Cytochrome P-450 and NADPH-Cytochrome P-450 Reductase Are Degraded in the Autolysosomes in Rat Liver

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Abstract. We have investigated the degradation in rat liver of two typical endoplasmic reticulum (ER) membrane proteins, phenobarbital (PB)-inducible cytochrome P-450 (P-450(PB)) and NADPH-cytochrome P-450 reductase (FP2). Autolysosomes, almost completely free from contamination by the other organelles such as ER, were prepared from leupeptin-treated rat livers according to the method of Furuno et al. (Furuno, K., T. Ishikawa, and K. Kato, 1982, J. Biochem., 91:1943–1950). Quantitative immunoblot analysis showed that these two proteins were found in large amounts in the autolysosomes regardless of PB treatment. The specific content of P-450 (PB) in the autolysosomes changed along with that in the microsomes during and after PB treatment, whereas hardly any P-450(PB) was detected in the cytosol fraction throughout the experiment. We also found a marked increase in the autolysosomal proteins 3 d after cessation of PB treatment when microsomal proteins are degraded most rapidly. Ferritin immunoelectron microscopy revealed directly that when the limiting membranes of the premature autolysosomes were partially broken the smooth vesicles segregated within the autolysosomes were heavily stained with ferritin anti-P-450(PB) conjugates. Thus, for the first time, we could present convincing evidence that P-450(PB) and FP2 are segregated to be degraded in the autolysosomes.

Cytochrome P-450 (P-450), together with NADPH-cytochrome P-450 reductase (FP2), constitutes the microsomal monooxygenase system in a hepatic cell that plays an important function in the oxidation of numerous endogeneous and xenobiotic compounds. It has been well-established that multiple forms of P-450 and specific forms of P-450 exist that are inducible by various drugs such as phenobarbital (PB), 3-methylcholanthrene, and β-naphthoflavone. For example, PB treatment causes rapid increases in the PB-inducible P-450 (P-450(PB)) and FP2 accompanied by remarkable proliferation of smooth endoplasmic reticulum (ER) (2, 11, 23).

Although in recent years many efforts have been made to clarify the mechanism for biosynthesis and drug induction of the microsomal monooxygenase enzymes (1, 5, 7, 9, 19, 20), the mechanism for their degradation is still poorly understood. The concentration of any enzyme in a cell is determined by the equilibrium between its biosynthesis and degradation, and it is very important to investigate the molecular and cellular mechanisms responsible for the degradation of the microsomal monooxygenase enzymes.

The turnover rate of several forms of P-450, together with the other microsomal proteins, has been previously reported (18, 21, 24, 26, 27). The wide differences in their half-lives have led some investigators to deny the important role of the autolysosomes in the degradation of microsomal proteins (21, 24, 26, 27). Bolender and Weibel (3), however, showed by morphometric analysis that the removal of PB-induced excess ER membranes was associated with increase in autophagic activity. We have previously shown that a decrease in the contents of microsomal proteins and P-450(PB) parallels an increase in the number of the autophagic vacuoles after the cessation of PB treatment (15). These results suggested the concomitant removal of the proliferated ER membranes and P-450(PB) via the autolysosomes.

No direct evidence, however, has been presented so far to show that microsomal enzymes such as P-450 and FP2 are indeed segregated and degraded in the autolysosomes. In this paper we isolated autolysosomes from leupeptin-treated rat livers and showed by quantitative immunoblot and immunoelectron microscopic analyses that the autolysosomes do contain large amounts of P-450(PB) and FP2 molecules to be degraded there.

Materials and Methods

Materials
PB was purchased from Sanko Seiyaku Kogyo Co., Ltd., Tokyo. 125I-Labeled protein A (30 mCi/mg protein) was obtained from Amersham International, England. Leupeptin was purchased from the Protein Research Foundation, Osaka, and Percoll and density marker beads were purchased from Pharmacia Fine Chemicals, Uppsala. All other chemicals were of the highest purity commercially available.

1. Abbreviations used in this paper: ER, endoplasmic reticulum; FP2, NADPH-cytochrome P-450 reductase; G-6-Pase, glucose-6-phosphatase; P-450, cytochrome P-450; PB, phenobarbital; P-450(PB), major form of cytochrome P-450 in hepatic microsomes of PB-treated rats.
PB Treatment of Rats

Male Sprague-Dawley rats (≈150 g) were fed ad libitum on laboratory chow and were given an intraperitoneal injection of PB (80 mg/kg body wt) once a day for 4 d.

Cell Fractionation

The autolysosome fraction was prepared from both PB-treated and untreated rats according to Furuno et al. (6) with a slight modification in that the Percoll density gradient centrifugation was performed twice for the purification.

Microsome fraction was prepared from the supernatant of the second centrifugation at 1,000 g for 10 min. After further centrifugation at 10,000 g for 20 min, the supernatant was centrifuged at 100,000 g for 90 min, and the resultant pellet was used as the microsome fraction. To obtain the cytosol fraction, the resultant supernatant was further centrifuged at 100,000 g for 5 h.

Preparation of Antibodies Against P-450(PB) and FP2

P-450(PB) and FP2 were purified from liver microsomes of PB-treated rats according to the procedures described by Masaki et al. (15) and Yasukochi and Masters (30), respectively.

Antisera against these two antigens were elicited in rabbits, and the IgG fractions of the antisera were purified by repeated ammonium sulfate fractionation followed by DEAE cellulose column chromatography (15). The specific antibody was purified by affinity chromatography using Sepharose 4B conjugated with purified P-450(PB) or FP2.

Immunoblot Procedures

SDS PAGE was performed according to the procedures of Laemmli (12). Immuno blotting with ~25I-protein A was done according to Burnette (4). The autoradiogram and the bands corresponding to P-450(PB) or FP2 were excised and counted in an autogamma spectrometer (model 5780; Packard Instrument Co., Inc., United Technologies, Downers Grove, IL). A standard curve based on purified P-450(PB) or FP2 run in the adjacent lanes was found to be linear up to 200 ng of each antigen.

Electron Microscopy

The autolysosome fraction was centrifuged at 15,000 g for 15 min. The pellet was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and postfixed in 1.0% osmium tetroxide in the same buffer, dehydrated, and embedded in Epon 812. The thin sections were cut and observed under an Hitachi electron microscope (model HU-11).

Ferritin Immunoelectron Microscopy

Ferritin antibody conjugates against P-450(PB) with the molar ratio of IgG to ferritin (≈1:1) were prepared previously described (16), and were concentrated to ≈2.5 mg of ferritin and 0.5 mg IgG/ml. The control conjugates were prepared in the same way by coupling ferritin with IgG from nonimmunized rabbits.

An aliquot of the rat liver autolysosomes (500 μg protein) was incubated for 30 min at 4°C with 100 μl of either antibody conjugates or control conjugates. After incubation, the specimens were washed repeatedly by centrifugation and processed for electron microscopic observation as described above.

Analytical Procedures

FP2 was assayed as described by Omura and Takesue (22). Glucose-6-phosphatase (G-6-Pase), cytochrome oxidase, cathepsin D, and acid phosphatase were determined by the methods of Leskes et al. (13), Wharton and Tzagoloff (31), Yamamoto et al. (28), and Pricer and Ashwell (25), respectively. Protein was measured by the method of Lowry et al. (14) using BSA.

Table 1. Marker Enzyme Activities in Liver Homogenate (Homog), Autolysosomes (AI), and Microsome (Ms) Fractions Prepared from PB-treated and Untreated Rats

<table>
<thead>
<tr>
<th>Cell fractions</th>
<th>Without PB treatment</th>
<th>With PB treatment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Homog</td>
<td>Al</td>
</tr>
<tr>
<td>G-6-Pase*</td>
<td>72.2 ± 9.5</td>
<td>2.1 ± 2.0</td>
</tr>
<tr>
<td>FP2</td>
<td>47.2 ± 7.1</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td>Cytochrome Oxidase†</td>
<td>0.66 ± 0.07</td>
<td>0.07 ± 0.05</td>
</tr>
<tr>
<td>Cathepsin D‡</td>
<td>1.4 ± 0.2</td>
<td>27.6 ± 13.1</td>
</tr>
<tr>
<td>Acid Phosphatase*</td>
<td>25.0 ± 1.7</td>
<td>323.2 ± 162.0</td>
</tr>
</tbody>
</table>

* moles of inorganic phosphate liberated/min/mg protein.
† moles of cytochrome C reduced/min/mg protein.
‡ moles of cytochrome C oxidized/min/mg protein.
§ μg of Tyrosine liberated/min/mg protein.
as the standard. The densities of Percoll fractions were determined with density gradient markers from Pharmacia Fine Chemicals.

**Results**

*Purification of Autolysosomes by Percoll Density Gradient Centrifugation*

Fig. 1 shows the distribution patterns of three marker enzymes after the first Percoll density gradient centrifugation. Cytochrome oxidase, a mitochondrial marker, was recovered in fractions with a density of 1.06–1.12 g/ml. G-6-Pase, an ER marker enzyme, was recovered in fractions of lower density (1.05–1.08 g/ml) than those of cytochrome oxidase and never recovered in the fractions of heavier density. Leupeptin did not change the distribution patterns of either cytochrome oxidase and G-6-Pase. Acid phosphatase, a lysosomal marker enzyme, was recovered in fractions with a density of 1.05–1.09 g/ml in the control rat. Upon treatment with leupeptin, another sharp peak of acid phosphatase activity appeared at the heavier region (1.13–1.15 g/ml), which was clearly separated from the mitochondria and ER markers. The latter fraction was further subjected to the second Percoll density gradient centrifugation to purify the autolysosomes.

*Biochemical and Morphological Characterization of the Purified Autolysosomes*

Table I shows the several marker enzyme activities of the autolysosome and microsome fractions from PB-treated and untreated rats. As reported by various investigators (7, 11, 20), PB treatment caused an increase in the microsomal FP2 activity (~twofold). The enzymatic profiles in the autolysosome fraction from PB-treated and untreated rats were nearly identical. As reported by Furuno et al. (6), the autolysosome fractions were enriched 15–20-fold and 13-fold in cathespin D and acid phosphatase activities, respectively. In contrast, the activities of two ER marker enzymes, G-6-Pase and FP2, were scarcely detectable in these fractions, as expected from the results shown in Fig. 1. The specific activity of cytochrome oxidase in the autolysosome fractions was ~10% of those of the homogenates. These results indicate a good separation of autolysosomes from ER and mitochondria.

The purity of the autolysosome fraction was confirmed by electron microscopic observations as shown in Fig. 2. The fraction consisted predominantly of autolysosomes, 99% of which were mature autolysosomes containing electron dense materials and 1% that were premature autolysosomes containing apparently intact cell organelles such as mitochondria and ER (Fig. 8). The contamination of the other organelles, especially of ER membranes, was hardly detectable.

*Imunochemical Detection of P-450(PB) and FP2 in the Liver Microsomes from PB-treated and Untreated Rats*

We prepared specific antibodies against P-450(PB) and FP2.

Figure 2. Electron micrograph of the purified autolysosome fraction. The autolysosome fraction consisted entirely of autolysosomes except for a few mitochondria shown by small arrows. Bar, 2.5 μm.
The specificity of the antibody preparations was tested by Ouchterlony double diffusion tests (data not shown) and further assessed by an immunoblot analysis. In the microsome fraction from PB-treated rats a single band at 53 kD corresponding to P-450(PB) was detected and no other component was labeled (Fig. 3). This result indicates the monospecificity of the antibody against P-450(PB). Although another band at ~50 kD was detected in the microsome fraction from PB-untreated rats, the ratio of the two bands varied with the microsome preparations. In addition, when poly (A)-containing mRNA prepared from PB-untreated rats was translated in a cell free system, the anti-P-450(PB) antibody precipitated only a single-labeled protein band at 53 kD corresponding to P-450(PB) (data not shown). These observations suggest that P-450(PB) in the microsomes of PB-untreated rats (53 kD) is easily degraded to ~50-kD component. An alternative possibility is that the ~50-kD component is a minor form of microsomal P-450 that cross-reacts with the antibody against P-450(PB).

It is quite evident from Fig. 3 that PB treatment dramatically increased P-450(PB). Fig. 3 also demonstrates that a single-labeled protein corresponding to FP2 in molecular mass (78 kD) was detected with the specific anti-FP2 antibody, indicating the monospecificity of this antibody. The induction of FP2 upon PB treatment was also confirmed.

**Detection of P-450(PB) and FP2 in the Autolysosome Fractions**

Next we tried to detect whether or not P-450(PB) was present in the autolysosomes using the following experiment. The gradients after the first Percoll density gradient centrifugation were analyzed for acid phosphatase activity and for the immunoblot. Fig. 4 shows the distribution patterns of acid phosphatase and P-450(PB) in the autolysosomes fractions with a density range of 1.13-1.15 g/ml. As shown in Fig. 4 A, the distribution pattern of P-450(PB) almost coincided with that of acid phosphatase. In contrast, no significant amount of acid phosphatase and P-450(PB) was detectable in the same fraction prepared from leupeptin-untreated rat liver (Fig. 4 B).

This result, together with those obtained by enzymatic and morphological characterizations of the purified autolysosomes, indicates clearly that P-450(PB) recovered in the autolysosome fraction cannot be attributed to the contaminating ER membranes. The immunoblot analysis of the autolysosome fractions prepared from PB-treated and untreated rats showed that both P-450(PB) and FP2 were detected by the corresponding specific antibodies (Fig. 5). Although the 53-kD band together with the ~50-kD band was detected in the autolysosomes from PB-untreated rats, the ~50-kD form was the main component in the autolysosomes fraction as shown in Fig. 5 B, suggesting that conversion of P-450(PB) in PB-untreated rats from 53 to 50 kD might be accelerated in the autolysosomes. Fig. 5 B also indicates that PB treatment produced an increase in the amounts of both P-450(PB) and FP2 in the autolysosomes.

**Quantitation of P-450(PB) and FP2 in the Microsome, Autolysosome, and Cytosol Fractions**

The amounts of P-450(PB) and FP2 in the microsome and autolysosome fractions can be estimated as described in Materials and Methods. The results of such quantitations are listed in Table II. The specific contents of the two proteins in the microsomes from PB-untreated rats were ~40 pmol/mg protein. Upon PB treatment, the amounts of P-450 (PB) and FP2 in microsomes increased ~15- and 2.5-fold, respectively, consistent with many previous reports (1, 2, 5, 7, 8, 11, 15, 20). Coincident with their increases in the microsomes, P-450(PB) and FP2 segregated in the autolysosomes increased 10- and twofold, respectively. The amounts of the two ER proteins recovered in the autolysosomes were ~5% of those in the microsomes.
Figure 4. Distribution patterns of acid phosphatase and P-450(PB) in the autolysosome fraction obtained by Percoll density gradient centrifugation. PB was previously administered to rats and crude lysosome fractions prepared from leupeptin-treated (A) and untreated (B) rat livers were subjected to Percoll density gradient centrifugation. Gradients were collected in fractions of 1 ml each, and the autolysosome fractions with a density of 1.13–1.15 were analysed for acid phosphatase activity (○) and P-450(PB) antigen (●). The latter was measured by quantitative immunoblot analysis as described in Materials and Methods. The corresponding immunoblot patterns of P-450(PB) in the autolysosome fractions together with that of purified P-450(PB) (arrow) are shown at the bottom of A and B.

The specific content of P-450(PB) in the cytosol fraction was only ~0.16 and 0.59% of those in the microsome fraction from PB-treated and untreated rat livers, respectively.

Effect of PB on the Protein and P-450(PB) Contents in Microsome and Autolysosome Fractions

Fig. 6 shows the changes in the protein contents of microsome and autolysosome fractions during and after PB treatment. Repeated injections of PB led to the increase in the microsomal protein corresponding to the proliferation of the smooth ER. After cessation of PB treatment, the microsomal proteins decreased drastically to the control level within 7 d as previously reported (15). The protein content in the autolysosome fraction did not increase during PB treatment but showed marked increase (~60%) on the third day after cessation of PB treatment when microsomal proteins were rapidly decreasing. This result suggests that the increase in the autophagic activity may be responsible for the removal of PB-induced ER membranes.

To determine whether induction of lysosomal enzymes occurred during and after PB treatment, the activities of cathepsin D and acid phosphatase were determined. The specific activities of the two enzymes in both the homogenate and autolysosome fractions were constant throughout the experimental period (data not shown).

Next, the levels of P-450(PB) in the microsome and autolysosome fractions were measured during and after PB treatment (Fig. 7). Increase in the specific contents of microsomal P-450(PB) was much more remarkable than that of microsomal proteins and the specific contents of P-450 (PB) segregated in the autolysosomes ran parallel to those in the microsomes.

Ferritin Immunoelectron Microscopy of the Autolysosomes Prepared from PB-treated and Untreated Rats

Ferritin immunoelectron microscopic techniques were applied to directly demonstrate the segregation of P-450(PB) of
ER membranes because previous studies from this laboratory (17) have shown that the antibodies used recognize the cytoplasmic domains of P-450(PB). Figure 8a shows an electron micrograph of an autolysosome fraction from PB-treated rat liver after incubation with ferritin antibody conjugates against P-450(PB). The mature autolysosomes completely covered by the limiting membranes were not stained at all by ferritin particles. In marked contrast, autophagosomes and premature autolysosomes are fragile and when their limiting membranes were partially broken, as shown in Fig. 8a, a number of ferritin particles were seen attached to the smooth vesicles segregated within the premature autolysosomes. The density of the ferritin particles attached to these smooth vesicles was comparable to that of the microsomal membranes prepared from PB-treated rat.

Fig. 8b shows an electron micrograph of the autolysosome fraction prepared from a PB-untreated rat. It is evident that the smooth vesicles in the immature autolysosomes are stained with much less ferritin particles than those from a PB-treated rat. Although the limiting membranes are apparently broken in this plane of sectioning, they are presumably broken in another plane.

As shown in Fig. 8c, practically no ferritin particles bound to the smooth vesicles when incubated with the control conjugates. The ferritin particles attached to the amorphous materials (arrows) in the broken premature autolysosomes are nonspecifically binding.

These results clearly demonstrate that the ER membranes containing P-450(PB) were in fact segregated into the autolysosomes. It is to be noted here that no ferritin particles were attached to the limiting membranes of the autolysosomes, regardless of their integrity and maturity. This topic will be the focus of a subsequent report. (Yamamoto, A., R. Masaki, and Y. Tashiro, manuscript in preparation.)

Discussion

We isolated autolysosomes from PB-treated and untreated rats according to Furuno et al. (6) and found, using immunoblot analysis, that both P-450 (PB) and FP2 were detected there in large amounts; the specific contents of these two proteins in the autolysosome fraction was ~5% of those in the microsomes (Table II). These two proteins recovered in the autolysosome fraction could not be attributed to the contamination by ER membranes. Enzymatic (Table I) and morphological (Fig. 2) characterization of the isolated autolysosomes indicated that the contamination of the autolysosome fractions by ER membranes is negligible. Assuming that G-6-Pase and FP2 are exclusively localized in the ER membranes and that the microsomes are derived from ER membranes, the contamination of the autolysosome fraction by ER membranes can be estimated from the enzyme activities of G-6-Pase and FP2 in the latter fraction to be ~0.4% on the average (Table I).

Thus there is a big discrepancy in the amount of the microsomal membrane proteins in the autolysosome fraction...
estimated from the enzyme activities of G-6-Pase and FP2 (≈4.0%) and from the immunoblot assays of P-450 (PB) and FP2 (≈5%). The most probable explanation for this discrepancy is that microsomal P-450 (PB) and FP2 are segregated to be degraded in the autolysosomes, but inhibition of lysosomal thiol proteases by leupeptin treatment might have led to the marked accumulation of immunoreactive, but enzymatically inactive, P-450(PB) and FP2 molecules in the autolysosomes. Some of the enzyme molecules segregated in the autolysosomes, especially in the premature ones, could be enzymatically active and may contribute to the enzyme activities found in the autolysosome fraction. The degree of contamination by the ER membranes of the autolysosome fraction estimated from the two marker enzyme activities, therefore, could be much less than the estimated value of 0.4%.

The present ferritin immunoelectron microscopic observations clearly revealed that the smooth vesicles segregated within the premature autolysosomes when the limiting membranes were broken are heavily stained with ferritin antibody conjugates (Fig. 8). The immunoelectron microscopic observations using a postembedding protein A colloidal gold method, however, clearly demonstrated that when treated with leupeptin P-450(PB) was detected not only in the premature autolysosomes but also in the mature autolysosomes in the rat hepatocyte. As shown in Table II and Fig. 5, P-450(PB) and FP2 were detected in the autolysosomes prepared from both PB-treated and untreated rat livers. These results strongly suggest that the autophagic process is responsible for the degradation of these enzymes regardless of PB treatment. That is, this process is likely to be a normal cellular process responsible for the degradation of the two ER membrane proteins. The increase in the amount of autolysosomal proteins at 3 d after cessation of PB treatment may indicate accelerated autophagocytosis in this special period.

Three possible routes could be suggested for degradation of the ER membrane proteins such as P-450 and FP2. The first is that the ER membrane proteins are transported to the lysosomal compartment via the Golgi apparatus to be degraded there. In the previous paper (29) we showed that P-450(PB) was not detectable on the membrane of Golgi cisternae either when P-450(PB) was maximally induced by PB treatment or when P-450 content in the microsomes rapidly decreased after cessation of the treatment. This route, therefore, could be ruled out.

The second is ER membrane proteins are released to the cytosol, then degraded either directly in the cytosol compartment or further sequestered to be degraded in the autolysosomes. Sequestration of several cytosolic proteins in an autophagosome–autolysosome system was clearly shown by Kominami et al. (10). In fact, we found a very small amount of P-450(PB) in the cytosol fractions. The possible participation of this route, therefore, can not be neglected at the present time.

The third is that ER membranes are directly segregated and then degraded in the autophagosome–autolysosome system. In this report we have demonstrated biochemical and immunoelectron microscopic evidences that convincingly show the existence of large amounts of P-450(PB) and FP2 associated with ER membranes in the autolysosomes.

Our results strongly suggest that the autophagosome–autolysosome system plays a very important role in the degradation of P-450(PB) and FP2. Recently, the half-lives of microsomal enzymes, including various molecular forms of P-450, have been reported by several investigators (18, 21, 24, 26, 27). The wide differences in the half-lives of microsomal enzymes, ranging from 3–4 h to >10 d were interpreted not to support the major contribution of the autophagic process to the degradation of ER enzymes in the liver cells (21, 24, 26, 27). This is because the autophagic digestion of the membranes as a whole will result in the uniform turnover rate of various microsomal enzymes if they are uniformly distributed in the ER membranes and no sorting of the membrane proteins occurs in the autophagic process.

A morphometric analysis of the content of the autolysosomes by Bolender and Weibel (3), however, showed that the ER membranes were preferentially segregated there and from this they concluded that the formation of autolysosomes was not a random process. It is possible that such a sorting mechanism also works at the level of the ER membrane proteins, preferentially segregating some membrane proteins, thus facilitating their degradation.

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