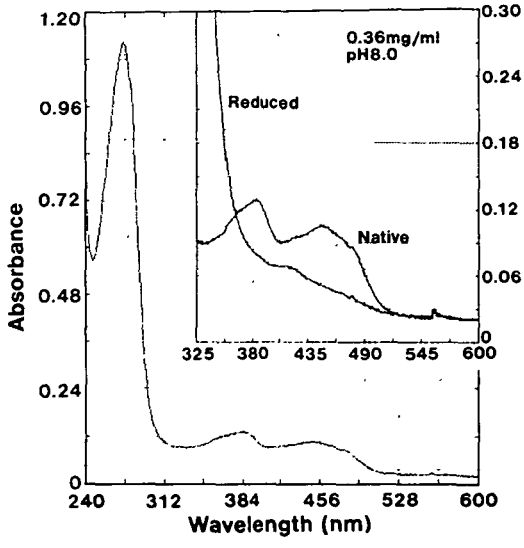

Fig. 4. **Absolute optical absorption spectra of Fpk in the oxidized and sodium dithionite-reduced forms.** The carat cell contained 60 μl of the enzyme preparation (protein concentration, 0.36 mg/ml) in 0.1 M Tris-acetate buffer, pH 8.0, containing 10% glycerol, 0.005% Triton X-100, 0.2 mM EDTA, 0.25 mM DTT, and 0.5 M NaCl, at room temperature. The magnified spectra in the visible region (inset) were recorded at 4-fold greater sensitivity.

TABLE I. **Purification of Fpk from pig liver mitochondrial outer membranes.** The Fpk activity was assayed spectrophotometrically at room temperature by the L-kynurenine-dependent disappearance of NADPH at 340 nm as described in "EXPERIMENTAL PROCEDURES."

	Volume (ml)	Total protein (mg)	Specific activity[a]	Total activity	Purification factor (fold)	Yield (%)
Outer membrane	109	1,846	8.7	16,044	1.0	100
Chaps-protamine sulfate precipitate	76	1,010	9.5	9,564	1.1	60
OGS supernatant	110	440	15.0	6,596	1.7	41
Ammonium sulfate precipitate (40-80%)	50	346	17.2	5,948	1.8	37
DEAE-Sepharose CL-6B OGS eluate	81	38	79.2	3,010	9.1	19
Matrex gel orange A KCl eluate and Centriplus-100	0.4	1.3	1,396[b]	1,815	160	11
HPLC gel filtration and Centriplus-100	0.3	0.1	1,353 763[c] (4,495) (2,553)[c]	135 (449)	156 (516)	0.8 (2.8)

[a]Specific activity: nmol of NADPH oxidized/min/mg protein. [b]The value is not true specific activity due to the activation by Triton X-100. It may be about one-third on the basis of the magnitude of activation by Triton X-100. [c]Specific activity in the absence of FAD. Numbers in parentheses are the values measured in the presence of Triton X-100 at a final concentration of 0.0133%.

min (marked by an arrow) onto the second column while the large peak appearing between 80 to 110 min due to Triton X-100 was drained since no enzyme activity was detected in these fractions. The solid lines (280 nm) and dotted line (450 nm) show the elution patterns from the second column into which only the peak suspected to be Fpk eluted from the first column had been introduced. As expected, the peak (solid line) appearing between 150 and 160 min was accompanied with a visible absorption peak (dotted line). This peak was collected and concentrated with an Amicon Centriplus-100 to a final volume of about 300 μl as the final purified enzyme preparation.

Table I summarizes the purification procedure resulting in an 160-fold increase of the specific activity with an overall yield of about 0.8%. Triton X-100 activated the enzyme activity, so that the specific activity of the final purified enzyme preparation was also assayed in the presence of Triton X-100 (values in parenthesis) for comparison with that of the concentrated eluate from the Matrex gel orange A column, since it contains micelles of Triton X-100 (see Fig. 3b). The specific activity further increased about 3 times at the final HPLC gel filtration step. Omission of FAD in the enzyme assay decreased the specific activity about 44% regardless of the presence of Triton X-100. This percentage decrease was almost the same in two separate preparations.

Absorption Spectra—Figure 4 shows optical absorption spectra of the Fpk. The native enzyme was pale yellow in color with absorption peaks at 275, 384, and 450 nm, and a shoulder at about 479 nm in the oxidized form. The ratio of the optical density of the peak at 275 nm to that at 450 nm was 10.4, like that of Ziegler's pig liver microsomal mixed function amine oxidase (*40*), and the ratio of the absorbance at 450 nm to that at 384 nm was 0.82. As shown in the inset, the addition of a few grains of solid sodium di-

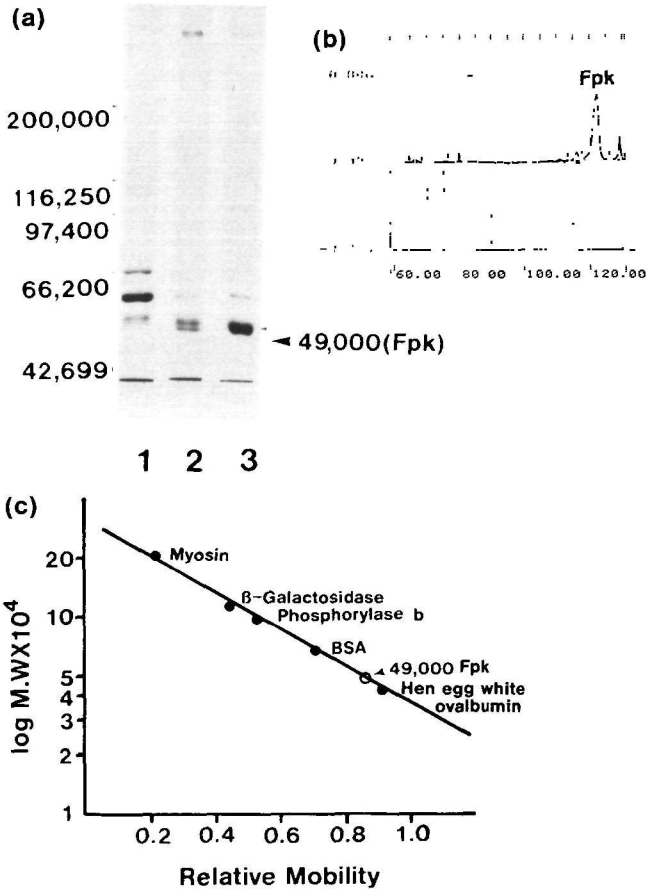

Fig. 5. **Electrophoretic pattern of the Fpk and estimation of molecular weight of the enzyme by SDS-PAGE.** (a) SDS-polyacrylamide gel (7.5%) electrophoresis profile of about 4 μg (1), 4 μg (2), and 2 μg (3) of Fpk protein. The gel was stained with Coomassie Blue R250. 1, DEAE-Sepharose CL-6B column eluate; 2, Matrex gel orange A column eluate; 3, HPLC gel filtration eluate. (b) Electrophoregram of Fig. (a—lane 3) which yields an estimate of the purity of the enzyme of about 86%. The main contaminant is a peak at about 60 kDa (about 7%), while the peak at the right margin is due to bromophenol blue. The abscissa is the distance (mm) from the starting point of the *y*-axis on the stage of the scanner and the ordinate is the absorbancy at 600 nm. (c) Estimation of the molecular weight of the Fpk plotted from (a). ●, Standard marker proteins; ○, Fpk.

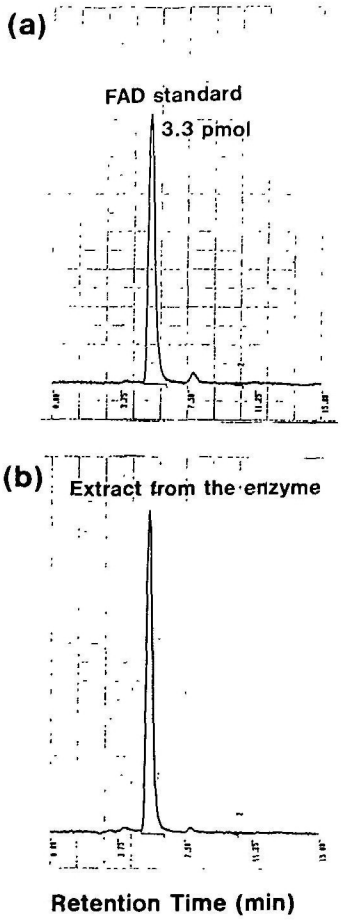

Fig. 6. **Quantification of FAD released from Fpk preparation by HPLC.** The chromatograms represent the injection of a 50 μl aliquot prepared from samples of (a) a standard solution containing 3.3 pmol FAD and (b) Fpk extract by heat treatment. The Fpk samples (about 4 μg of protein in 10 μl) were diluted by addition of 190 μl of H_2O and were incubated at 85°C for 20 min and then cooled. After centrifugation at 16,500×*g* for 15 min, the supernatant was filtered and diluted four times with HPLC elution buffer and an aliquot (50 μl) was subjected to HPLC analysis with a reversed-phase column (Cosmosil 5C 18-AR) and a Gilson 121 filter fluorometric detector. The mobile phase was 30% methanol containing 20 mM MES-NaOH buffer (pH 6.0). The analysis was performed at room temperature with a flow rate of 0.5 ml/min.

thionite aerobically caused a decrease in the visible absorption peaks. Total flavin determined spectrophotometrically using $\varepsilon_{ox\text{-}red} = 10.3\ mM^{-1} \cdot cm^{-1}$ from the decrease in absorbance at 450 nm after reduction with sodium dithionite was 5.4 nmol/ml. The enzyme concentration in the cuvette being 0.31 mg/ml on the basis of the purity (86%, see Fig. 5b). Hence, we can calculate a minimum molecular weight per molecule of flavin of about 57,000.

Homogeneity and Molecular Weight—The native holoenzyme was found to have a molecular mass of about 300 kDa on HPLC gel filtration in the presence of 0.005% Triton X-100 as shown in Fig. 3. Electrophoresis on SDS-PAGE showed one major band with three minor bands (Fig. 5a, lane 3). The purity appeared to be about 86% by quantitative gel scanning (Fig. 5b). The minimum molecular weight of the enzyme was estimated from its electrophoretic mobility to be about 49,000 (Fig. 5c).

Flavin Quantification—FAD was released by denaturing the enzyme with heat treatment (*27*) and flavins associated with the Fpk preparations were determined by HPLC with a reversed-phase column. Figure 6 shows the elution patterns of standard FAD (Fig. 6a) and FAD released from the enzyme (Fig. 6b). The extract from the Fpk preparation showed only one peak which corresponded to the standard FAD, and no other peaks were seen. Its concentration was calculated to be 5.8 nmol/ml of enzyme, which agrees quite well with the spectrophotometrically determined value (Fig. 4). The FAD content was determined to be 16.8 nmol/mg protein based on the purity (86%). Assuming that the molecular weight of the native enzyme is about 300,000 (Fig. 3b), this corresponds to a minimum molecular weight of 59,000 per molecule of flavin, which again agrees with the spectrophotometrically estimated value (Fig. 4). The enzyme appears to be polymeric in its native form.

Monoclonal Antibodies to FpK—Table II shows the properties and the effect of mAbs in hybridoma cell supernatant on the Fpk activity. All mAbs belonged to the IgG class. The four mAbs (Nos. 1-4) in group I strongly inhibited the enzyme activity (70-90%) but the immunoblot showed double or multiple protein bands stained very weakly. The five mAbs (Nos. 5-9) in group II inhibited the enzyme activity by about 30-60% and yielded a moderately stained single band having an apparent molecular mass of 49 kDa in the Western blot. The three mAbs (Nos. 10-12) in group III inhibited the enzyme activity less than those in the previous two groups but yielded a main, strongly stained 49 kDa band with two other minor weakly stained bands. The inhibition rate was unaffected by secondary reagents. Figure 7 shows the Western blot patterns of representatives of the three groups. MAb No. 2 of group I stained double bands very weakly (lane 1). MAb No. 6 of group II stained a single band moderately (lane 2) and mAb No. 11 of group III stained one band at 49 kDa strongly and

TABLE II. **Properties and effect of monoclonal antibodies in hybridoma cell supernatants on the Fpk activity.**

Name of No. clones	Subclass	Inhibition of enzyme activity (%)		Western blot	
		−Protein A agarose beads	+Protein A agarose beads	Band[a]	Color intensity[b]
Group I					
1. Fpk1G4	IgG1k	84	75	m	w
2. Fpk3H11	IgG1k	88	71	d	vw
3. Fpk7C8	IgG1k	88	72	d	vw
4. Fpk8E7	IgG1k	87	72	d	vw
Group II					
5. Fpk2B7	IgG1k	49	58	s	md
6. Fpk2G1	IgG1k	47	40		md
7. Fpk4A1	IgG1k	64	57	s	md
8. Fpk5G5	IgG1k	55	57	s	md
9. Fpk1A10	IgG2bk	37	31	s	md
Group III					
10. Fpk2G5	IgG2bk	42	27	t?	st
11. Fpk4F9	IgG1k	38	27	t?	st
12. Fpk7C1	IgG1k	29	12	t?	st

[a]s, single; d, double; t, triple; m, multiple (5 bands). [b](v)w, (very) weakly; md, moderately; st, strongly stained. Western blot was performed using the same antigen as that used for immunization. The inhibition of enzyme activity was measured twice by HPLC-ECD as described in "MATERIALS AND METHODS" and is shown as per cent inhibition calculated from the mean value.

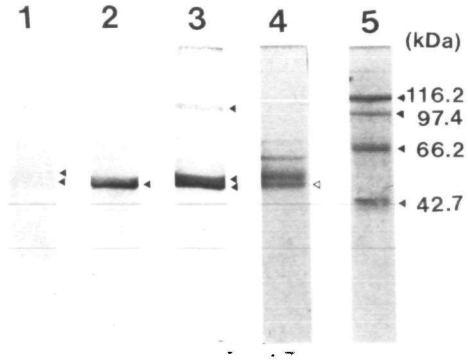

Fig. 7. **Western blot patterns of three representative mAbs from groups I, II, and III in Table II.** Western blot was performed using the same antigen as that used for immunization. Lane 1, mAb No. 2; lane 2, mAb No. 6; lane 3, mAb No. 11; lane 4, Fpk preparation (about 6 μg/lane). The open triangle shows the Fpk band whose molecular mass is 49 kDa; lane 5, molecular weight standards (Bio-Rad). Lanes 4 and 5 were stained with Coomassie Brilliant Blue.

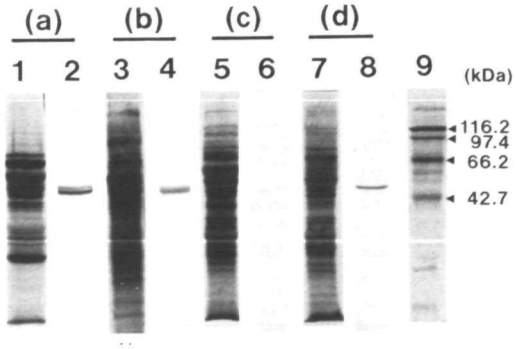

Fig. 8. **Immunoblot analysis of hepatic and renal mitochondrial Fpk prepared from pig and rabbit.** Partially purified Fpk or purified mitochondrial OMs prepared as described in Ref. *10* were subjected to 10% SDS-PAGE. Proteins were blotted onto PVDF membrane, reacted with mAb (No. 12) and stained with a POD immunostaining set. Lanes 1, 3, 5, and 7 show the proteins stained with Coomassie Brilliant Blue R-250. Lanes 2, 4, 6, and 8 show the immunoblot of Fpk. Lane 9 shows molecular weight standards (Bio-Rad) stained with Coomassie Brilliant Blue. (a) 15 μg/lane of partially purified Fpk (preparation of step 5); (b) 34 μg/lane of purified mitochondrial OM from pig kidney; (c) 23 μg/lane of purified mitochondrial OM from rabbit liver; (d) 23 μg/lane of purified mitochondrial OM from rabbit kidney.

two other bands moderately or weakly (lane 3).

Immunoblot Analysis of Hepatic and Renal Mitochondrial Fpk Prepared from Pig and Rabbit—Figure 8 shows immunoblot detection of pig and rabbit mitochondrial Fpk from liver and kidney using cell culture supernatant No. 12 in group III. In order to visualize bands clearly, a mAb of group III was used, despite the fact that the bands detected by mAbs in group III was not single but triple with the other minor bands weakly stained (Fig. 7 and Table II). It detected a darkly stained, single protein band (49 kDa) with a band of a light shade just above this band when partially purified Fpk from pig liver was electrophoresed on SDS-gels (Fig. 8a, lane 2). The band of a light shade was stained non-specifically since it was seen also in the control lane where no primary mAb was added (data not shown). When mitochondrial OMs purified by a swelling method from pig kidney were electrophoresed on SDS-gels (Fig. 8b), this mAb detected again only one band at molecular mass of 49 kDa (lane 4) among various protein bands (lane 3). When purified mitochondrial OMs from rabbit liver were separated on SDS-gels, this mAb also detected a single protein band (49 kDa) weakly (Fig. 8c, lane 6), but the same protein band could be stained clearly with this mAb when purified mitochondrial OMs from rabbit kidney were separated on SDS-gels (Fig. 8d, lane 8). Almost the same staining pattern was obtained with one of the mAbs in group II, but the staining was weaker than that with group III (data not shown).

DISCUSSION

In the present study we have obtained for the first time a purified preparation of Fpk nearly completely free of heme contamination, and we present definitive evidence that Fpk is a simple flavoprotein. The 275 nm/450 nm absorption ratio was greater than that of most flavoproteins, a characteristic also observed in Ziegler's mixed function amine oxidase (*40*). The absorption at about 420 nm of the reduced enzyme may be due to the presence of a trace of hemoprotein contaminant, but the oxidized form showed no noticeable Soret band of heme (Fig. 4), so that our spectrum is superior to that reported for the rat liver enzyme (*17*), which still showed a Soret band originating from heme contamination and lacked a peak near 380 nm due to FAD. Thus, our spectrum clearly demonstrates that Fpk is a simple flavoprotein.

Our enzyme preparation shows very high specific activity (Table I) compared with the best rat liver enzyme preparation reported previously (*16*). The mechanism of the decrease in the specific activity by omission of FAD is presently not known, but the more than 200 times higher concentration of added FAD (40 μM) compared to the K_m for FAD (*11*) may have had a non-specific effect on the enzyme. Although the dissociation constant of FAD has not been reported because of the difficulty of reconstitution with FAD (*17*), it might be small enough for FAD not to dissociate, since diluted enzyme solution at a final concentration of 3.5×10^{-9} M (calculated on the basis of the molecular mass of the native enzyme being 300 kDa) showed still the linearity (data not shown). In other words, the activation by added FAD (Table I) is not due to the presence of apoenzyme.

As to FAD content, whereas the enzyme from rat liver was reported to contain 4 mol of FAD/mol of monomeric enzyme (*17*), we found only 1 mol of FAD/mol of monomeric enzyme from pig liver. This estimation was obtained from the content of FAD, determined spectrophotometrically from the redox difference of the absorbance at 450 nm (Fig. 4) or measured by HPLC (Fig. 6) and from the purity of the enzyme preparation, which was considered to be 86% pure (Fig. 5b). The molecular weight of the native enzyme in the presence of 0.005% Triton X-100 determined by HPLC gel filtration was about 300,000 (Fig. 3), and that of the monomer determined by SDS-PAGE (Fig. 5a) and from the flavin content (Figs. 4 and 6) was about 49,000 and 57,000-59,000, respectively. A predicted molecular mass of 55,762 Da has been reported very recently based on the nucleotide sequence of the cloned cDNA of the human enzyme (*41*). The lower estimation of molecular weight by SDS-PAGE may be due to the hydrophobic nature of the enzyme. In its native form the enzyme may have some bound detergent. Nevertheless, UV/VIS spectra could be well recorded with the final enzyme preparation (Fig. 4) using HPLC gel filtration buffer as a reference, because a large amount of Triton X-100 could be removed by HPLC gel filtration (Fig. 3b).

The monomer of the Fpk from pig liver mitochondrial outer membrane is extremely different from that reported for rat liver mitochondria with respect to molecular weight and FAD content. The monomeric molecular mass of the rat enzyme has been reported to be 200 kDa (*16*), 160 kDa (*17*), and 145 kDa (*18*), too high for a simple flavoprotein, since the monomeric molecular mass of any simple external flavoprotein monooxygenase generally falls in the range between 46 and 93 kDa (*42, 43*). However, this may be due to the difference of animal species, since such species difference in molecular weight has been reported for another liver mitochondrial enzyme, NAD^+-glycohydrolase (*44*). We are now purifying the Fpk from rat liver mitochondrial outer membrane.

The present purification method is more reproducible than our previous method in which two detergents, sodium cholate and Lubrol PX were used (*19*). In the present method we used OGS in place of sodium cholate and Lubrol PX since OGS has two major advantages: (i) it can be removed more easily than Lubrol PX from the enzyme preparation; (ii) Lubrol PX shows a strong inhibitory effect on the enyzme activity unless freshly prepared Lubrol PX solution is used (*19*). A point of great difficulty during purification of the Fpk from pig liver mitochondria is to remove heme contamination and MAO activity without inactivation of the Fpk. Since an organomercurial-Sepharose was used to purify pig brain mitochondrial MAO (*45*), we tried an Aff-gel 501 (an organomercurial agarose) to remove MAO activity, but without success (*21*). The SDS-PAGE electrophoregram of purified Fpk (Fig. 5a, lane 3) shows a weakly stained band at a molecular mass of about 60 kDa just above the main protein band at a molecular mass of 49 kDa. The former (about 7% as quantified by scanning) may be MAO B subunit since the molecular mass of MAO B subunit from pig liver mitochondra has been reported to be about 60 kDa (*46*). However, the yield of MAO activity in the final preparation was 0.005% and no trace of an unusual absorption band at 412 nm due to the anionic flavin semiquinone of MAO B (*47*) could be seen in our spectrum (Fig. 4).

Concerning the removal of heme contamination without loss of the enzyme activity, the importance of the use of a small amount of Triton X-100 (0.005%) at the HPLC gel filtration step must be emphasized. This step served to remove not only heme contamination, but also micelles of Triton X-100 formed during ultrafiltration by an Amicon Centriplus-100 (*48*) of the eluate from dye ligand chromatography which was performed in the presence of 0.1% Triton X-100. This detergent forms micelles at concentrations above its critical micellar concentration (0.25 mM = 0.015%) (*49*). We previously used OGS at this step during purification of the enzyme from rat liver mitochondria to avoid interference with monitoring of elution at 280 nm (*50*). However, protein peaks could also be detected in the presence of 0.005% Triton X-100. Despite the much lower molecular weight of hemoproteins, such as outer membrane cytochrome b_5 (*51, 52*), than that of Fpk, a visible spectrum having no heme contamination could not be obtained when OGS was used in place of Triton X-100. Both proteins seem to be present as protein-protein complexes held together *via* very tight hydrophobic interaction and only Triton X-100 could disrupt this interaction, while OGS could not. Although the method described in this paper yields an enzyme preparation of high purity, the low yield (0.8%) remains a problem. We are currently trying to improve recovery of the enzyme.

Since monospecific antibodies can be prepared against a component of an impure antigen preparation by using hybridoma technology (*53*), we tried to make a mouse mAb to pig liver mitochondrial Fpk. We have obtained for the first time mAbs against Fpk with different properties. All of these mAbs directly inhibited the enzyme activity without secondary reagents (Table II). MAbs in group I showing a strong inhibitory effect but almost negligible Western blot, perhaps resemble mAb 3-29-9 to human cytochrome P-450 3A4 (*54*). Some mAbs in groups II and III may be useful as probes for the screening of cDNA expression libraries to isolate cDNA of pig liver Fpk. In fact, these mAbs in groups II and III could stain only one protein band at 49 kDa among various protein bands of mitochondrial OMs (Fig. 8).

All enzymes of the kynurenine pathway beginning from tryptophan and leading to NAD *via* Fpk also exist in the brain (*54-57*). Kynurenine is situated at the branch point for the formation of quinolinic acid, a potent endogenous neuroexcitotoxin, and of kynurenic acid, an endogenous neuroprotective factor (*6, 58*). Tryptophan catabolism is very much enhanced in the case of viral infection in the brain (*59*) and brain damage following cerebral ischemia (*60*). Fpk is the first step enzyme for the biosynthesis of quinolinate from kynurenine. Information about the molecular structure of Fpk will serve to elucidate the regulatory mechanism of the Fpk activity in the brain.

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