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Purification and Characterization of Two Mevalonate Pyrophosphate Decarboxylases from Rat Liver: A Novel Molecular Species of 37 kDa

Akihiro Michihara,[1] Makoto Sawamura, Yasuo Nara, Katsumi Ikeda, and Yukio Yamori

Graduate School of Human and Environmental Studies, Kyoto University, Sakyo-ku, Kyoto 606

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The biosynthesis of cholesterol is regulated mainly by HMG-CoA reductase, however, recent studies indicated the pivotal role of another enzyme in cholesterol homeostasis. A previous report showed a marked decrease in the activity of mevalonate pyrophosphate decarboxylase (MPD) in stroke-prone spontaneously hypertensive rats and its possible involvement in the pathogenesis of the disorder. In this study, we purified liver MPD from rats fed a diet containing cholestyramine and pravastatin (CP diet) using conventional chromatographic techniques. We obtained two electrophoretically homogeneous enzyme preparations; 45 and 37 kDa proteins with specific activities of 8.0 and 7.4 μmol/min/mg, respectively. The enzymes showed similar molecular weights of 90 kDa, as judged on gel permeation chromatography. A kinetic study indicated apparent K_m values for mevalonate pyrophosphate and ATP of 22.7 μM and 0.71 mM, respectively, for the 45 kDa MPD, and 20.0 μM and 0.80 mM, respectively, for the 37 kDa MPD. Half maximum activities were observed at 1.5 mM and 1.1 mM Mg^{2+} for the 45 and 37 kDa MPDs, respectively. Both enzymes required ATP as a phosphate acceptor, and in addition Mg^{2+}, Mn^{2+}, and Co^{2+} were effective as divalent cations. The optimum pH for both enzymes was 7.0. The isoelectric points for the 45 and 37 kDa MPDs were 5.6 and 5.4, respectively. Polyclonal antiserum raised against the 45 kDa enzyme detected both the 45 and 37 kDa bands on immunoblots with CP diet-induced liver crude extract as an antigen. However, non-induced liver contained the 45 kDa protein but not the 37 kDa protein. These results indicated that the CP diet induced a new species, 37 kDa, of MPD which is characteristically and immunologically very similar to the well-known 45 kDa MPD.

Key words: characterization, decarboxylase, mevalonate pyrophosphate, pravastatin, purification.

One of the first steps in the biosynthesis of cholesterol from acetic acid is catalyzed by MPD. Although HMG-CoA reductase is the rate limiting enzyme of this pathway (*1-3*). MPD is also considered to be regulated by dietary cholesterol or cholesterol-lowering drugs (*4-7*). This decarboxylase catalyzes a bimolecular reaction between MVAPP and ATP to form isopentenyl pyrophosphate, inorganic phosphate, ADP and CO_2.

The enzyme has been purified from various sources including yeast (*8, 9*), latex of Hevea brasiliensis (*10*), pig liver (*11, 12*), rat liver (*13, 14*), and chicken liver (*15*). The chicken liver enzyme has been purified 5,800-fold and has a specific activity of 6.3 μmol/min/mg protein. It is an enzyme of a molecular weight of 85,400, and consists of two indistinguishable subunits. Toth and Huwyler recently reported the cDNA sequences of MPDs from human liver and yeast (*16*). The recombinant human enzyme is a homodimer of a 43 kDa subunit with 400 amino acids.

SHRSP is a strain of rat suffering from severe hypertension and cerebral strokes (*17, 18*). Recently, we reported reduced MPD activity in tissues of SHRSP, and the resultant low biosynthetic activity of cholesterol might contribute to the pathophysiology of the disorder (*19*). However, the mechanism underlying the lower activity of MPD in SHRSP remains unclear.

To address this issue, we planned to purify the MPDs of SHRSP and the parental control rat, WKY, to compare their molecular properties. In the course of this project, we first purified MPD from WKY rats fed a diet containing cholesterol-lowering drugs in this study, and unexpectedly found two molecular species of MPD.

MATERIALS AND METHODS

Materials—Ampholine PAG plates, an isoelectric focusing calibration kit, Blue Sepharose 6FF, Phenyl Sepharose HP, Superose 12HR 10/30, and molecular weight marker

[1] To whom correspondence should be addressed.

Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MPD, mevalonate pyrophosphate decarboxylase; MVAPP, mevalonate pyrophosphate; MVK, mevalonate kinase; NADH, β-nicotinamide-adenine dinucleotide, reduced form; PEP, phosphoenolpyruvic acid; PMSF, phenylmethylsulfonyl fluoride; QAE, quaternary aminoethyl (diethyl mono-2-hydroxybutylaminoethyl); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SHRSP, stroke-prone spontaneously hypertensive rat; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; WKY, Wistar Kyoto rat.

proteins were purchased from Pharmacia Biotech; QAE TOYOPEARL 550 and TOYOPAK ODS cartridges were from TOSOH (Tokyo); Macro-Prep Ceramic Hydroxyapatite and protein assay dye reagent were from Bio-Rad; pravastatin was from Sankyo (Tokyo); cholestyramine was from Bristol Laboratories; and RS-[^{3}H]mevalonolactone was from Du Pont-New England Nuclear. All other chemicals were of reagent grade and purchased from commercial sources.

Animals—Male WKY rats (12 weeks old, about 310 g in body weight) were housed in a light-controlled room (light phase, 6:00-18:00). The rats were fed on powdered rat chow containing cholestyramine and/or pravastatin as indicated. The rats were fasted for one day before sacrifice (10:00).

Radioactive Assay—The enzyme activities of the crude extract as well as the purified enzyme were measured according to Sawamura *et al.* (*19*). Unless otherwise indicated, the reaction mixture comprised 100 mM Tris-HCl (pH 7.0), 5 mM ATP, 5 mM $MgCl_2$, 10 mM iodoacetamide, 10 mM NaF, 30 mM nicotinamide, 250 nmol MVAPP, and 50 ng of purified enzyme. Preparation of [^{3}H]MVAPP from [^{3}H]mevalonic acid was carried out based on the method described by Cardemil and Jabalquinto (*20*).

Spectrophotometric Assay—Enzyme activity was measured spectrophotometrically during the purification (*20*). The reaction mixture (1 ml final volume) contained 0.1 M Tris-HCl buffer (pH 7.0), 0.1 M KCl, 5 mM ATP, 5 mM $MgCl_2$, 0.5 mM PEP, 0.25 mM NADH, 10 units of pyruvate kinase, 10 units of lactic dehydrogenase, MPD, and 250 nmol MVAPP.

Purification of MPD—Purification of rat liver MPD was carried out at 4°C essentially as described previously by Alvear *et al.* (*15*).

(a) Homogenization: Rat livers (102 g wet weight, 15 rats) were washed with 100 mM sodium phosphate (pH 7.0) containing 10 mM 2-mercaptoethanol and 1 mM EDTA (Buffer A), and then homogenized in 3 volumes of ice cold Buffer A containing 0.5 mM PMSF using a Polytron motor driven homogenizer. The homogenate was centrifuged at 20,000×g for 30 min to obtain a crude extract.

(b) Ammonium sulfate [$(NH_4)_2SO_4$] fractionation: Solid $(NH_4)_2SO_4$ was added to the supernatant and the 35-60% precipitate fraction was obtained. The precipitate was resuspended in 50 ml of 10 mM sodium phosphate (pH 7.0) buffer containing 10 mM 2-mercaptoethanol and 0.1 mM EDTA (Buffer B), and then dialyzed for 18 h against 6 liters of the same buffer.

(c) QAE column chromatography: The dialyzate was applied to a column (3.0×32 cm, V=226 ml) of QAE equilibrated with Buffer B. After washing with one column volume of Buffer B, the enzyme was eluted with five column volumes of the buffer containing a linear gradient of 10-160 mM sodium phosphate at the flow rate of 2 ml/min (18 ml/tube). MPD was eluted at 100 mM and the enzyme was concentrated with 80% $(NH_4)_2SO_4$, followed by dialysis against 20 mM Tris-HCl (pH 7.0) buffer containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol (Buffer C).

(d) Blue Sepharose 6FF column chromatography: The dialyzate was applied to a column (1.5×20 cm, V=35 ml) of Blue Sepharose equilibrated with Buffer C. The column was washed with one column volume of Buffer C and then the enzyme was eluted with five column volumes of the same buffer containing a linear gradient of 0-0.6 M NaCl at the flow rate of 1.0 ml/min (3 ml/tube). The active fractions (at 0.2 M) were pooled, concentrated as above, and then dialyzed against 5 mM sodium phosphate (pH 7.0) containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol (Buffer D).

(e) Hydroxyapatite column chromatography: The dialyzate was applied to a column (1.5×11 cm, V=19 ml) of hydroxyapatite equilibrated with Buffer D. The column was washed with one column volume of Buffer D and then the enzyme was eluted with seven column volumes of the same buffer containing a linear gradient of 10-70 mM sodium phosphate (pH 7.0) at the flow rate of 0.5 ml/min (1.5 ml/tube). 1.5 ml fractions were collected. Three active peaks were observed, which were designated as fractions A, B, and C, respectively. On SDS-PAGE, each fraction was found to contain different molecular species; a 45 kDa band for A, a 45 kDa band and a 37 kDa one for B, and mainly a 68 kDa band and a 37 kDa one for C. Fractions A and B were stored at −20°C in Buffer D containing 20% glycerol until use. Fraction C was concentrated and then NaCl added to a final concentration of 3 M for further purification.

(f) Phenyl Sepharose HP column chromatography: Fraction C was applied to a column (1.5×15 cm, V=26 ml) of phenyl Sepharose equilibrated with Buffer D containing 3 M NaCl. The column was washed with one column volume of the same buffer, and then the enzyme was eluted with five column volumes of the same buffer containing a linear gradient of 3-0 M NaCl at the flow rate of 0.5 ml/min (1 ml/tube). MPD was eluted with 1.7 M NaCl, however, SDS-PAGE analysis showed trace contamination by the 45 kDa protein. Therefore, the 37 kDa protein was further purified by hydroxyapatite column chromatography as described in (d), which resulted in a homogeneous preparation of the 37 kDa protein on SDS-PAGE. The purified 37 kDa protein was stored as described above.

Molecular Weight Determination—The molecular weight of the purified MPD was determined at room temperature on a Superose 12 column (1.0×30 cm, V=23.6 ml) equilibrated with 50 mM sodium phosphate (pH 7.0), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.15 M NaCl. The standard molecular weight markers were as follows: ferritin (445 kDa), catalase (238 kDa), aldolase (158 kDa), BSA (68 kDa), and ovalbumin (45 kDa).

Isoelectric Focusing—The isoelectric points (pIs) of the pure MPDs were determined using Ampholine PAG plates (pH range, 3.5-9.5). Three micrograms of a purified enzyme was electrofocused at 200 V for 1 h, 400 V for 1 h, and 1,000 V for 2 h at 10°C. The anode solution was 0.1 M phosphoric acid, and the cathode solution was 0.1 M NaOH. After electrofocusing, the gels were fixed in 0.7 M trichloroacetic acid and 0.16 M 5-sulfosalicilic acid, and then the protein bands were visualized by Coomassie Blue staining. The pI values of the 45 and 37 kDa enzyme were calculated by comparison with the following isoelectric focusing standards : aminoglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin-acidic band (6.85), horse myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65), and trypsinogen (9.30).

Preparation of Anti-MPD Antiserum and Antibody Purification—The purified enzyme (200 μg) emulsified with complete Freund's adjuvant was injected subcutaneously into a New Zealand White rabbit (2.0 kg) every 2 weeks. Two weeks after the third injection, the animal was bled from the ear vein.

Most of the experiments in this study were carried out with this antiserum, however, the antibody was further purified with the antigen when indicated in the results using the method described by Coudrier *et al.* (*21*). The purified 45 kDa MPD was subjected to SDS-PAGE followed by blotting on to a nitrocellulose membrane. Strips of nitrocellulose containing the relevant polypeptides were excised and reacted with the antiserum. The antibodies were eluted at 4°C from the nitrocellulose strips with glycine-HCl, pH 3.0, containing 0.2% gelatin, and then neutralized immediately with a 1 M Tris base solution.

Immunoblot Procedures—Western blot analysis was carried out using ECL. The Western blotting detection kit (Amersham, Bucks, UK) was used according to the manufacturer's instructions. When the crude extract was analyzed, rat liver was homogenized in three volumes of 0.1 mM phosphate buffer alone or Buffer H (H.B.; Buffer A containing 1 mM EGTA, 0.5 mM PMSF, 0.1 mM antipain, 0.1 mM pepstatin A, 0.1 mM chymostatin, 0.1 mM leupeptin, and 0.15 M NaCl). After being centrifuged at 20,000 × *g* for 20 min, the supernatant was subjected to SDS-PAGE.

Analysis of the N-Oligosaccharide Chain—The purified 45 kDa (25 ng) and 37 kDa (4 ng) MPDs were incubated with 10 mU/ml neuraminidase containing 0.05% BSA in acetate buffer (pH 6.5) or 10 mU/ml *N*-glycanase in Tris-HCl buffer (pH 8.0) for 12 h at 37°C. The proteins were then subjected to SDS-PAGE, followed by immunoblotting.

Gel Electrophoresis—SDS-PAGE was carried out on a 10% acrylamide gel as described by Laemmli (*22*).

Protein Determination—Protein concentrations were determined by means of the Bio-Rad protein assay using BSA as the standard.

RESULTS

Induction of MPD Activity—Since mevalonate kinase (MVK) was reported to have been purified from liver treated with pravastatin and cholestyramine (*23*), we attempted to purify MPD from the livers of rats fed a diet containing these drugs. Figure 1 shows the typical increasing activity of MPD in the drug-treated rats. MPD activity in the liver crude extract was markedly increased by treatment with cholestyramine and/or pravastatin. When treated with 5% cholestyramine and 0.1% pravastatin (CP diet) for 12 days, maximum activity of up to 10-fold that in rats given normal chow was observed. Therefore, MPD was purified from the livers of rats treated with the CP diet for 12 days in this study.

Purification of MPD—Rat liver MPD was purified as described under "MATERIALS AND METHODS." On hydroxyapatite chromatography, we found three active peaks (Fig. 2A, fractions A, B, and C). SDS-PAGE analysis revealed that fraction A contained a protein of 45 kDa, fraction B ones of 45 and 37 kDa, and fraction C ones of 37 and 68 kDa (Fig. 2B). From the results in Fig. 2, A and B, it was found that the 45 kDa protein exhibits MPD activity. However, both the 68 and 37 kDa proteins were suggested to be

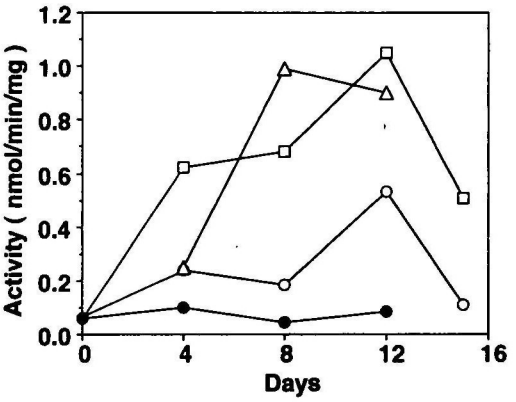
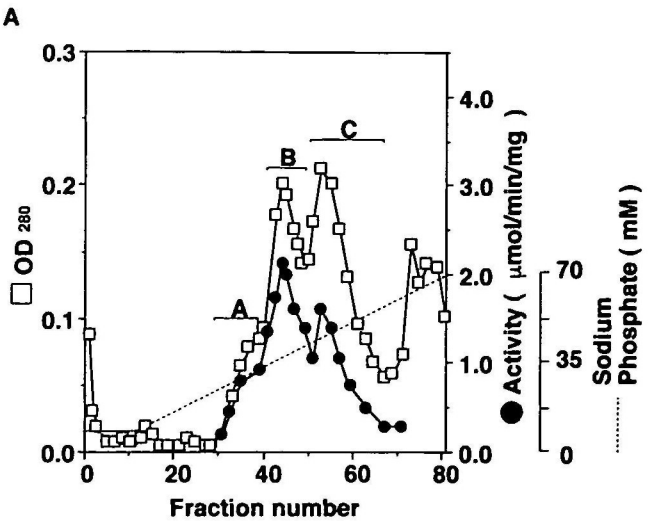


Fig. 1. **Induction of MPD activity in rat liver by cholestyramine and pravastatin.** Rats were fed powdered chow without (●) or with 5% cholestyramine (○), or 5% cholestyramine and either 0.1% (□) or 0.5% (△) pravastatin for the indicated periods. MPD activities in the liver crude extracts were measured by means of a radioactive assay as described under "MATERIALS AND METHODS."



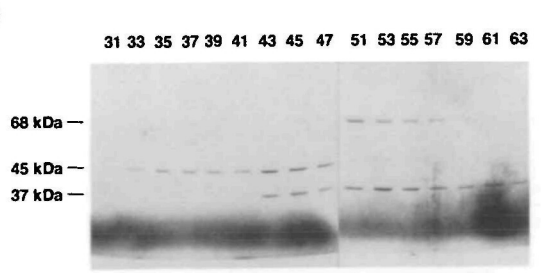


Fig. 2. **Purification of MPD by hydroxyapatite column chromatography.** A: After the active peak material obtained on Blue Sepharose had been dialyzed, it was applied to a column of hydroxyapatite. Absorbance at 280 nm (□) and enzyme activity (●) were measured for each fraction by means of a spectrophotometric assay. B: Each fraction eluted from the hydroxyapatite column was analyzed by SDS-PAGE.

MPDs, too. Therefore, fraction C was further purified by phenyl Sepharose chromatography (Fig. 3). The 68 kDa protein eluted with 0.65 M NaCl was purified to electrophoretic homogeneity (data not shown). However, the 68 kDa protein did not exhibit MPD activity. The 37 kDa protein having MPD activity was further purified by second hydroxyapatite chromatography. Consequently, we obtained two different MPDs from the CP diet-treated rat liver, a 45 kDa MPD and a 37 kDa MPD that has not been previously reported. Figure 4 and Table I show the results of the overall purification steps. As compared with the QAE fraction, the 45 and 37 kDa MPDs were purified approximately 20- and 18.5-fold, with specific activities of 8.0 and 7.4 μmol/min/mg protein, and 7.1 and 1.0% recoveries, respectively. The specific activities were the highest among the values reported so far (*8-15*).

Molecular Weights of the MPDs—The molecular weights of the native enzymes were determined on Superose 12 (Fig. 5). The 45 kDa enzyme had a molecular weight of 90 kDa, indicating a homodimer structure. Surprisingly, the 37 kDa enzyme was eluted at a position identical to that of the 45 kDa enzyme. Indeed, when a mixture of the two enzymes was applied on the column, a single peak corresponding to 90 kDa was observed.

Initial Velocity Studies—The apparent K_m and V_{max} values for MVAPP were 22.7 μM and 7.1 μmol/min/mg, respectively, for the 45 kDa MPD, which were comparable to those for the 37 kDa MPD (K_m, 20 μM; V_{max}, 6.1 μmol/min/mg; Fig. 6A). When the concentration of ATP was changed, the K_m and V_{max} values were very similar for the 45 and 37 kDa MPDs (K_m, 0.71 mM; V_{max}, 5.4 μmol/min/mg for 45 kDa; and K_m, 0.80 mM; V_{max}, 5.3 μmol/min/mg for 37 kDa; Fig. 6B). These K_m values were in consistent with those reported by other investigators (*14, 15*). Figure 6C shows the dependence of the MPD activity on Mg^{2+}. The half maximum activities were observed at 1.5 mM for 45 kDa MPD, and at 1.1 mM for 37 kDa MPD. The "maximum activities" at high concentrations of Mg^{2+} were also similar for the two enzymes.

Effect of pH on the MPD Activity—Figure 7 shows the pH dependency of the 45 and 37 kDa MPDs. The maximum activity was observed at pH 7.0 for both MPDs, while the

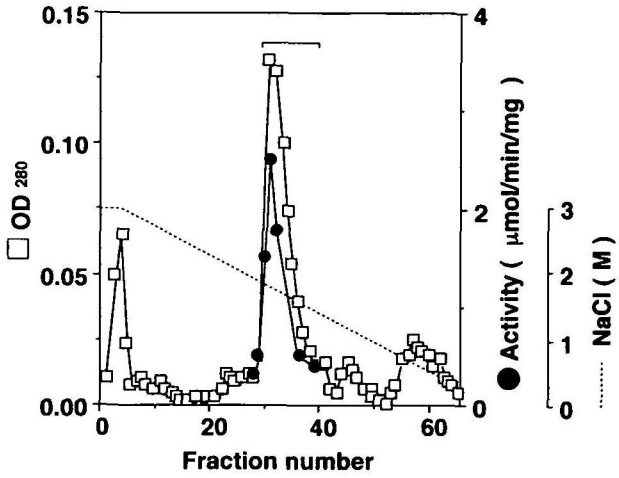


Fig. 3. **Purification of MPD by phenyl Sepharose column chromatography.** NaCl was added to a final concentration of 3 M to fraction C obtained on hydroxyapatite, and then the fraction was applied to a column of phenyl Sepharose.

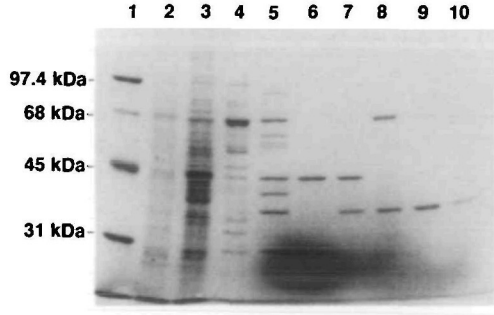


Fig. 4. **Proteins at each purification step were analyzed by SDS-PAGE.** The active fractions at each step were subjected to SDS-PAGE, and then the proteins were visualized by Coomassie Blue staining. The relative positions of the molecular weight standards are shown in the left margin. Lane 1, markers (5 μg); 2, crude extract (2 μg); 3, 35-60% $(NH_4)_2SO_4$ (5 μg); 4, QAE (5 μg); 5, Blue Sepharose (5 μg); 6, hydroxyapatite, fraction A (1 μg); 7, hydroxyapatite, fraction B (1 μg); 8, hydroxyapatite, fraction C (1 μg); 9, phenyl Sepharose—fraction C obtained on hydroxyapatite was applied (1 μg); 10, second hydroxyapatite—active peak obtained on phenyl Sepharose was applied (0.5 μg).

TABLE I. **Purification of MPD from rat liver.** MPD was purified from 15 livers (102 g) of rats fed on the CP diet for 12 days.

Purification step	Total protein (mg)	Total units (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
QAE	310	126	0.4		100
Blue Sepharose	31.6	65	2.9	5.0	52
Hydroxyapatite A	1.13	9.0	8.0	20.0	7.1
B	3.00	27.9	9.3	23.3	22.1
C	5.46	25.1	4.6	11.5	19.9
Phenyl Sepharose	0.81	9.89	12.4	31.0	7.8
2nd Hydroxyapatite	0.17	1.26	7.4	18.5	1.0

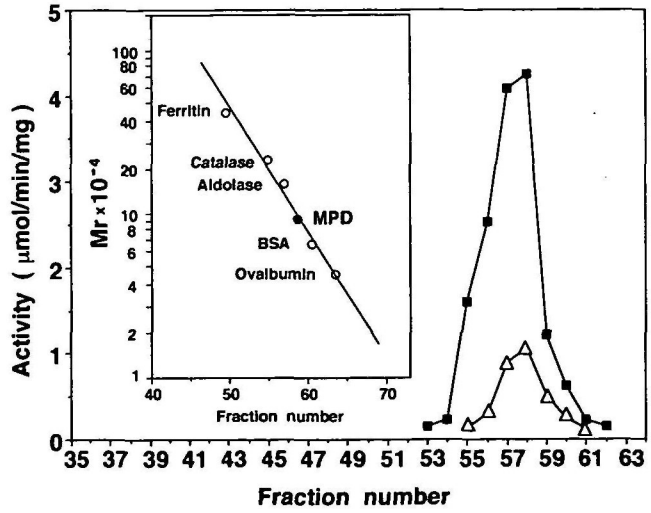


Fig. 5. **Native molecular weights of the MPDs.** The purified 45 kDa (34 μg) and 37 kDa (11 μg) MPDs were loaded onto a Superose 12 column. The MPD activity in each fraction was measured. The molecular weights of the purified enzymes were determined using the molecular weight standards shown in the inset. 45 kDa (■) and 37 kDa (△).

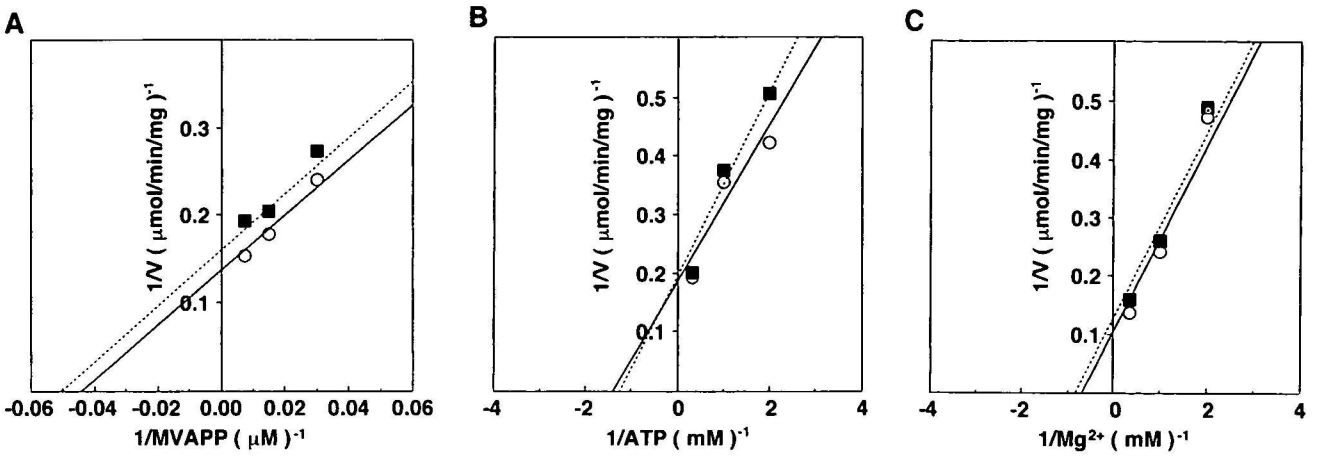


Fig. 6. **Kinetics of MPDs.** Fifty nanograms of the purified 45 kDa (○) and 37 kDa (■) enzymes was incubated with various amounts of MVAPP (A), ATP (B), and Mg (C), after which the initial velocities were estimated.

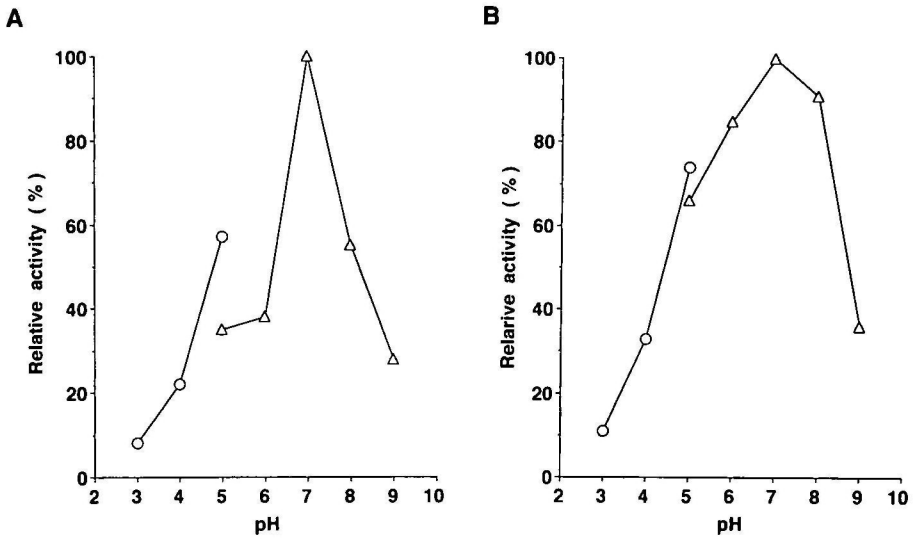


Fig. 7. **The effect of pH upon the activities of the MPDs.** Fifty nanograms of the purified 45 kDa (A) and 37 kDa (B) enzymes was assayed in 0.1 M glycine-HCl (○) or 0.1 M Tris-HCl (△). Each point represents the average of triplicate determinations.

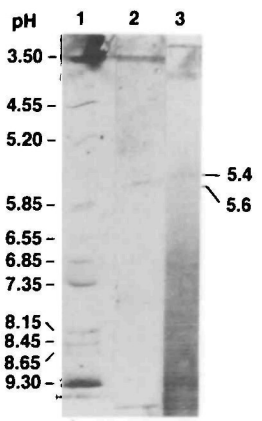


Fig. 8. **Isoelectric points of the MPDs.** The relative positions of the isoelectric focusing standards are shown in the left margin (lane 1). The purified 45 kDa (lane 2) and 37 kDa (lane 3) enzymes were electrofocused, and the proteins were visualized by Coomassie Blue staining.

TABLE II. **Effects of nucleotides and cations on the activity of the 45 and 37 kDa MPD purified from rat liver.** Activity in the presence of ATP and Mg is taken as 100%. Data are the means of four identical experiments and each value varies within 10%.

Nucleotide and cation (5 mM)	Relative activity (%)	
	45 kDa	37 kDa
ATP+$MgCl_2$	100	100
ATP+$MnCl_2$	126	149
ATP+$CoCl_2$	125	91
ATP+$CaCl_2$	55	38
ATP+$CdCl_2$	33	25
ATP+$BaCl_2$	21	16
ATP+KCl	16	15
ATP+NaCl	21	13
GTP+$MgCl_2$	15	15
CTP+$MgCl_2$	10	11
UTP+$MgCl_2$	8	11
None	9	9

37 kDa enzyme showed a broad pH dependency when compared with that of the 45 kDa. Previous reports indicated lower optimum pH values (4.0-6.5) for the 45 kDa MPD (*13, 15*).

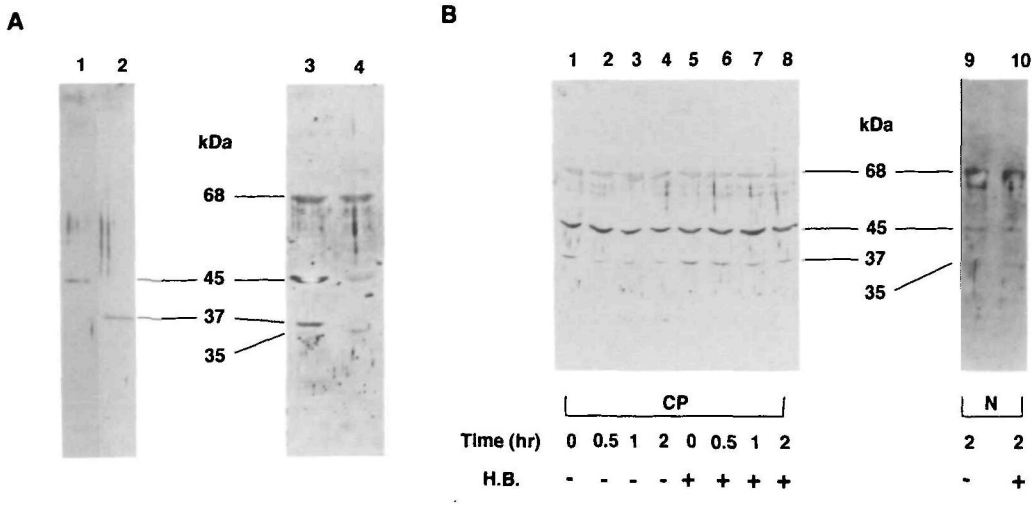


Fig. 9. **Immunoblot analysis of the MPDs.** A: Immunoblot analysis of the purified 45 kDa (lane 1, 4 ng) and 37 kDa (lane 2, 4 ng) enzymes, the CP diet-treated crude extract (lane 3, 10 μg), and the non-treated crude extract (lane 4, 20 μg) in H.B. B: Immunoblot analysis of liver soluble fractions. CP diet-treated (CP) or non-treated (N) rat liver was homogenized in 3 volumes of 0.1 M phosphate buffer alone (lanes 1-4 and 9) or H.B. (lanes 5-8 and 10). The homogenates were incubated for the indicated periods, followed by centrifugation at 20,000$\times g$ for 20 min. The supernatants (10 μg) were subjected to Western blot analysis.

Isoelectric Points of the MPDs—The isoelectric points of the 45 kDa and 37 kDa enzymes were almost identical (5.4 and 5.6, respectively), as determined on an Ampholine PAG plate (Fig. 8). These values were slightly higher than those of chicken liver MPD, as determined by chromatofocusing.

Cofactor Requirements—As shown in the Table II, Mg^{2+} could be replaced by Mn^{2+} or Co^{2+} with similar or slightly higher activity, so far as examined. Mn^{2+} was the best cation for the maximum activity for the 37 kDa MPD, while Mn^{2+} and Co^{2+} gave the same activity for the 45 kDa MPD. ATP was the only nucleotide required for the MPD activity. There was no difference between the 37 kDa and 45 kDa MPDs concerning the requirements for nucleotides and cations. Alvear *et al.* reported MPD was sensitive to sulfhydryl-directed reagents (*24*), however, our 37 and 45 kDa MPDs showed full activities even in the presence of 10 mM iodoacetamide (data not shown).

Immunochemical Studies—The results shown above indicated the very similar properties of the two MPDs. To distinguish the 45 and 37 kDa enzymes, anti-MPD antiserum was produced in a rabbit by multiple injections of the purified 45 kDa enzyme. The antiserum detected not only the purified 45 kDa but also the purified 37 kDa enzyme (Fig. 9A, lanes 1 and 2), even with the affinity-purified antibody (data not shown). Indeed, when the crude extract (20,000$\times g$, sup) of rat liver with the CP diet was subjected to Western blot analysis using this antiserum, both the 45 and 37 kDa enzymes were detected (Fig. 9A, lane 3). However, the crude extract of non-treated rat liver contained only the 45 kDa enzyme, *i.e.* not the 37 kDa MPD (Fig. 9A, lane 4). Also, the amount of the 45 kDa enzyme was markedly increased by the CP diet, as compared with the non-treated rat liver (Fig. 9A, lanes 3 and 4).

Since the 45 and 37 kDa proteins were immunologically related, the 37 kDa protein could be proteolytically cleaved from the 45 kDa protein during purification. In order to exclude this possibility, we preincubated the crude extract of CP diet-treated rat liver in the presence or absence of various protease inhibitors, and the two MPDs were analyzed by Western blotting (Fig. 9B). Both MPDs were detected even in the presence of protease inhibitors (Fig. 9B, lanes 5-8), and preincubation for up to two hours in the phosphate buffer alone did not increase the amount of the 37 kDa MPD (Fig. 9B, lanes 1-4). Moreover, the 37 kDa MPD was not detected in the crude extract of non-treated rat liver with preincubation in the presence or absence of protease inhibitors (Fig. 9B, lanes 9 and 10). Therefore, we concluded that the 37 kDa MPD was not proteolytically cleaved from the 45 kDa MPD during purification.

In this series of experiments the antiserum detected a 68 kDa protein in the crude extract which comigrated with BSA among the molecular marker standards. Since BSA was also detected by the antiserum (data not shown), the 68 kDa protein is considered to be serum albumin. A 35 kDa band was also detected by the antiserum in the crude extract of non-treated rat liver (Fig. 9A, lane 4, and 9B,

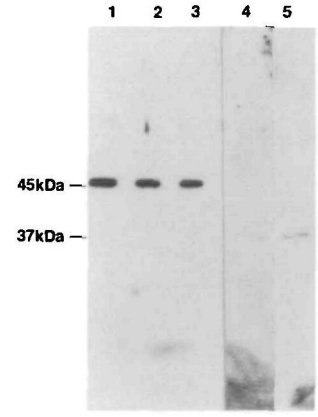


Fig. 10. **Analysis of glycosylation of the MPDs.** The purified MPDs were incubated with neuraminidase or *N*-glycanase, and then subjected to Western blot analysis. Lane 1, neuraminidase-treated 45 kDa; 2, *N*-glycanase-treated 45 kDa; 3, untreated 45 kDa; 4, *N*-glycanase-treated 37 kDa; 5, untreated 37 kDa.

lanes 9 and 10). A weak signal of this band was sometimes found in the CP diet-treated rat liver (data not shown). However, since it was lost during the purification procedure, the 35 kDa band was an immunologically-related protein with no MPD activity.

To eliminate the possibility that multiple polypeptides (45, 37, 35, and 68 kDa) were detected because of the impurity of the antiserum, we affinity-purified the antiserum with the purified 45 kDa MPD as an antigen. Although background signals were markedly reduced, essentially the same results were obtained with the purified antibody (data not shown).

Analysis of the N-Oligosaccharide Chain—*N*-Glycosylation is a well-known posttranslational modification of proteins, and may contribute to the difference between the 45 and 37 kDa proteins. To characterize the structures of the *N*-linked oligosaccharides of the MPDs, we treated the purified enzymes with *N*-glycanase, which released *N*-oligosaccharide chains from the *N*-linked complex form and high mannose form, and neuraminidase, which released sialic acid from the *N*-linked complex form. As shown in Fig. 10, neither neuraminidase nor *N*-glycanase treatment shifted the bands on SDS-PAGE. Furthermore, the periodate-Shiff procedure detected neither the 45 kDa nor the 37 kDa band (data not shown). These results suggest that intracellular glycosylation does not contribute to the difference between the 45 and 37 kDa species of MPD.

DISCUSSION

We isolated two distinct MPDs, a 45 kDa enzyme and a 37 kDa one, from the livers of rats subjected to the CP diet. These preparations had specific activities of 8.0 and 7.4 μmol/min/mg protein, respectively (Table I), which were higher than previously reported. The two molecular species of MPD could be partially separated only on hydroxyapatite column chromatography (Fig. 2). We failed to separate the 37 kDa enzyme from the 45 kDa one on an ATP agarose column or a phosphocellulose column (data not shown).

As in previous studies, analysis by Superose 12 column chromatography demonstrated that the native MPD had a molecular weight of 90 kDa, *i.e.* it consisted of two identical subunits of 45 kDa (Fig. 4). We also found a 37 kDa protein as a subunit of MPD, however, unexpectedly the native molecular weight of this enzyme was also 90 kDa. However, molecular weight estimation by gel permeation chromatography is not so accurate, so this type of MPD might be composed of two 37 kDa subunits constituting a 74 kDa enzyme.

The 37 kDa enzyme appeared only when the rats were fed the CP diet. A kinetic study demonstrated that the 37 kDa MPD had very similar characteristics to the 45 kDa MPD with respect to the affinity to substrates and divalent cations (Fig. 6 and Table II). The pH dependency and pI values of the 37 kDa MPD were almost identical to those of the 45 kDa MPD (Figs. 7 and 8). Moreover, the structural similarity between the 37 and 45 kDa enzymes was strongly suggested on Western blot analysis as well as copurification by several chromatographic procedures. However, the 37 kDa enzyme is not proteolytically cleaved from the 45 kDa enzyme during purification (Fig. 9). At least under our experimental conditions, the proteolytic activity of the 45 kDa MPD was not found in the crude extract (Fig. 9B). Posttranslational glycosylation can not explain the difference between the two enzymes (Fig. 10). These results suggested that the 37 kDa MPD was transcribed from a different gene from the 45 kDa MPD with the CP diet or both MPDs resulted from alternative splicing of a single gene product.

The role of the 37 kDa MPD in cholesterol biosynthesis is an issue of great interest. Recent studies demonstrated that some cholesterol-lowering drugs induced the proliferation of peroxisomes through the peroxisome proliferator-activated receptor (PPAR) (*25, 26*). Peroxisomes have been reported to contain enzymes involved in cholesterol biosynthesis including MPD, though MPD is generally believed to be a cytosolic enzyme (*27*). The role of the activities in peroxisomes is unclear, however, the intracellular compartmentation of mevalonate metabolism suggests different roles of these biosynthetic systems. Therefore, it might be that the 45 kDa enzyme is a cytosolic enzyme, and when the CP diet is fed not only the 45 kDa enzyme in cytosol, but also the 37 kDa enzyme is induced in peroxisomes. Stamellos *et al.* reported the presence of MVK in rat liver peroxisomes and cytosol (*28*). Each of these compartments contains a different form of MVK with respect to molecular weight and pI. The peroxisomal MVK was released into the cytosol by a number of different hypolipidemic drugs. Our purified 37 kDa MPD might be located in peroxisomes and released from peroxisomes into the cytosol by the CP diet.

Physiological agonists that produce the 37 kDa MPD are of great interest. The biosynthetic activity of cholesterol is known to have a diurnal rhythm, with maximum activity at midnight. However, in a preliminary experiment no 37 kDa MPD was detected on Western blot in rat liver removed at midnight (data not shown). Other cholesterol-lowering drugs should be examined to determine whether or not they produce the 37 kDa MPD.

Antiserum raised against the 45 kDa enzyme detected the 35 kDa protein in the livers of rats fed on an ordinary chow diet and sometimes fed on the CP diet. Shama Bhat and Ramasarma reported that rat liver MPD was composed of four subunits of a molecular weight of 35 kDa (*13*). However, the 35 kDa protein was not detected throughout our purification from rat liver with the CP diet. The 35 kDa protein may be cleaved from the 37 kDa enzyme intracellularly, and when induced by the CP diet, the 37 kDa enzyme may be produced in large amounts. However, the 35 kDa protein was not increased even when the homogenate of CP diet- or non-treated rat liver was incubated in phosphate buffer at 37°C for 2 h (Fig. 9). Therefore, at present, we consider the 35 kDa protein is immunologically related to but different from MPD.

The pathological significance of the 37 kDa MPD in SHRSP needs to be elucidated. Several questions emerged in this respect in the present study. (1) Does SHRSP have the same amount of 45 kDa MPD in the absence of the CP diet? (2) If this is the case, are the kinetical properties identical to those in WKY rats? (3) Is the 37 kDa MPD induced by the CP diet in SHRSP? (4) Are the amount and properties of the 37 kDa MPD comparable to those in WKY rats? Purification of MPD from SHRSP is necessary to answer these questions.

In conclusion, we found a 37 kDa MPD in CP diet-treated rat liver which is structurally and characteristically similar to the well-known 45 kDa MPD. Further studies on the

structure and regulation of the genes will shed light on the relationship between the two MPDs.

REFERENCES

1. Kennelly, P.J. and Rodwell, V.W. (1985) Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by reversible phosphorylation-dephosphorylation. *J. Lipid Res.* **26**, 903-914
2. Beg, Z.H., Stonik, J.A., and Brewer Jr., H.B. (1987) Modulation of the enzymic activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase by multiple kinase systems involving reversible phosphorylation: a review. *Metabolism* **36**, 900-917
3. Goldstein, J.L. and Brown, M.S. (1990) Regulation of the mevalonate pathway. *Nature* **343**, 425-430
4. Ramachandran, C.K. and Shah, S.N. (1976) Decarboxylation of mevalonate pyrophosphate is one rate-limiting step in hepatic cholesterol synthesis in suckling and weaned rats. *Biochem. Biophys. Res. Commun.* **69**, 42-47
5. Ramachandran, C.K. and Shah, S.N. (1977) Studies on mevalonate kinase, phosphomevalonate kinase and pyrophosphomevalonate decarboxylase in developing rat brain. *J. Neurochem.* **28**, 751-757
6. Jabalquinto, A.M. and Cardemil, E. (1980) Secondary regulatory sites in rat liver cholesterol biosynthesis: role of 5-pyrophosphomevalonate decarboxylase. *Lipids* **15**, 196-199
7. Jabalquinto, A.M. and Cardemil, E. (1981) The effect of diabetes, nutritional factors, and sex on rat liver and kidney mevalonate kinase, mevalonate-5-phosphate kinase, and mevalonate-5-pyrophosphate decarboxylase. *Arch. Biochem. Biophys.* **210**, 132-139
8. Bloch, K., Chaykin, S., Phillips, A.H., and de Waard, A. (1959) Mevalonic acid pyrophosphate and isopentenylpyrophosphate. *J. Biol. Chem.* **234**, 2595-2604
9. Dhe-Paganon, S., Magrath, J., and Abeles, R.H. (1994) Mechanism of mevalonate pyrophosphate decarboxylase: evidence for a carbocationic transition state. *Biochemistry* **33**, 13355-13362
10. Skilleter, D.N. and Kekwick, R.G.O. (1971) The enzymes forming isopentenyl pyrophosphate from 5-phosphomevalonate (mevalonate 5-phosphate) in the latex of hevea brasiliensis. *Biochem. J.* **124**, 407-417
11. Popjak, G. (1969) Enzymes of sterol biosynthesis in liver and intermediates of sterol biosynthesis. *Methods Enzymol.* **15**, 393-454
12. Chiew, Y.E., O' Sullivan, W.J., and Lee, C.S. (1987) Studies on pig liver mevalonate-5-diphosphate decarboxylase. *Biochim. Biophys. Acta* **916**, 271-278
13. Shama Bhat, C. and Ramasarma, T. (1980) Purification & properties of mevalonate pyrophosphate decarboxylase of rat liver. *Indian J. Biochem. Biophys.* **17**, 249-254
14. Toth, M.J., Huwyler, L., and Park, J. (1996) Purification of rat liver mevalonate pyrophosphate decarboxylase. *Prep. Biochem. Biotechnol.* **26**, 47-51
15. Alvear, M., Jabalquinto, A.M., Eyzaguirre, J., and Cardemil, E. (1982) Purification and characterization of avian liver mevalonate-5-pyrophosphate decarboxylase. *Biochemistry* **21**, 4646-4650
16. Toth, M.J. and Huwyler, L. (1996) Molecular cloning and expression of the cDNAs encoding human and yeast mevalonate pyrophosphate decarboxylase. *J. Biol. Chem.* **271**, 7895-7898
17. Okamoto, K., Yamori, Y., and Nagaoka, A. (1974) Establishment of the stroke-prone spontaneously hypertensive rat (SHR). *Cir. Res.* **34/35**, Suppl. 1, 143-153
18. Yamori, Y. (1977) in *Gene-Environment Interaction in Common Diseases* (Inoue, E. and Nishimura, H., eds.) pp. 141-154, University of Tokyo Press, Tokyo
19. Sawamura, M., Nara, Y., and Yamori, Y. (1992) Liver mevalonate 5-pyrophosphate decarboxylase is responsible for reduced serum cholesterol in stroke-prone spontaneously hypertensive rat. *J. Biol. Chem.* **267**, 6051-6055
20. Cardemil, E. and Jabalquinto, A.M. (1985) Mevalonate 5-pyrophosphate decarboxylase from chicken liver. *Methods Enzymol.* **110**, 86-92
21. Coudrier, E., Reggio, H., and Louvard, D. (1983) Characterization of an integral membrane glycoprotein associated with the microfilaments of pig intestinal microvilli. *EMBO J.* **2**, 469-475
22. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
23. Tanaka, R.D., Schafer, B.L., Lee, L.Y., Freudenberger, J.S., and Mosley, S.T. (1990) Purification and regulation of mevalonate kinase from rat liver. *J. Biol. Chem.* **265**, 2391-2398
24. Alvear, M., Jabalquinto, A.M., and Cardemil, E. (1989) Inactivation of chicken liver mevalonate 5-diphosphate decarboxylase by sulfhydryl-directed reagents: evidence of a functional dithiol. *Biochim. Biophys. Acta* **994**, 7-11
25. Gottlicher, M., Widmark, E., Li, Q., and Gustafsson, J.A. (1992) Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. USA* **89**, 4653-4657
26. Bocos, C., Gottlicher, M., Gearing, K., Banner, C., Enmark, E., Teboul, M., Crickmore, A., and Gustafsson, J.A. (1995) Fatty acid activation of peroxisome proliferator-activated receptor (PPAR). *J. Steroid Biochem. Mol. Biol.* **53**, 467-473
27. Biardi, L. and Krisans, S.K. (1996) Compartmentalization of cholesterol biosynthesis. Conversion of mevalonate to farnesyl diphosphate occurs in the peroxisomes. *J. Biol. Chem.* **271**, 1784-1788
28. Stamellos, K.D., Shackelford, J.E., Tanaka, R.D., and Krisans, S.K. (1992) Mevalonate kinase is located in rat liver peroxisomes. *J. Biol. Chem.* **267**, 5560-5568