# Immunochemical Identity of Peroxisomal Enoyl-CoA Hydratase with the Peroxisome-Proliferation-associated 80,000 mol wt Polypeptide in Rat Liver

M. KUMUDAVALLI REDDY, SAEED A. QURESHI, PAUL F. HOLLENBERG, and JANARDAN K. REDDY

Department of Pathology, Northwestern University Medical School, Chicago, Illinois 60611

ABSTRACT Peroxisome proliferators, which induce proliferation of hepatic peroxisomes, have been shown previously to cause a marked increase in an 80,000 mol wt polypeptide predominantly in the light mitochondrial and microsomal fractions of liver of rodents. We now present evidence to show that this hepatic peroxisome-proliferation-associated polypeptide, referred to as polypeptide PPA-80, is immunochemically identical with the mutifunctional peroxisome protein displaying heat-labile enoyl-CoA hydratase activity. This conclusion is based on the following observations: (a) the purified polypeptide PPA-80 and the heat-labile enoyl-CoA hydratase from livers of rats treated with the peroxisome proliferator Wy-14,643 {[4-chloro-6(2,3-xylidino)-2-pyrimidinylthio]acetic acid} exhibit identical minimum molecular weights of  $\sim$ 80,000 on SDS polyacrylamide gel electrophoresis; (b) these two proteins are immunochemically identical on the basis of Ouchterlony double diffusion, immunotitration, rocket immunoelectrophoresis, and crossed immunoelectrophoresis analyses; and (c) the immunoprecipitates formed by antibodies to polypeptide PPA-80 when dissociated on a Sephadex G-200 column yield enoyl-CoA hydratase activity. Whether the polypeptide PPA-80 exhibits the activity of other enzyme(s) of the peroxisomal  $\beta$ -oxidation system such as fatty acyl-CoA oxidase activity or displays immunochemical identity with such enzymes remains to be determined.

The availability of antibodies to polypeptide PPA-80 and enoyl-CoA hydratase facilitated immunofluorescent and immunocytochemical localization of the polypeptide PPA-80 and enoyl-CoA hydratase in the rat liver. The indirect immunofluorescent studies with these antibodies provided direct visual evidence for the marked induction of polypeptide PPA-80 and enoyl-CoA hydratase in the livers of rats treated with Wy-14,643. The present studies also provide immunocytochemical evidence for the localization of polypeptide PPA-80 and the heat-labile enoyl-CoA hydratase in the peroxisome, but not in the mitochondria, of hepatic parenchymal cells. These studies, therefore, provide morphological evidence for the existence of fatty acyl-CoA oxidizing system in peroxisomes. An increase of polypeptide PPA-80 on SDS polyacrylamide gel electrophoretic analysis of the subcellular fractions of liver of rodents treated with lipid-lowering drugs should serve as a reliable and sensitive indicator of enhanced peroxisomal  $\beta$ -oxidation system.

Several structurally unrelated hypolipidemic drugs and certain industrial plasticizers such as di-(2-ethylhexyl)phthalate which cause profound proliferation of peroxisomes in hepatic parenchymal cells of rodents (37, 38, 50, 51), also cause a marked increase in the quantity of a polypeptide with an approximate molecular weight of 80,000 in the postnuclear, large particle and microsomal fractions of liver (39, 47). This polypeptide has recently been designated as PPA-80 (PPA, for peroxisomeproliferation-associated; 80 for an approximate 80,000 mol wt) because of its increase in livers with peroxisome proliferation (39, 47). Although studies from our laboratory indicate that the polypeptide PPA-80 is selectively induced by hepatic peroxisome proliferators and not by compounds that induce the synthesis of smooth endoplasmic reticulum (39), it is not certain whether the polypeptide PPA-80 is localized in peroxisomes or induced in all cytoplasmic organelles. The relative abundance of this polypeptide in peroxisome-rich light mitochondrial fraction and its absence in the highly purified mitochondrial fractions of liver strongly favor peroxisomal localization (47).

We recently have purified the polypeptide PPA-80 from the livers of rats treated with the peroxisome proliferators, Wy-14,643 {[4-chloro-6-(2,3-xylidino)2-pyrimidinylthio]acetic acid}; tibric acid [2-chloro-5-(3,5-dimethylpiperiodino-sulphonyl)benzoic acid], nafenopin {2-methyl-2-[p-(1,2,3,4tetrahydro-1-naphthyl)-phenoxy]propionic acid}, and clofibrate [ethyl- $\alpha$ -( $\rho$ -chlorophenoxy)isobutyrate] and showed by immunodiffusion that the purified PPA-80 polypeptides induced by these different hypolipidemic agents are immunologically identical (47). Furthermore, immunodiffusion studies indicated that the polypeptide PPA-80 induced by the peroxisome proliferators is neither catalase, a marker enzyme for peroxisomes, nor NADPH-cytochrome P-450 reductase, which has the same approximate molecular weight of 80,000 (47). Because all hepatic peroxisome proliferators possess lipid-lowering properties (38), induce polypeptide PPA-80, and cause a marked enhancement of carnitine acetyltransferase and of peroxisomal  $\beta$ -oxidation in liver (16, 26, 31, 38), we entertained the possibility that polypeptide PPA-80 may be involved in peroxisomal lipid metabolism (39). In the present report we show that the polypeptide PPA-80 displays immunochemical identity with the heat-labile peroxisomal enoyl-CoA hydratase, a multifunctional protein (31) involved in the peroxisomal  $\beta$ oxidation of fatty acids (17, 18). We also provide direct visual evidence for the induction and presence of polypeptide PPA-80 and peroxisomal enoyl-CoA hydratase in peroxisomes by immunofluorescent and immunocytochemical procedures.

#### MATERIALS AND METHODS

#### Chemicals

The hypolipidemic compound, Wy-14,643, was generously supplied by Dr. R. M. Tomarelli, Wyeth Laboratories (Radnor, Pa.). Crotonyl-CoA, ATP, EDTA, hexamethylphosphoric triamide,  $\beta$ -mercaptoethanol, carboxymethyl (CM)-cellulose, cellulose phosphate, horseradish peroxidase, and 3,3'-diaminobenzidine HCl were purchased from Sigma Chemical Co. (St. Louis, Mo.). Nonidet P40 was obtained from Bethesda Research Laboratories (Rockville, Md.). Acrylamide (enzyme grade), N,N'-bismethylene acrylamide, and dithiothreitol were purchased from Eastman Kodak Co. (Rochester, N.Y.). Molecular weight standards for SDS polyacrylamide gel electrophoresis were obtained from BioRad Laboratories (Richmond, Calif.). Freund's adjuvant was purchased from Difco Laboratories (Detroit, Mich.). Goat anti-rabbit globulin conjugated with fluorescein isothiocyanate was obtained from Behring Diagnostics, American Hoechst Corp. (Sommerville, N.J.). All other chemicals were obtained from commercial sources and were of the highest purity available.

### Animals

Inbred male F344 rats, weighing 120-150 g were obtained from A. R. Schmidt/ Sprague Dawley, (Madison, Wis.). The hypolipidemic peroxisome proliferator, Wy-14,643 was administered to these animals at 0.1% (wt/wt) level in powdered rat chow *ad libitum* for 4 wk and the rats were killed by cervical dislocation.

#### Subcellular Fractionation

The livers were homogenized (10% [wt/vol]) in 0.25 M sucrose in a Potter-Elvehjem homogenizer. Postnuclear, large particle, light mitochondrial, and microsomal fractions (4, 15) were prepared as described elsewhere (39, 47).

### SDS Polyacrylamide Gel Electrophoresis

Electrophoresis was performed on SDS polyacrylamide slab or tube gels by the method of Laemmli (15) as previously described (47). Isoelectric focusing was done using slab gels (pH 3.5-10.0 range) or tube gels (pH 9-11 range).

#### Enzyme Assays

Enoyl-CoA hydratase (EC 4.2.1.17) activity was assayed as described by Steinman and Hill (49) in 0.3 M Tris-HCl buffer (pH 7.4), containing 5 mM EDTA, 0.05 mg/ml bovine serum albumin, and 200  $\mu$ M crotonyl-CoA as substrate. The heat-labile peroxisomal enoyl-CoA hydratase activity was assayed after diluting the enzyme preparations with 50 mM potassium phosphate (pH 7.0) and heating at 57°C for 5 min. Catalase (EC 1.11.1.6) activity was assayed by the method of Lück (22) as described elsewhere (43). Carnitine acetyltransferase (EC 2.3.1.7) was measured by the method of Markwell et al. (24). Protein content was determined by the method of Lowry et al. (21) using bovine serum albumin as the standard. When protein concentration was low, absorbance at 280 nm was used to measure the protein.

### Purification of Polypeptide PPA-80

This peroxisome-proliferation-associated polypeptide was purified as recently described (47) from the livers of rats treated with 0.1% Wy-14,643 for 4 wk.

### Purification of Peroxisomal Enoyl-CoA Hydratase

The steps employed in the peroxisomal enoyl-CoA hydratase purification from the livers of Wy-14,643-treated rats were those outlined by Osumi and Hashimoto (31) with some modifications. Briefly, the frozen liver tissue (10 g) was thawed, homogenized with 100 ml of 10 mM K<sub>3</sub>PO<sub>4</sub> containing 0.1% hexamethylphosphoric triamide, 2 mM mercaptoethanol, and 5 mM EDTA in a Potter-Elvehjem homogenizer. The supernate obtained after centrifugation in a Beckman J-21C centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 17,000 g for 15 min, was adjusted to pH 7.0 with 1 M potassium phosphate, pH 6.0, and loaded on a phosphocellulose column (2.5 × 25 cm) previously equilibrated with 50 mM potassium phosphate, pH 7.0, containing 0.1% hexamethylphosphoric triamide, 2 mM mercaptoethanol, and 5 mM EDTA. The column was eluted with a linear gradient (50-500 mM phosphate buffer in a total volume of 400 ml), the fractions with enoyl-CoA hydratase activity were pooled, and fractionated with ammonium sulfate. The protein that precipitated between 20 and 40 g ammonium sulfate/100 ml volume was dissolved in the above buffer and the ammonium sulfate removed by chromatography on a column of Sephadex G-25 or by extensive dialysis. The enzyme solution was diluted with an equal volume of cold water containing 2 mM mercaptoethanol, 0.1% hexamethylphosphoric triamide, and 5 mM EDTA, and loaded on a CMcellulose column, with a 25-ml bed volume, previously equilibrated with 25 mM potassium phosphate, pH 7.0. The column was washed with 1 vol of 50 mM potassium phosphate buffer, pH 7.0, and eluted with a linear gradient of 50-300 mM phosphate in a total volume of 300 ml. The enoyl-CoA hydratase-active fractions were pooled and the enzyme precipitated by addition of 35 g of ammonium sulfate/100 ml eluate. The pure enzyme was separated from ammonium sulfate by passing through a Sephadex G-25 column as above.

Enoyl-CoA hydratase was also purified as described above from microsomal fractions prepared from the liver of normal and Wy-14,643-treated rats. The purity of the sample was assessed by SDS polyacrylamide gel electrophoresis.

# Preparation of Monospecific Antibodies against Purified Polypeptide PPA-80 and Enoyl-CoA Hydratase

Monospecific antibodies to purified polypeptide PPA-80 were raised in white New Zealand male rabbits as described previously (47). Antibodies to enoyl-CoA hydratase were similarly raised in four white New Zealand rabbits by injecting purified rat peroxisomal enoyl-CoA hydratase (2 mg/animal) in 1 ml of complete Freund's adjuvant, as described previously for the production of antibodies to polypeptide PPA-80 (47).

### Double Immunodiffusion Assays

Immunodiffusion assays according to Ouchterlony and Nilsson (33) were performed at  $22 \pm 2^{\circ}$ C for 30-35 h in 1% agarose in 0.08 M Tris-HCl (pH 8.6) containing 1 mM EDTA, 1 mM NaN<sub>3</sub>, and 0.15 M NaCl. The antigens were solubilized in SDS and sequestered with 20% Triton X-100 as described by Chua and Blomberg (2). After the immunoprecipitates had developed, the immunodiffusion plates were washed with 0.15 M NaCl for 2 days and distilled water for an additional 1 day, dried, and then stained (47).

#### Rocket Immunoelectrophoresis Method

The purified polypeptide PPA-80 and enoyl-CoA hydratase were analyzed by rocket immunoelectrophoresis using the method described by Weeke (53).

# Crossed Immunoelectrophoresis of SDS Polypeptides of Microsomal and Light Mitochondrial Fractions of Livers from Wy-14,643-treated Rats

Crossed immunoelectrophoresis was performed by the method of Converse and Papermaster (3) as modified by Chua and Blomberg (2). The polypeptides of either microsomal (47) or light mitochondrial (4, 47) fractions of liver from Wy-14,643-treated rats were separated on 1.5-mm-thick SDS polyacrylamide slab gels containing a linear gradient of 5-15% acrylamide concentration. Samples containing 20  $\mu$ g protein were separated by electrophoresis. After electrophoresis, the slab gels were cut longitudinally to yield 10-mm-wide parallel strips, some of which were stained to obtain a reference polypeptide profile. The unstained strips were used for immunoelectrophoresis in the second dimension, as described by Chua and Blomberg (2). The antibody gel contained either antipolypeptide PPA-80 or anti-enoyl-CoA hydratase antibodies. After immunoelectrophoresis, the gel was soaked in 0.15 M NaCl for 1 d and washed with water for an additional 1 d before staining with Coomassie Brilliant Blue as before (47).

# Immunotitration of Enoyl-CoA Hydratase with Antibodies to Polypeptide PPA-80 and Enoyl-CoA Hydratase

Enoyl-CoA hydratase purified from livers of rats treated with Wy-14,643 was used for immunotitration with antipolypeptide PPA-80 antibodies. Enoyl-CoA hydratase (78  $\mu$ g/ml) was incubated for 1 h at 37°C in 0.05 M phosphate buffer (pH 7.4), containing 0.15 M NaCl and increasing volumes of either antipolypeptide PPA-80 or anti-enoyl-CoA hydratase antiserum, in a final volume of 1 ml. After incubation the mixture was allowed to stand at 4°C for 20 h, then centrifuged to sediment the immunoprecipitates. The residual enoyl-CoA hydratase activity was assayed as before. The immunoprecipitates recovered were analyzed by SDS polyacrylamide gel electrophoresis. In addition, immunoprecipitates obtained after incubating light mitochondrial fractions with antipolypeptide PPA-80 antibodies, were separated on Sephadex G-200 column (1.5 × 90 cm) in the presence of 0.1 M glycine/HCl buffer (pH 2.5) with 0.15 M NaCl. The fractions collected were neutralized to pH 7.0 and assayed for enoyl-CoA hydratase activity.

### Quantitative Precipitin Assay

A constant amount of anti-enoyl-CoA hydratase antiserum was added to varying amounts of purified heat-labile enoyl-CoA hydratase or polypeptide PPA-80. The immunoprecipitates obtained as described above were analyzed for the protein content by the method of Lowry et al. (21).

### Immunofluorescence Procedure

For the immunofluorescent method, small  $3 \times 4 \times 3$  mm pieces of liver from normal and Wy-14,643-treated rats were fixed in ice-cold 96% ethyl alcohol for 24 h and then in cold xylene for 40 h before embedding in paraffin (48). Sections, ~2 µm thick, were deparaffinized, hydrated, and washed with phosphate-buffered saline containing 2% bovine plasma albumin for 2 h at room temperature. The sections were then incubated with rabbit antiserum (1:10 dilution) to polypeptide PPA-80 or enoyl-CoA hydratase for 1 h at room temperature in a moist chamber. After three consecutive 15-min washes with phosphate-buffered saline, the sections were covered with goat anti-rabbit gamma globulin conjugated with fluorescein isothiocyanate and permitted to react for 30 min. The sections were then thoroughly washed with phosphate-buffered saline, mounted in 50% glycerol in the above buffer, and examined in a Lietz fluorescence microscope. The specificity of immunofluorescence was ascertained (a) by using nonimmunized rabbit serum, (b) by omitting the incubation with specific antibody, and (c) by adsorption with specific antibody.

### Immunocytochemical Method

Fab fragments were prepared according to the method of Porter (34) from (a)

rabbit nonspecific IgG, (b) rabbit anti-rat polypeptide PPA-80, and (c) rabbit anti-rat enoyl-CoA hydratase. Horseradish peroxidase was coupled to the Fab fragments by the procedure outlined by Avrameas and Ternynck (1). For the immunocytochemical localization, the livers of Wy-14.643-treated rats were fixed according to Karnovsky (13) and the nonfrozen, 15- $\mu$ m-thick sections prepared with an Oxford Vibratome (Oxford Laboratories, Foster City, Calif.) were incubated in the medium containing peroxidase coupled to Fab fragments of the specific and nonspecific IgG and processed essentially by the method outlined by Novikoff et al. (30) for their studies on the immunocytochemical localization of epoxide hydrase in hyperplastic liver nodules. We also carried out additional incubations in the presence of 3-amino-1.2,4-triazole, a specific inhibitor of catalase, when sections exposed to peroxidase-coupled Fab fragments were incubated in the 3,3'-diaminobenzidine medium (pH 7.6) of Graham and Karnovsky (6). The addition of aminotriazole ensured elimination of nonspecific reaction because of the peroxidatic activity of peroxisomal catalase.

### RESULTS

### **Enzyme Activities**

In earlier reports from this laboratory (25, 27, 36–38), we demonstrated that several hypolipidemic peroxisome proliferators, including Wy-14,643, increased the activities of hepatic catalase and carnitine acetyltransferase when administered to the rat. The results in Table I confirm the extent of enhancement of the activities of both these peroxisomal enzymes in rats fed Wy-14,643. Furthermore, the data in Table I also demonstrate a 13-fold increase in the activity of heat-labile enoyl-CoA hydratase activity in the livers of rats that were fed Wy-14,643 for 4 wk when compared to controls. The increase in the enoyl-CoA hydratase activity is comparable to the increase in the activity of the cyanide-insensitive hepatic peroxisomal palmitoyl-CoA oxidizing system observed by Lazarow (16) in Wy-14,643-treated rats.

As expected, SDS polyacrylamide gel electrophoresis of the postnuclear, large particle, light mitochondrial and microsomal fractions obtained from the livers of Wy-14,643-treated rats showed a marked increase in the content of polypeptide PPA-80. The electrophoretic profiles of the light mitochondrial fractions from normal and Wy-14,643-treated rat livers are presented in Fig. 1 (slots 1 and 2, respectively).

### Purity of Enoyl-CoA Hydratase

A summary of the typical purification of heat-labile enoyl-CoA hydratase from whole liver homogenates, achieved in the present investigation is given in Table II. The specific activity of the enzyme increased by ~12.5-fold, and the yield ranged from 35 to 70%. All the enzyme activity thus purified was heatlabile; heating the sample for 5 min at 57°C completely abolished the enzyme activity. These results are comparable to the data on the purification of heat-labile enoyl-CoA hydratase

TABLE 1 Effect of Wy-14,643 Administration on Peroxisomal Catalase, Carnitine Acetyltransferase, and Enoyl-CoA Hydratase Activities

	Enzyme activity		
Parameter	Control	Wy-14,643	ρ
Catalase (U/mg protein)	42.9 ± 0.36	78 ± 6.9	<0.001
Carnitine acetyltransfer- ase (U/mg protein)	$3.3 \pm 0.69$	212 ± 18.47	<0.001
Enoyl-CoA hydratase, heat-labile (μmol/ min/mg protein)	15.0 ± 4.00	190 ± 39.0	<0.001

Two groups of four rats were fed a control or Wy-14,643 (0.1% [wt/wt])-containing diet for 4 wk. The enzyme activities were determined on liver extracts as described in Materials and Methods. Values are the means  $\pm$  SD.



FIGURE 1 SDS polyacrylamide slab gel electrophoretograms of the (1) light mitochondrial fraction from normal rat liver; (2) light mitochondrial fraction from the liver of Wy-14,643-treated rat; (3) liver extract used for enoyl-CoA purification; (4) after first ammonium sulphate precipitation; and (5) after second ammonium sulphate precipitation. Samples 3, 4, and 5 correspond to samples obtained after purification steps 1, 3, and 4 outlined in Table II. Arrow indicates the position of polypeptide PPA-80 in gels 1 and 2 and to the position of enoyl-CoA hydratase during its purification in gels 3-5. Note that the two low molecular weight polypeptides present in slot 5 were removed by crystallization (see Fig. 2, slot 1). Slots 1 and 2 had ~15  $\mu$ g protein; slots 3 and 5 ~10  $\mu$ g protein, and slot 4 ~20  $\mu$ g protein.

from the livers of di-(2-ethylhexyl)-phthalate-treated rats reported by Osumi and Hashimoto (31).

Fig. 1 (slots 3-5) shows the SDS polyacrylamide gel electrophoretic analysis of the individual fractions obtained during purification. The enoyl-CoA hydratase after CM-cellulose chromatography and ammonium sulphate fractionation (step 4, Table II) often showed two low molecular weight polypeptides (Fig. 1, slot 5), which were removed by crystallization (see Fig. 2, slot 1) in the presence of ethanol according to the method of Steinman and Hill (49). This crystallization step, however, did not appreciably alter the specific activity of the purified enzyme. Highly purified enoyl-CoA hydratase was stable for at least 3 mo when stored at  $-20^{\circ}$ C in the presence of 50% glycerol and 1 mM  $\beta$ -mercaptoethanol in 0.01 M phosphate buffer (pH 7.0). Frequent freezing and thawing of purified enoyl-CoA hydratase resulted in a gradual loss of enzyme activity and appeared to degrade the protein into low

TABLE II Purification of Peroxisomal Enoyl-CoA Hydratase from Livers of Rats Treated with Wy-14,643

Purification step	Total protein	Total activity	Enzyme specific activity	Recov- ery
	mg	U	U/mg protein	%
Step 1: Frozen liver ex- tract	1,100	62,000	56	100
Step 2: Phosphocellulose	158	58,000	367	93
Step 3: Ammonium sul- phate fractionates	140	50,000	360	80
Step 4: Ammonium sul- phate fractionation after CM-cellulose chromatography	68	48,000	700	70

Groups of male F344 rats were fed Wy-14,643 (0.1% [wt/wt]) for 4 wk to induce peroxisome proliferation. Heat-labile enoyl-CoA hydratase from the frozen livers was purified essentially according to the procedure outlined by Osumi and Hashimoto (31). The results of specific enzyme activity recovered are representative of five similar purification attempts.



FIGURE 2 SDS polyacrylamide cylindrical gels of the (1) purified heat-labile enoyl-CoA hydratase (10  $\mu$ g); (2) purified polypeptide PPA-80 (5  $\mu$ g); and (3) standard proteins (20  $\mu$ g). Arrow indicates the position of enoyl-CoA hydratase and polypeptide PPA-80, both of which show identical mobility. The molecular weights in kilodaltons are indicated in gel 3.

molecular weight fragments as was observed with polypeptide PPA-80 (47).

# Molecular Weight Comparison of Enoyl-CoA Hydratase and Polypeptide PPA-80

The highly purified enoyl-CoA hydratase and the purified polypeptide PPA-80 were electrophoretically homogeneous

and revealed the same mobility on SDS polyacrylamide gel electrophoresis (Fig. 2.) Both proteins had the same approximate molecular weight of 80,000 (78,500  $\pm$  1,200; n = 12). This is comparable to the molecular weight of 77,000 reported for the heat-labile enoyl-CoA hydratase purified from rat liver after the administration of the peroxisome proliferator, di-(2ethylhexyl)phthalate (31). This slight difference in the molecular weight of enoyl-CoA hydratase is most likely caused by minor differences in the SDS electrophoretic conditions. When these two proteins, enoyl-CoA hydratase and polypeptide PPA-80, were mixed and applied to the tube gel, only one major protein-staining band was visualized, indicating identical minimum molecular weights in SDS polyacrylamide gels.

The isoelectric points of purified polypeptide PPA-80 and of heat-labile enoyl-CoA hydratase were at pH 9.9 (Fig. 3).

# Immunochemical Relationship between Polypeptide PPA-80 and Heat-labile Enoyl-CoA Hydratase

IMMUNODIFFUSION: Because the SDS polyacrylamide gel electrophoretic profile indicated that polypeptide PPA-80 might be the heat-labile enoyl-CoA hydratase, it was necessary to compare the immunological properties of these proteins. The immunological comparison is essential, in view of our earlier observation that, although polypeptide PPA-80 and the liver microsomal NADPH-cytochrome P-450 reductase had migrated similarly on SDS polyacrylamide gels, they are antigenically dissimilar (47).

The purified enoyl-CoA hydratase produced precipitating antibodies when injected into rabbits. The antibodies gave a single precipitin line with the purified protein as well as with crude solubilized light mitochondrial and microsomal fractions of liver prepared from Wy-14,643-treated rats. As shown in Fig. 4, the purified polypeptide PPA-80 and enoyl-CoA hydratase from Wy-14,643-treated rats were immunochemically identical in Ouchterlony double diffusion analyses. The immunoprecipitin bands produced by the reaction of each protein against antipolypeptide PPA-80 or anti-enoyl-CoA hydratase formed a continuous precipitin arc, indicating immunological identity of the two proteins (Fig. 4). The absence of multiple precipitin lines in these double diffusion studies provides strong support for the contention that the purified proteins used in these studies to raise antibodies consist of a single homogeneous protein.

ROCKET IMMUNOELECTROPHORESIS: To investigate further the immunochemical relationship, the purified polypeptide PPA-80 and enoyl-CoA hydratase were analyzed by rocket immunoelectrophoresis using anti-polypeptide PPA-80 or anti-enoyl-CoA hydratase and polypeptide PPA-80 or by both enoyl-CoA hydratase and polypeptide PPA-80 antigens on antipolypeptide PPA-80 antiserum containing gel, are illustrated in Fig. 5A. Fig. 5B shows rockets developed by polypeptide PPA-80 and enoyl-CoA hydratase on anti-enoyl-CoA antibody containing gel. These results further support the



FIGURE 4 Ouchterlony double-diffusion analysis using rabbit antirat polypeptide PPA-80 (10  $\mu$ l serum; well 1) and anti-rat enoyl-CoA hydratase (20  $\mu$ l serum; well 2) antiserum. Wells 3 and 4 contained 10  $\mu$ l of purified polypeptide PPA-80 (0.7 mg/ml), and 10  $\mu$ l of purified enoyl-CoA hydratase (0.9 mg/ml), respectively.



FIGURE 5 Rocket immunoelectrophoresis of (A) purified enoyl-CoA hydratase (wells 1–3) and polypeptide PPA-80 (wells 4–6) in 1% agarose gel containing anti-polypeptide PPA-80 antiserum (1%) and (B) polypeptide PPA-80 (well 1) and enoyl-CoA hydratase (well 2) on anti-enoyl-CoA hydratase antiserum (1%) containing gel. Protein concentration in A: 5  $\mu$ g (wells 1 and 4), 10  $\mu$ g (wells 2 and 5), and 15  $\mu$ g (wells 3 and 6). Protein concentration in B: wells 1 and 2 each contained ~8  $\mu$ g protein. The immunoprecipitates were visualized by staining gels with Coomassie Blue.



FIGURE 3 Isoelectric focusing of the purified polypeptide PPA-80 (renatured) (A) and purified peroxisomal heat-labile enoyl-CoA hydratase (B) in tube gels in the range of pH 9-11. Both proteins appear to have identical isoelectric points of  $\sim$ 9.9.

immunological identity of these two proteins.

CROSSED IMMUNOELECTROPHORESIS: To demonstrate that the polypeptide PPA-80 induced in the livers of rats by hepatic peroxisome proliferators is identical to heat-labile enoyl-CoA hydratase, we utilized the crossed immunoelectrophoretic procedure (2). The antibodies to enoyl-CoA hydratase gave a precipitin peak at the position of the polypeptide PPA-80 when examined by crossed immunoelectrophoresis (Fig. 6). Similar results were also obtained when antibodies to polypeptide PPA-80 were characterized by the crossed immunoelectrophoresis technique. Within the limit of sensitivity of this procedure, no other polypeptide on the SDS polyacrylamide gel yielded a perceptible precipitin peak with either antipolypeptide PPA-80 or anti-enoyl-CoA hydratase antibodies, indicating that only polypeptide PPA-80 possesses the antigenic site(s) and that the two antibodies employed in these studies are highly specific.

IMMUNOTITRATION: Purified enoyl-CoA hydratase was incubated with increasing quantities of either anti-enoyl-CoA hydratase or antipolypeptide PPA-80 antiserum, and the resulting immunoprecipitates were sedimented (Fig. 7A). The enoyl-CoA hydratase activity remaining in the supernate was determined. The results showed that excess amounts of both antipolypeptide PPA-80 and anti-enoyl-CoA hydratase antibodies precipitated completely the enoyl-CoA hydratase activity (Fig. 8A), suggesting that antigens (i.e., polypeptide PPA-80, and enoyl-CoA hydratase) used for raising these antibodies are immunochemically identical. The differences in the amount of antiserum required to precipitate completely the enoyl-CoA hydratase activity is most likely caused by the difference in the concentration of specific IgG present in these two antisera. Quantitative precipitin assays performed with anti-enoyl-CoA hydratase antibody showed similar protein yields with both polypeptide PPA-80 and enoyl-CoA hydratase (Fig. 8 B).

Because polypeptide PPA-80 was purified in the presence of SDS, we could not readily identify the purified polypeptide PPA-80 as the enoyl-CoA hydratase by measuring the enzyme activity. Therefore, in this study we have attempted to separate the immunoprecipitate formed by antipolypeptide PPA-80 on a Sephadex G-200 column. For this purpose Nonidet P40 solubilized microsomal fraction from the liver of Wy-14,643-treated rats was incubated with either antipolypeptide PPA-80 or anti-enoyl-CoA hydratase. Both antisera precipitated identical 80,000 mol wt polypeptides (Fig. 7 B and C). The immunoprecipitate obtained after incubation with antipolypeptide



FIGURE 6 Crossed immunoelectrophoresis of polypeptides of light mitochondrial fraction from the liver of Wy-14,643-treated rat with antiserum raised against heat-labile enoyl-CoA hydratase. The first-dimensional strip contained 20  $\mu$ g protein of light mitochondrial protein. The antibody gel contained 1% antiserum (0.5 mg protein/cm<sup>2</sup>). The precipitin peak is at the position of polypeptide PPA-80 (arrow).



FIGURE 7 SDS polyacrylamide tube gel electrophoretic profiles of immunoprecipitates separated on 7.5% separating gel with 3% stacking gel. The immunoprecipitates were obtained by incubating either purified enoyl-CoA hydratase (A) or Nonidet P40 solubilized microsomal fractions of liver (B and C) from Wy-14,643-treated rats with anti-enoyl-CoA hydratase (C) or antipolypeptide PPA-80 (A and B) antiserum. Approximately 20  $\mu$ g immunoprecipitate was loaed on the gels. The arrow indicates the position of enoyl-CoA hydratase or polypeptide PPA-80. The positions of rabbit IgG (1) and of two low molecular proteins (2) co-precipitated from the solubilized microsomal fractions are shown.



FIGURE 8 (A) Immunotitration of purified rat liver peroxisomal enoyl-CoA hydratase activity with antienoyl-CoA hydratase ( $\bullet$ ) and antipolypeptide PPA-80 (O) antiserum. Increasing amounts of antiserum were added to constant amount of enoyl-CoA hydratase (activity 78 U/ml) and the resulting immunoprecipitates sedimented. The enoyl-CoA hydratase activity remaining in the supernate was determined and expressed as percent of control. The data show that both antisera are capable of immunoprecipitating the purified enoyl-CoA hydratase to zero activity. The difference in the amount of antiserum needed to immunoprecipitate is caused by variability in the specific IgG concentration of the two antisera. (B) Quantitative precipitin assay of polypeptide PPA-80 (O) and enoyl-CoA hydratase ( $\bullet$ ) with anti-enoyl-CoA hydratase antibody. As described in the text, the reaction mixtures contained 0.25 ml of antiserum.

PPA-80 was then dissociated on a Sephadex G-200 column. Under these conditions the dissociated polypeptide PPA-80 protein displayed enoyl-CoA hydratase activity. These results further confirm that polypeptide PPA-80, identifiable in SDS polyacrylamide gels, is immunochemically identical to enoylCoA hydratase or composed predominantly of enoyl-CoA hydratase.

# Immunocytochemical Localization of Polypeptide PPA-80 and Heat-labile Enoyl-CoA Hydratase

Differences in the intensity of cytoplasmic immunofluorescent staining for polypeptide PPA-80 and enoyl-CoA hydratase in the liver between normal and Wy-14,643-treated rats are striking (Fig. 9). In the liver of normal rat, the cytoplasmic fluorescent staining was punctate and spotty with both antipolypeptide PPA-80 and anti-enoyl-CoA hydratase antibodies. In Wy-14,643-treated rats, the enlarged liver cells showed intense and rather diffuse cytoplasmic fluorescence; focal nonfluorescent spots, probably representing mitochondria, were discerned on higher magnification. No nuclear fluorescent staining was visualized. Sinusoidal cells, bile duct epithelium, and other nonhepatic parenchymal cells did not reveal any immunofluorescence. Adsorbing these antisera with either antigen abolished the immunofluorescent staining suggesting antigenic identity. These immunofluorescent studies clearly demonstrate that treatment of rats with Wy-14,643 results in a marked increase in the concentration of enoyl-CoA hydratase and polypeptide PPA-80 in liver parenchymal cells.

The availability of antibodies to enoyl-CoA hydratase and polypeptide PPA-80 made it possible to visualize the localization of these proteins, for the first time, in the liver cells at the ultrastructural level by an immunocytochemical method. After exposure of 10- to 15-µm-thick vibratome cut sections to peroxidase-coupled Fab fragments from either anti-enoyl-CoA hydratase or antipolypeptide PPA-80 antibodies, the liver parenchymal cells of Wy-14,643-treated rats showed a granular 3,3'-diaminobenzidine reactivity in the cytoplasm when examined by a light microscope (Fig. 10). Electron microscope examination of thin sections cut through the exposed sections demonstrated reaction product in peroxisomes and some peroxisome-associated smooth endoplasmic reticulum channels (Fig. 11 A and B). The subcellular localization of enoyl-CoA hydratase and polypeptide PPA-80 was identical. The nuclei, mitochondria, and the Golgi complex were negative. The nonparenchymal cells in the liver were also negative. No hepatic parenchymal cell staining was observed when sections were exposed to Fab fragments of nonspecific IgG coupled to peroxidase (Fig. 11 C). The presence of aminotriazole during the incubation of sections with either Fab fragments of specific IgG coupled to peroxidase or with the 3,3'-diaminobenzidine medium after exposure to specific Fab coupled to peroxidase did not prevent the specific immunocytochemical staining.

## DISCUSSION

The observation that hepatic peroxisome proliferation can be induced by clofibrate and other peroxisome proliferators in the absence of catalase synthesis, prompted Reddy et al. (44) to suggest 10 yr ago that increase of the catalase-deficient peroxisome population might be caused by possible induction of other unidentified peroxisomal components. Although the peroxisomes induced in the presence of allylisopropylacetamide (a potent inhibitor of catalase synthesis) were deficient in catalase activity, they nevertheless exerted a substantial serum lipid-lowering effect in these rats fed peroxisome proliferators (45). These studies, therefore, indicated that peroxisomal catalase is not essential for the hypolipidemic effect of peroxisome proliferators and emphasized the need to determine the identity of those enzymes in peroxisomes that might be involved in lipid metabolism. The studies of Lazarow and de Duve (18) 2and Lazarow (17) demonstrated the presence of a novel fatty acyl-CoA  $\beta$ -oxidation system in hepatic peroxisomes. This enzyme system is enhanced markedly in the livers of animals treated with several peroxisome proliferators (9-11, 16, 23, 31, 32, 37). Other workers have confirmed these findings and have further characterized the nature of this cyanide-insensitive system of peroxisomal  $\beta$ -oxidation of fatty acids (9, 10, 31, 32). The presence of a number of other enzymes in peroxisomes has also been recognized in recent years (7, 9, 10, 24, 25). On the basis of SDS polyacrylamide gel electrophoretic analysis of the polypeptide composition, we reported induction of a polypeptide with a molecular weight of ~80,000 in the postnuclear, large particle, and microsomal fractions of liver of rats and mice treated with various structurally unrelated peroxisome proliferators (39, 47). The enhanced synthesis of this peroxisome-proliferation-associated polypeptide in the liver of rats, in which peroxisome proliferation has been induced by hypolipidemic agents, has since been confirmed by other investigators (8, 10-12, 31, 52). Furthermore, the hepatic peroxisome proliferation induced in rats by high dietary fat has also been shown to be associated with a remarkable increase in the content of polypeptide PPA-80 (12).

The results described in this paper show that the hepatic peroxisome-proliferation-associated 80,000 mol wt polypeptide (polypeptide PPA-80), is immunochemically identical to the heat-labile enoyl-CoA hydratase, a peroxisomal protein that participates in the  $\beta$ -oxidation of fatty acids (31). This conclusion is based on the following observations: (a) the purified polypeptide PPA-80 and heat-labile enoyl-CoA hydratase exhibit identical minimum molecular weights of ~80,000 on SDS polyacrylamide gel electrophoresis and display similar isoelectric points; (b) these two proteins are immunochemically identical on the basis of Ouchterlony double-diffusion, immunotitration of enoyl-CoA hydratase activity, rocket immunoelectrophoresis, and crossed-immunoelectrophoresis analyses; and (c)immunoprecipitates formed by antibodies to polypeptide PPA-80, when dissociated on a Sephadex G-200 column, yield enoyl-CoA hydratase activity. The identical minimum molecular weight of ~80,000 found in these studies for polypeptide PPA-80 and enoyl-CoA hydratase purified from rats treated with the peroxisome proliferator Wy-14,643, correlate well with the minimum molecular weights of 74,000, 77,000, and 78,000 observed for enoyl-CoA hydratase isolated respectively from glyoxysomes (5), di-(2-ethylhexyl)phthalate-treated rat liver (31), and Escherichia coli (35). Although the polypeptide PPA-80 was purified by preparative SDS gel electrophoresis, it does not appear to contain any co-migrating proteins as evidenced by isoelectric focusing and Ouchterlony double-diffusion analysis. This may be because rat livers that contained an abundance of this polypeptide PPA-80 due to Wy-14,643-induced peroxisome proliferation, were used for purification.

The results of indirect immunofluorescence studies with either antipolypeptide PPA-80 or anti-enoyl-CoA hydratase antibodies presented here provide visual evidence for the marked induction of polypeptide PPA-80 (i.e., heat-labile enoyl-CoA hydratase) in the livers of rats treated with the peroxisome proliferator, Wy-14,643. The results obtained by immunofluorescence are in good agreement not only with the peroxisome proliferation observed by electron microscopy but



FIGURE 9 Fluorescent immunohistochemical localization of heat-labile enoyl-CoA hydratase and polypeptide PPA-80 in the liver of normal and Wy-14,643-treated (0.1% in the diet for 4 wk) rats. (A) Normal rat liver shows the punctate fluorescent staining with anti-enoyl-CoA hydratase antibody. Antipolypeptide PPA-80 antiserum gave a similar cytoplasmic staining (C) Wy-14,643-treated rat shows intense, somewhat diffuse, fluorescent staining for enoyl-CoA hydratase (B) and polypeptide PPA-80 (D). Note some round structures in the cytoplasm of liver cells, presumably mitochondria, in D that do not reveal fluorescence. (A) × 820; (B) × 480; (C) × 640; (D) × 820.





also with the SDS polyacrylamide gel electrophoresis data of increase in polypeptide PPA-80 concentration and the enoyl-CoA hydratase activity in the treated livers. The availability of these antibodies also facilitated the localization of enoyl-CoA hydratase and polypeptide PPA-80 at the ultrastructural level. It is evident from the immunocytochemical studies that the polypeptide PPA-80 and heat-labile enoyl-CoA hydratase are located in peroxisomes and not in mitochondria. These studies provide, for the first time, morphological evidence for the localization of polypeptide PPA-80 and of heat-labile enoyl-CoA hydratase in peroxisomes, thereby lending further support to the existence of the  $\beta$ -oxidation system in peroxisomes (17, 18, 31). The endoplasmic reticulum also showed positive but variable immunocytochemical staining. This variability may, in part, be attributable to suboptimal preservation of the fine structure of liver cells. The importance of better fixation and milder steps in tissue preparation has been emphasized recently by Novikoff (28). Because these cytochemical observations indicate that peroxisomes are the major cytoplasmic constituents containing substantial quantity of polypeptide PPA-80, it would then appear that the marked increase in the polypeptide PPA-80 content observed in microsomes, when analyzed by SDS polyacrylamide gel electrophoresis, is caused, in part, by contaminant smaller-sized peroxisomes as discussed elsewhere (47). The possibility of partial or complete extraction of peroxisomal matrix proteins occurring during liver homogenization (19, 27, 40) can be envisaged from the nearly empty peroxisomes showing a peripheral rim of polypeptide PPA-80 reaction product in a nonfrozen section of liver cell illustrated in Fig. 11 B (inset). These morphological observations on the localization of polypeptide PPA-80 and enoyl-CoA hydratase in peroxisomes are consistent with the previous observations of tbe cytochemical localization of peroxisomal marker enzyme catalase (29).

The direct visual demonstration of enoyl-CoA hydratase in all of the proliferated peroxisomes, provides strong evidence for the major contribution of peroxisomes to fatty acid oxidation in hypolipidemic drug-treated animals (16, 17). The leaching-out of peroxisomal matrix proteins, including the loss of enzymes responsible for the fatty acid oxidation from these organelles, should be considered as a major problem, particularly in studies that attempt to compare rates of fatty acid oxidation by peroxisomes and mitochondria in the liver cells isolated from animals treated with peroxisome proliferators. The relatively low rates of peroxisomal fatty acid oxidation, when compared to mitochondrial oxidation, observed in isolated hepatocytes of clofibrate-treated rats by Mannaerts et al. (23), might be attributable to the leakage of peroxisomal matrix enzymes during the prolonged perfusion of liver which is necessary to obtain isolated hepatocytes.

The studies of Osumi and Hashimoto (31, 32) demonstrate that heat-labile enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase constitute a co-purifiable multifunctional protein. Our studies now show that this multifunctional protein is immunochemically identical with the peroxisome-proliferation-specific polypeptide PPA-80. It is also considerable interest to point out that the polypeptide PPA-80 also appears as a component of the purified peroxisomal fatty acyl-CoA oxidase (10). These observations suggest that the polypeptide PPA-80 may constitute or play a pivotal role in the fatty acid oxidizing multienzyme complex. Additional studies are needed to ascertain whether the polypeptide PPA-80 is immunochemically identical to the purified peroxisomal fatty acyl-CoA oxidase and other enzymes of the fatty acid oxidizing system. It is also essential to characterize these various components of the peroxisomal fatty acid oxidizing system by determining the amino acid composition, peptide mapping, and ascertaining the Nterminal sequences. The availability of antibodies to polypeptide PPA-80 and peroxisomal enoyl-CoA hydratase may facilitate studies on the synthesis of these proteins in in vitro translational systems. Such studies may provide necessary insight into the nature of this multienzyme complex. An increase of polypeptide PPA-80 on SDS polyacrylamide gel electrophoretic analysis of the subcellular fractions of liver of rodents treated with lipid-lowering drugs should serve as a reliable indicator of enhanced peroxisomal  $\beta$ -oxidation system.

Finally, recent studies from our laboratory indicate that sustained proliferation of peroxisomes induced by peroxisome proliferators in rodents leads to the development of hepatocellular carcinomas (42, 46). It is important to point out that the lack of mutagenicity of these compounds strongly suggests that the carcinogenicity may be related directly to the metabolic



FIGURE 11 Portions of hepatocytes from sections of liver of rats fed Wy-14,643 which were exposed to Fab fragments of antibody to enoyl-CoA hydratase (A) or polypeptide PPA-80 (B) coupled to peroxidase; incubated in diaaminobenzidine medium (pH 7.6) for 15 min at room temperature. Reaction product is seen in peroxisomes (P) and in some smooth endoplasmic reticulum channels (ser). Note that some of the peroxixomes (P) in inadequately fixed tissue appear as empty vesicles (*inset B*) because of leaching out of peroxisomal matrix proteins during perfusion with the fixative. The mitochondria show no reactivity. Control sections (C) incubated as in Fig. 10 B show no immunocytochemical staining. (A) Not stained en bloc with uranyl acetate,  $\times$  31,000; (B and C) stained en bloc with uranyl acetate (B) and *inset*,  $\times$  29,000 (C)  $\times$  31,000.

perturbations associated with peroxisome proliferation. The continued administration of these compounds results in a persistent elevation of polypeptide PPA-80 in the livers (46). The primary hepatocellular carcinomas that develop in these livers also maintain a very high level of polypeptide PPA-80 (46). Whether this is caused by the continued presence of peroxisome proliferator in the diet or whether the induced polypeptide PPA-80 level becomes "fixed" in these hypolipidemic drug-induced tumors, similar to that encountered in the case of the microsomal enzyme epoxide hydrase in acetylaminofluorene-induced hyperplastic liver nodules (20, 30), remains to be ascertained.

The sustained proliferation of peroxisomes and the elevation of polypeptide PPA-80 in the livers of hypolipidemic drugtreated rats and in hepatocellular carcinomas developing in such livers raises an important question of the significance of peroxisomes and peroxisomal enzymes in liver carcinogenesis. The persistent increase in liver cell peroxisome proliferation in rodents chronically fed peroxisome proliferators appears to cause an excessive generation of H<sub>2</sub>O<sub>2</sub> as a result of increased peroxisomal  $\beta$ -oxidation of fatty acids (41). The H<sub>2</sub>O<sub>2</sub> thus generated may react with superoxide radical  $(O_2^-)$  to form hydroxyl radical (•OH). The rate of production of these highly toxic oxygen radical may determine the relationship between peroxisome proliferation and hepatocarcinogenicity in rodents chronically exposed to peroxisome proliferators. Therefore, it is of great importance to ascertain the patho-physiological role of polypeptide PPA-80 and other peroxisomal enzymes in the regulation of cell proliferation and neoplasia, particularly in the livers of rodents treated with peroxisome proliferators.

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