SITE OF BIOSYNTHESIS OF CYTOCHROME P450 IN HEPATOCYTES OF PHENOBARBITAL TREATED RATS

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Summary: Localization of nascent cytochrome P450 was studied using immunochemical techniques. [125-I]-labeled specific antibody to the purified cytochrome P450 was found to bind preferentially with membrane bound ribosomes, while only a small amount of radioactivity was found in free ribosomes. When nascent polypeptides in free and membrane bound ribosomes were labeled with [3H]-puromycine in the presence of a high salt buffer, and were incubated separately with the specific antibody and carrier cytochrome P450, the [3H]-radioactivity was found exclusively in the immunoprecipitates from bound ribosomes, thus indicating the preferential presence of nascent cytochrome P450 peptides in bound ribosomes. These two lines of evidence strongly suggest that cytochrome P450 is exclusively synthesized on membrane bound ribosomes.

There is a number of evidence that secretory proteins are exclusively synthesized by membrane-bound polyribosomes, whereas most cytosol proteins are made by free polyribosomes (see review by Rolleston (1)). The site of synthesis of membrane-associated proteins is however, controversial. Originally it was suggested that constitutive membrane proteins are synthesized on rough endoplasmic reticulum with subsequent translocation to smooth membrane (2-4). However, an alternative proposal is that some membrane proteins are synthesized on free polyribosomes and inserted into membrane from a cytoplasmic pool (5-7). Indeed, Ragnotti et al (8) reported that NADPH-cytochrome c reductase was made equally by the two classes of ribosomes, and Lowe and Hallinan (9) reported that isolated free polyribosomes from rat liver synthesized the reductase in vitro four times as efficiently as membrane-bound polyribosomes.

Cytochrome P450 is one of the major microsomal membrane

proteins, comprising ~ 5 % and 15 % of the protein of non-induced and phenobarbital "induced" liver microsomes, respectively, and composes the microsomal mixed function oxidase system with NADPH-cytochrome c reductase and lipid.

We purified cytochrome P450 from liver microsomes of phenobarbital treated rats and the specific antibodies to the cytochrome were prepared by affinity chromatography. Free and membrane bound ribosomes were purified from rat liver and the existence of nascent cytochrome P450 molecules on these ribosomes were studied using immunochemical techniques. Preferential existence of nascent cytochrome P450 molecules in the membrane bound ribosomes strongly suggests that cytochrome P450 is synthesized on membrane bound ribosomes.

Materials and Methods.

Male Sprague-Dawley rats weighing 200 g were given a single daily i.p. injection of phenobarbital (10 mg per 100 g body weight) for seven days and were fasted for 24 h before sacrifice. The

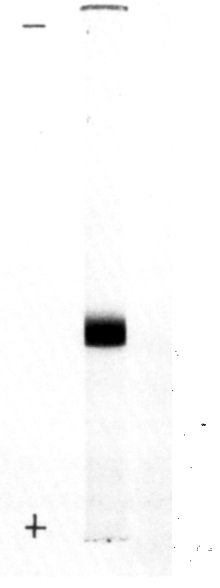

FIGURE 1. SDS-polyacrylamide gel electrophoresis of purified cytochrome P450. Cytochrome P450 preparation (~ 20 μg) was dissolved in 1 % SDS solution containing sample buffer and applied onto a 5 % acrylamide gel column. After electrophoresis, the gel column was stained with Coomassie blue.

cytochrome was purified according to the procedures of Imai and Sato (10) with slight modifications, details of which will be reported elsewhere. The specific content of cytochrome P450 in the purified preparation was 14 nmol/mg of protein with an overall yield of about 6 % of total content in microsomes. SDS-acrylamide gel electrophoresis showed that the purified cytochrome P450 gave a single band as shown in Fig. 1.

Rabbit antiserum against the purified cytochrome P450 was prepared by repeated injection of 2 mg of cytochrome P450 mixed with complete Freund's adjuvant into the foot pad of the rabbit. The IgG fraction of the rabbit antiserum was obtained by fractionation with ammonium sulfate. Specific antibody to the purified cytochrome P450 was isolated by affinity chromatography (11, 12) using cytochrome P450 conjugated Sepharose 4 B and the immunological specificity of the antibody was checked by Ouchterlony double diffusion analysis. Iodination of IgG was carried out according to the method of Hunter and Greenwood (13).

Free and membrane bound ribosomes were prepared from post-mitochondrial supernatant of rat liver according to the procedures of Blobel and Potter (14). Male Sprague-Dawley rats weighing 160-180 g and fasted for 20 h were sacrificed 5 h after single i.p injection of phenobarbital (10 mg/100 g body weight).

These two classes of ribosomes were resuspended separately in 0.25 M sucrose TKM buffer (50 mM Tris-HCl pH 7.5, 25 mM KCl and 5 mM $MgCl_2$), treated with 1 % deoxycholate and 5 ml aliquots were overlayered on a discontinuous sucrose density gradient of 1.65 M sucrose-TKM buffer (2 ml) and 2.25 M sucrose-TKM buffer (4-5 ml), and centrifuged at 105,000 g for 20 h in a Hitachi RP-40 rotor. The pellets were used as purified free and membrane bound ribosomes, respectively.

After receiving a single dialy i.V. injection of phenobarbital (10 mg/100 g body weight) for two days, male Sprague-Dawley rats weighing 150 g were sacrificed 30 min after an i.V. injection of ^{14}C-L-Leucine (10 μCi/100 g body weight). Liver microsomes were prepared by a previously reported method (10). The labeled microsomes were suspended in distilled water, solublized with 1 % of DOC, and the DOC-soluble supernatant was obtained by centrifugation at 50,000 rpm for 2 h, in a Hitachi RP-65T preparative centrifuge.

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Hinman and Philips (15). Cytochrome P450 was assayed following the method of Omura and Sato (16). The concentration of protein was determinated according to the method of Lowry et al (17). The amount of ribosomes was estimated using $E^{260\ nm}_{1\%} \approx 135$ (18). [3H]-puromycin (3 μCi/mmole) and ^{125}I were purchased from New England Nuclear Co., Boston. [14 C]-L-Leucine (57 mCi per mole) was purchased from Department des Radio Elements, France.

Results

Specificity of the Anti-cytochrome P450 Antibody. Ouchterlony double diffusion technique show a single distinct precipitin line between the purified rat cytochrome P450 and the rabbit specific antibody (not shown). As another criterion for the specificity, the specific antibody was added to DOC-soluble supernatant previously

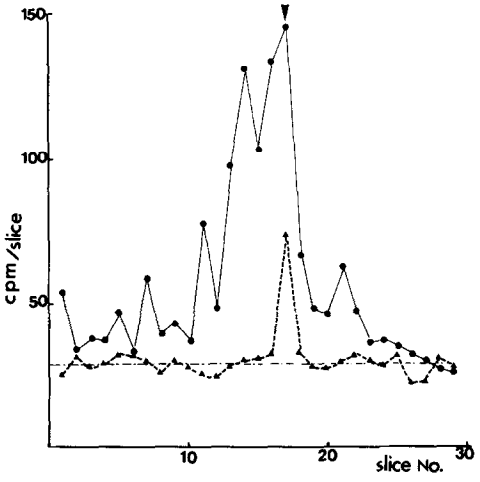

FIGURE 2. SDS-polyacrylamide gel electrophoretic analysis of the immuno-precipitate made between anti-cytochrome P450 antibody and DOC-soluble supernatant of microsomes previously labeled in vivo with [14 C]-L-Leucine. DOC-soluble supernatant of the labeled microsomes (0.5 mg protein) was incubated with about 4 mg of anti-cytochrome P450 antibody for 30 min at 25°C and then for 12 h at 4°. The medium of incubation contained 50 mM Tris-HCl buffer, pH 8, 1 % DOC. The precipitate formed was collected by centrifugation at 3,000 rpm, washed with the above medium, and again washed with 50 mM Tris-HCl pH 8 buffer. The washed precipitates were dissolved in SDS-sample buffer and analyzed in 7.5 % gel column. After run, the column was cut into thin slices 2 mm in thickness, which were dissolved in H_2O_2 and the radioactivity was measured in a Toluene-Triton X-100 scintillator (solid circles). Another aliquot of the microsomal supernatant (0.3 mg of protein/column) was analyzed by gel electrophoresis without immuno-precipitation and the radioactivity in the slices was measured (solid triangles).

labeled in vivo with [14 C]-L-Leucine (see Materials and Methods). The immuno-precipitates formed were analyzed by SDS-polyacrylamide gel electrophoresis, and the radioactivity in the sliced gel discs was measured. As shown in Fig. 2, there was only a single radioactive peak of which electrophoretic mobility was exactly the same as seen in the purified cytochrome P450 (arrow point), indicating that the specificity of the anti-cytochrome P450 antibody is sufficient to precipitate only cytochrome P450 from the DOC-soluble supernatant.

Binding of ^{125}I-labeled Anti-cytochrome P450 Antibody with Free and Membrane-bound Ribosomes. Free and membrane bound ribosomes prepared as described in Material and Methods were incubated separately with [125-I]-labeled anti-cytochrome P450 antibody, and then

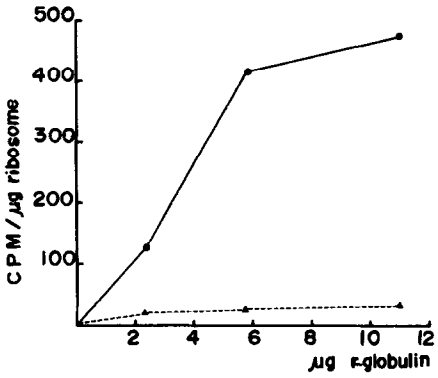

FIGURE 3. The binding of ^{125}I-labeled specific anti-cytochrome P450 antibody with free or membrane bound ribosomes. Free or membrane bound ribosomes (250 μg) were incubated at 25° for 15 min with 350 μg of normal rabbit IgG and various amounts of ^{125}I-labeled specific antibody in TKM buffer. After incubation, ribosomes in the incubation mixtures were pelleted down by centrifugation at 60,000 rpm for 4 h through 3 ml 1.65 sucrose and 3 ml of 2.0 M sucrose in TKM buffer. Radioactivities of the ribosomal pellets were measured by the auto-gamma scintillation spectrometer. The ribosomes incubated with ^{125}I-labeled normal rabbit IgG served as a control and the radioactivities in these fractions were subtracted from the above radioactivities.
●—● membrane bound ribosome, ▲----▲ free ribosome.

the ribosomes were isolated by sedimentation through 2.0 M sucrose-TKM solution. As shown in Fig. 3, a larger amount of the labeled antibodies bound with membrane bound ribosomes than with free ribosomes. The maximum number of the antibody molecules bound with the former was $\sim 5 \times 10^{-2}$ per ribosome. This result strongly suggests that cytochrome P450 is exclusively synthesized on membrane bound ribosomes in rat hepatocytes. The possibility that the radioactivity may be simply due to the artificial binding of the complete molecules of cytochrome P450 with membrane bound ribosomes had to be ruled out and so we designed the following experiment.

Immunochemical Precipitation of ^{3}H-puromycin Labeled Nascent Cytochrome P450 Chains. Free and membrane bound ribosomes were incubated separately with [3H] puromycin in the presence of 750 mM KCl, and after extensive dialysis against 0.1 M Tris buffer, pH 8, the anti-cytochrome P450 antibody was added to the incubation mixtures and the radioactivity in the precipitates formed was measured. ^{3}H-radioactivity was preferentially found in the precipi-

tate from membrane bound ribosomes, while only one tenth or less of the radioactivity was precipitated from the free ribosomes as shown in Fig. 4.

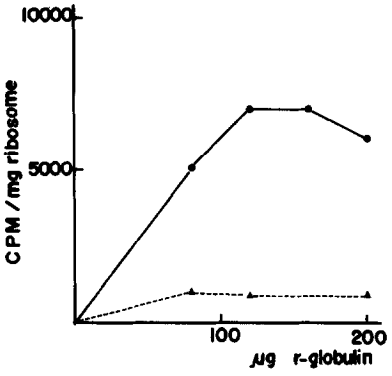

FIGURE 4. Immuno-precipitation of ^{3}H-puromycin labeled nascent P450 chains. Free or membrane bound ribosomes (~ 4 mg) were incubated with ^{3}H-puromycin (1 μCi per mg of ribosomes) in 2 ml of high salt buffer; 50 mM Tris-HCl buffer, pH 7.5, 750 mM KCl and 5 mM $MgCl_2$ (20). After overnight dialysis against 0.1 M Tris-Cl pH 8, 30 μg of carrier purified cytochrome P450 and various amounts of anti-cytochrome P450 antibody were added to 250 μl of the dialysed ribosome solutions. The final volume of the mixtures was adjusted to 500 μl with 0.1 M Tris-Cl, pH 8. After incubation for 30 min at 25°C and then for about 12 h at 4°C, the immuno-precipitates formed were collected by centrifugation at 3,000 rpm and washed with the 0.1 M Tris-HCl buffer, and then washed with 0.1 M Tris-HCl buffer pH 8 containing 1 % DOC. The washed precipitates were treated with 5 % hot TCA and then with ethanol-ether (3 : 1), and finally were dissolved in Soluen-350 (Packard) for counting in a toluene scintillator. As a control, normal rabbit IgG was added to an aliquot of the dialyzed ribosome solution. After mixing, goat IgG against rabbit Fab fragment was added and the radioactivity in the precipitates was measured. This value was subtracted as a control (about 300 cpm/mg ribosome).
●—● membrane-bound ribosome, ▲···▲ free ribosome.

Discussion

Ichikawa and Mason (5) spectrophotometrically measured hepatic cytochrome P450 on free and membrane bound polyribosome fraction after administration of phenobarbital to rabbits and found preferential association of cytochrome P450 with free polyribosomes. These results, however, have been claimed by Craft et al (19), who asserted that cytochrome P450 found in free ribosome fraction was

associated with membraneous contamination and the cytochrome in the free ribosome fraction was removed when the free ribosomal fraction passed repeatedly through 2.0 M sucrose layer.

In such work, the amount of cytochrome P450 was estimated by spectrophotometry which definitely necessitates existence of completed holoenzymes on the site of synthesis——ribosomes. As it is well known that proteins synthesized on ribosomes are rapidly released from the ribosomes, existence of holo-cytochrome P450 on ribosomes itself is questionable. It is well known that ribosomes have unusual properties which bind many types of molecules (20), and it is possible that cytochrome P450 detected on ribosomes is not due to the newly synthesized and/or the nascent molecules but rather to artificial binding of the completed molecules during the isolation procedures.

In a different approach to this problem, we purified cytochrome P450 from the livers of phenobarbital treated rats and prepared a specific antibody to cytochrome P450. This antibody precipitated specifically cytochrome P450 molecules as shown in Fig. 2. Free and membrane bound ribosomes were prepared by the procedures of Blobel and Potter (14) and further purified by deoxycholate treatment and subsequent sedimentation through 2.25 M sucrose layer to eliminate as little as possible the contamination of membrane. Using these preparations it could be clearly demonstrated that nascent cytochrome P450 synthesized immediately after phenobarbital treatment is localized preferentially on membrane bound ribosomes and suggests that cytochrome P450 is exclusively synthesized on membrane bound ribosomes.

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