Topological Arrangement in Microsomal Membranes of Hepatic Haem Oxygenase Induced by Cobalt Chloride

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1. The microsomal haem oxygenase activity induced by the administration of CoCl_2 was found mainly in the smooth-surfaced microsomal fraction, whereas that of the untreated control animals was widely distributed in smooth-surfaced microsomal, rough-surfaced microsomal and Golgi fractions. 2. When microsomal preparation was incubated and the time course of the distribution of biliverdin between the membranes and the medium was followed, most of the biliverdin formed was found first in the medium. This suggests that the active site of haem oxygenase is exposed on the cytoplasmic surface of the membranes. The possible localization of the enzyme at the outer surface of the membranes was also supported by a digestion experiment with trypsin. The haem oxygenase activity was greatly decreased even at low concentration of the proteinase, which did not affect the NADPH–cytochrome *c* reductase activity. 3. When microsomal preparation was further fractionated by isopycnic centrifugation in the presence of deoxycholate or by partitioning of sonicated microsomal preparation in aqueous-polymer two-phase systems, most of the haem oxygenase activity was found in a fraction different from the main fraction of the NADH- and NADPH–cytochrome *c* reductase and NADH–ferricyanide reductase activities. This indicates the different distribution of haem oxygenase from the other enzymes mentioned, on the lateral plane of microsomal membranes, and suggests the different localization of the haem oxygenase system from the electron-transport system linked with cytochrome *b*₅ and cytochrome *P*-450.

The initial step in the catabolism of both endogenous and exogenous haem is catalysed by the haem oxygenase system present in microsomal fraction of spleen, liver and other tissues (Tenhunen *et al.*, 1968; Maines & Kappas, 1974; Gerns *et al.*, 1974; Raffin *et al.*, 1974). Immunochemical and kinetic studies reported in the preceding paper (Hino & Minakami, 1979) have shown that the same haem oxygenase system, consisting of NADPH–cytochrome *c* reductase (EC 1.6.2.4) and haem oxygenase (EC 1.14.99.3), catalyses both the NADPH-dependent and NADH-dependent haem catabolism in the microsomal preparation *in situ*.

Many microsomal enzymes have been known to be distributed asymmetrically and heterogeneously in microsomal membranes (Depierre & Dallner, 1975), which makes it possible to exert a regional control of the enzyme functions. Thus the topological information about the haem oxygenase system seems to be of some help for the elucidation of the control mechanism of haem catabolism. In this paper, we studied the submicrosomal, transverse and lateral distribution of the rat liver microsomal haem oxygenase system induced by CoCl_2 administration. We showed that the induced haem oxygenase activity was found

mainly in the smooth-surfaced microsomal fraction, that the active site of haem oxygenase was exposed to the cytoplasmic surface of the membranes, and that the lateral distribution of haem oxygenase was different from that of NADPH–cytochrome *c* reductase.

Experimental

Treatment of animals and subcellular fractionation

Administration of CoCl_2 to rats and the preparation of microsomal fraction were carried out as described in the preceding paper (Hino & Minakami, 1979). Rough- and smooth-surfaced microsomal fractions were obtained by layering the supernatant (9000g, 15 min, r_{av} 8.25 cm) over 1.23 M-sucrose solution and centrifuged as described by Rothschild (1963). Golgi fraction was prepared from the liver homogenate, which was obtained by pressing liver mince through a stainless-steel sieve (100 mesh), as described previously (Hino *et al.*, 1978a), and the microsomal fraction prepared from the same homogenate was used for comparison. Particulate preparations thus obtained were suspended in excess of ice-cold 0.15 M-KCl, recovered by centri-

fugation at 77000g for 120min and resuspended in ice-cold water.

Analytical and assay methods

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard, and RNA was determined as described by Fleck & Munro (1962). NADH- and NADPH-cytochrome *c* reductase activities were assayed as described by Okuda *et al.* (1972) and Phillips & Langdon (1962) respectively, NADH-ferricyanide reductase (EC 1.6.99.3) activity was assayed as described by Mihara & Sato (1972) and glucose 6-phosphatase (EC 3.1.3.9) activity as described by Swanson (1955). Cytochrome *P*-450 was determined by the method of Omura & Sato (1964). Haem oxygenase activity was assayed by measuring the formation of biliverdin or bilirubin with NADPH at 0.5 mM (Hino & Minakami, 1979).

Distribution of biliverdin in the medium and in the membranes

A mixture containing 0.1M-potassium phosphate buffer, pH 7.4, 50 μ M-haematin, bovine serum albumin (1mg/ml) and 8.0mg of microsomal protein, in a final volume of 6.0ml, was preincubated at 37°C for 5min and the reaction was started by adding 0.06ml of 50mM-NADPH. After a given time of incubation, the reaction was stopped by the addition of 0.3mM-HgCl₂ and cooled in ice. The reaction mixture was then centrifuged at 100000g (Hitachi 55P, RP65TA rotor, r_{av} . 5.6cm) for 90min at 2°C to separate the membranes from the medium. The medium (about 6.2ml) was acidified by adding 0.8ml of 80% (w/v) trichloroacetic acid, and biliverdin was extracted with 3 $\times$ 4ml of diethyl ether/ethanol (4:1, v/v). Membrane fraction obtained by the centrifugation was suspended in about 1ml of water and was acidified by adding 0.5ml of 20% (w/v) trichloroacetic acid. Biliverdin was extracted with 3 $\times$ 2ml of the organic solvent. The pooled extracts were condensed by evaporation *in vacuo* and the volumes were adjusted to 1.5ml with ethanol. Biliverdin was measured by the A_{690} – A_{800} difference as described in the preceding paper (Hino & Minakami, 1979).

Trypsin digestion of the microsomal preparation

A mixture containing 50mM-potassium phosphate buffer, pH 7.4, 10.4mg of microsomal protein and various amounts of trypsin (less than 10 μ g), in a final volume of 1.0ml, was incubated at 30°C for 1h and then cooled in ice. The reaction mixture was diluted to 9ml with ice-cold 10mM-potassium phosphate buffer, pH 7.4, and centrifuged at 105000g (Hitachi RP40A rotor, r_{av} . 5.9cm) for 90min at 2°C. The precipitate was suspended in ice-cold water and the remaining activities of NADH- and NADPH-

dependent haem oxygenase and NADPH-cytochrome *c* reductase were determined. The concentrations of NADH and NADPH used for the haem oxygenase reaction were 1.0 and 0.5mM respectively.

Isopycnic centrifugation of microsomal preparation in a sucrose density gradient containing deoxycholate

Microsomal preparation was centrifuged in a sucrose density gradient containing low concentrations of deoxycholate by the method of Winqvist & Dallner (1976), as follows. Microsomal fraction suspended in water (3ml) was layered on a linear sucrose density gradient (0.36–1.7M; 28ml) that contained various concentrations of deoxycholate. After centrifugation at 64000g for 48h at 2°C in a swing-out rotor (Hitachi RPS 25, r_{av} . 9.1cm), the five distinct bands formed were separately collected by Pasteur pipettes. Protein concentration and enzyme activities of each fraction were determined and the distribution was expressed as the percentage of each activity in the total activity recovered. The percentages recovered of the added activities were 79% for haem oxygenase, 99% for NADPH-cytochrome *c* reductase, 85% for NADH-ferricyanide reductase and 60% for NADH-cytochrome *c* reductase.

Partition in an aqueous-polymer two-phase system

Microsomal fraction suspended in water at a concentration of 5.2mg of protein/ml was sonicated for 90s in a Tomy model UR-150P sonicator and partitioned in an aqueous-polymer two-phase system (Albertsson, 1971). The recovery of the haem oxygenase activity after the sonication was 73%, whereas other enzyme activities were not affected by the treatment. A two-phase system was prepared in a test tube by mixing various amounts of poly(ethylene glycol) 4000 and dextran T500, containing 0.10g of 0.4M-Tris/maleate buffer, pH 7.0, 0.50g of the sonicated microsomal suspension and water to make the final weight of the system 4.20g. The concentrations of two polymers were the same, and a series of phase systems, in which the concentration of each polymer varied from 0.57 to 0.65% (w/w), was used. The phase system was equilibrated at 4°C and the contents were mixed vigorously. Each system was then centrifuged for 10min at 1000g to give complete phase separation. The partition behaviour of each enzyme activity was expressed as the percentage of the activity at the top phase in the total activity recovered after the separation. The total recovery of the haem oxygenase activity after the separation was about 80%, whereas other activities were essentially not affected by the treatment.

Chemicals

Dextran T500 (lot no. 7693) and haemin (bovine, type I) were purchased from Sigma Chemical Co.,

St. Louis, MO, U.S.A., and poly(ethylene glycol) 4000 (lot no. TCK 0759) was from Wako Pure Chemical Industries, Osaka, Japan. NADPH and NADH were purchased from Oriental Yeast Co., Tokyo, Japan, horse heart cytochrome *c*, glucose 6-phosphate and trypsin from Boehringer und Söhne, Mannheim, Germany, and bovine serum albumin (fraction V) was from Armour Pharmaceutical Co., Kankakee, IL, U.S.A. All other reagents used were of analytical grade.

Results

Submicrosomal distribution of haem oxygenase activity induced by the administration of CoCl₂

Because no data are available on the submicrosomal distribution of microsomal haem oxygenase, we surveyed the distribution of hepatic haem oxygenase activity in untreated control rats and in rats treated with CoCl₂. Table 1 shows the distribution of RNA, cytochrome *P*-450 and the haem oxygenase activity in submicrosomal fractions. Low but significant haem oxygenase activity (about 4 nmol of bilirubin/h per mg of protein) was detected in the microsomal fraction of untreated control rats and it was rather uniformly distributed in smooth-surfaced and rough-surfaced microsomal subfractions. Specific contents of RNA in rough- and smooth-surfaced microsomal fractions showed a proper separation of these subfractions. Recoveries of haem oxygenase activity and cytochrome *P*-450 in the microsomal fraction were essentially the same, suggesting similar subcellular localization of both proteins.

After treatment with CoCl₂, the haem oxygenase activity in whole microsomal fraction increased about

4-fold (to 17 nmol of bilirubin/h per mg of protein). Most of the induced haem oxygenase activity was found in the smooth-surfaced microsomal fraction, where the activity increased about 7-fold (to 30.1 nmol of bilirubin/h per mg of protein) compared with the control value, but the increase in the rough-surfaced microsomal fraction was not significant. The total activity of haem oxygenase recovered in the smooth-surfaced microsomal fraction was about 76% of that of whole microsomal fraction in CoCl₂-treated rats, whereas it was only 35% in untreated control rats. Although the content of cytochrome *P*-450 was decreased by the CoCl₂ treatment (Tephly & Hibbeln, 1971; Yasukochi *et al.*, 1977), the submicrosomal distribution pattern of the cytochrome in the CoCl₂-treated animals was similar to that of untreated controls.

Because a close functional and structural relationship exists between Golgi apparatus and endoplasmic reticulum, and smooth-surfaced microsomal fraction contains Golgi-derived vesicles, we compared the haem oxygenase activities in the Golgi and microsomal fractions isolated from the same liver homogenate (Table 2). The specific activity of haem oxygenase in the Golgi fraction of CoCl₂-treated rats was about 22% of that in the microsomal fraction, whereas the specific activity in the Golgi fraction of untreated control rats was about 48% of that in the microsomal fraction. Although the specific activities of NADPH-cytochrome *c* reductase, NADH-cytochrome *c* reductase and glucose 6-phosphatase in the Golgi fraction were in the range 22–25%, this does not mean that the Golgi fraction was contaminated with smooth endoplasmic reticulum to this extent (Hino *et al.*, 1978b). Therefore at

Table 1. *Submicrosomal distribution of haem oxygenase activity, cytochrome P-450 and RNA*

Microsomal preparation was subfractionated by the method of Rothschild (1963) and haem oxygenase activity was measured by the formation of bilirubin. The values given are means $\pm$ s.d. of three preparations for control rats and seven preparations for CoCl₂-treated rats.

Fractions	Haem oxygenase		Cytochrome <i>P</i> -450		RNA specific content (μ g/mg of protein)
	Activity (nmol of bilirubin/h per g wet wt. of liver)	Specific activity (μ mol of bilirubin/h per mg of protein)	Content (nmol/g wet wt. of liver)	Specific content (nmol/mg of protein)	
Untreated rats					
Homogenate	0.15 $\pm$ 0.08	1.3 $\pm$ 0.6	30.6 $\pm$ 11.2	0.28 $\pm$ 0.11	79 $\pm$ 16
Whole microsomal	0.09 $\pm$ 0.05	4.7 $\pm$ 2.4	15.9 $\pm$ 6.6	0.83 $\pm$ 0.34	238 $\pm$ 79
Rough microsomal	0.03 $\pm$ 0.02	4.0 $\pm$ 2.0	4.0 $\pm$ 2.1	0.63 $\pm$ 0.24	316 $\pm$ 10
Smooth microsomal	0.03 $\pm$ 0.01	4.2 $\pm$ 2.0	7.5 $\pm$ 4.1	0.93 $\pm$ 0.64	29 $\pm$ 1
CoCl ₂ -treated rats					
Homogenate	0.97 $\pm$ 0.4	7.3 $\pm$ 1.9	14.9 $\pm$ 4.2	0.12 $\pm$ 0.04	87 $\pm$ 4
Whole microsomal	0.36 $\pm$ 0.1	17.4 $\pm$ 4.7	6.3 $\pm$ 2.6	0.39 $\pm$ 0.22	158 $\pm$ 25
Rough microsomal	0.06 $\pm$ 0.04	7.8 $\pm$ 5.6	1.74 $\pm$ 1.2	0.24 $\pm$ 0.18	415 $\pm$ 12
Smooth microsomal	0.28 $\pm$ 0.12	30.1 $\pm$ 13.2	4.5 $\pm$ 1.2	0.66 $\pm$ 0.29	45 $\pm$ 12

Table 2. *Activities of haem oxygenase and microsomal marker enzymes in microsomal and Golgi fractions*
Golgi and whole microsomal fractions were prepared from the same homogenate by the method of Hino *et al.* (1978a) and haem oxygenase activity was measured by the formation of biliverdin. The values given are either means with individual observations in parentheses or means $\pm$ s.d. with the numbers of preparations in parentheses.

Fraction	Haem oxygenase (nmol of bilirubin/h per mg of protein)	NADPH-cytochrome <i>c</i> reductase (μ mol/min per mg of protein)	NADH-cytochrome <i>c</i> reductase (μ mol/min per mg of protein)	Glucose 6-phosphatase (μ mol/min per mg of protein)
Untreated rats				
Microsomal	5.52 (4.2, 6.83)	0.25 (0.23, 0.26)	0.84 (0.88, 0.79)	0.11
Golgi	2.63 (2.2, 3.06)	0.051 (0.060, 0.042)	0.209 (0.142, 0.276)	0.027
CoCl ₂ -treated rats				
Microsomal	27.8 $\pm$ 4.5 (4)	0.20 $\pm$ 0.01 (4)	0.84 $\pm$ 0.05 (4)	0.16 (0.20, 0.12)
Golgi	6.2 $\pm$ 1.6 (4)	0.039 $\pm$ 0.003 (4)	0.17 $\pm$ 0.03 (4)	0.033 (0.023, 0.041)

Fig. 1. *Distribution of biliverdin in the medium and in the membranes as a function of incubation time*

Microsomal preparation was incubated with haemin, and biliverdin in the medium (○) and in the membranes (●) was determined.

least a part, if not all, of the haem oxygenase found in the Golgi fraction may be indigenous to Golgi apparatus. Although the haem oxygenase activity in the Golgi fraction was increased 2.4-fold by the CoCl₂ treatment, the contribution of the Golgi apparatus to the total activity of hepatic haem oxygenase seems to be of little importance, because the amount of Golgi protein is less than 10% of microsomal protein.

Localization of biliverdin formed by the microsomal haem oxygenase system

This experiment was designed to study the transverse localization of haem oxygenase in microsomal membranes. If the conversion of haem into biliverdin takes place on the outer surface of the membranes, the biliverdin formed should be released directly into the medium. On the contrary, if the conversion takes place in the membranes or on the inside surface of

the membranes, the biliverdin formed should appear first in the membrane fraction rather than in the medium.

Fig. 1 shows the time course of the biliverdin distribution in the medium and in the membrane fraction. Biliverdin appeared first in the medium, but the concentration in the medium reached a plateau within 10 min, probably owing to the limited solubility of biliverdin in aqueous medium. The appearance of biliverdin in the membrane fraction was initially slower than that in the medium, though biliverdin continued to accumulate in the membrane

Fig. 2. *Effects of trypsin digestion on the activities of haem oxygenase and NADPH-cytochrome c reductase*

Microsomal preparation was treated with various amounts of trypsin as indicated on the abscissa and the remaining enzyme activities were measured. ○, NADPH-dependent haem oxygenase (biliverdin formation); ●, NADPH-cytochrome c reductase.

Fig. 3. *Isopycnic centrifugation of microsomal preparation in a sucrose density gradient containing deoxycholate* Microsomal preparation was subjected to isopycnic centrifugation in a linear sucrose density gradient (0.36–1.7 M) containing deoxycholate. Five bands (1–5, from top to bottom) were separately collected and the enzyme activities in each band were measured. The distribution pattern was expressed as the percentage of each activity in the total activity recovered. The amounts of microsomal protein and concentrations of deoxycholate used were (a) 20 mg, 0.18 %, (b) 30 mg, 0.18 %, and (c) 30 mg, 0.10 %, respectively. ○, NADPH-dependent haem oxygenase (bilirubin formation); ●, NADPH-cytochrome *c* reductase; △, NADH-cytochrome *c* reductase; ▲, NADH-ferricyanide reductase.

fraction even after the increase of biliverdin in the medium virtually stopped. Biliverdin in the membrane fraction may be regarded as being due to the adsorption of the hydrophobic compound from the medium into the lipid portion of the membrane during incubation. The observation may imply that biliverdin formed by the microsomal haem oxygenase system is released directly into the medium from haem oxygenase, whose active site is localized on the outer surface of the membranes. This idea was further supported by the following experiment involving trypsin digestion.

Effect of trypsin digestion on the components of the microsomal haem oxygenase system

Fig. 2 shows the effect of trypsin digestion on the NADPH-dependent microsomal haem oxygenase

and NADPH-cytochrome *c* reductase activities. The haem oxygenase activity was more susceptible to trypsin digestion than the reductase was. The former activity was greatly decreased (30 % of control) by the treatment, even at a low concentration of trypsin (0.1 µg/mg of protein), which did not affect the latter activity, indicating that the inactivation of the former activity is not due to the inactivation of the latter, but that haem oxygenase itself is attacked by trypsin. Because trypsin cannot penetrate into the microsomal vesicles and the vesicles remain closed even after the trypsin digestion (Ito & Sato, 1969), it is likely that trypsin acts only on the outer surface of the membranes. This again indicates that haem oxygenase is exposed on the cytoplasmic surface of microsomal membranes. The NADH-dependent haem oxygenase activity was inactivated by trypsin similarly to the NADPH-dependent reaction, whereas the NADH-

ferricyanide reductase activity was hardly affected by the treatment (results not shown).

Isopycnic centrifugation of microsomal preparation in a sucrose density gradient containing low concentrations of deoxycholate

The experiment was undertaken to obtain information about the distribution of the haem oxygenase system in the lateral plane of microsomal membranes. Microsomal preparation was subfractionated by centrifugation in a sucrose density gradient containing deoxycholate by the method of Winqvist & Dallner (1976), and the five subfractions obtained were analysed for haem oxygenase, NADPH-cytochrome *c* reductase and other activities. Fig 3 shows the distribution pattern of various enzyme activities among these subfractions, at three different protein/deoxycholate ratios. When suitable protein/deoxycholate ratios were used (Figs. 3*a* and 3*b*), the haem oxygenase activities were distributed differently from the activities of NADPH-cytochrome *c* reductase, NADH-cytochrome *c* reductase and NADH-ferricyanide reductase, indicating that the lateral distribution of haem oxygenase was different from that of NADPH-cytochrome *c* reductase as well as those of NADH-cytochrome *c* reductase and NADH-ferricyanide reductase. This conclusion was further supported by the following partition experiment in an aqueous-polymer two-phase system.

Fig. 4. Partition behaviour of haem oxygenase activity in an aqueous-polymer two-phase system

Sonicated microsomal preparation was partitioned in a two-phase system containing poly(ethylene glycol) and dextran. The concentrations of the two polymers were the same and the concentration of one component is given on the abscissa. The enzyme activity in the top phase is given on the ordinate as the percentage in the total activity recovered. ○, NADPH-dependent haem oxygenase (bilirubin formation); ●, NADPH-cytochrome *c* reductase; △, NADH-cytochrome *c* reductase; ▲, NADH-ferricyanide reductase.

Partition behaviour of various enzyme activities in an aqueous-polymer two-phase system

When sonicated microsomal preparation was added to a mixture of poly(ethylene glycol) and dextran, the fragments were partitioned in the top phase, bottom phase or at the interface according to their surface properties. Fig. 4 shows the percentages of various enzyme activities partitioned in the top phase of aqueous two-phase systems of various polymer concentrations as indicated on the abscissa. The partition behaviour of the haem oxygenase activity was different from those of the NADPH-cytochrome *c* reductase, NADH-cytochrome *c* reductase and NADH-ferricyanide reductase activities. This again indicates that haem oxygenase was distributed in the lateral plane of microsomal membranes differently from NADPH-cytochrome *c* reductase, NADH-cytochrome *c* reductase and NADH-ferricyanide reductase.

Discussion

The following three conclusions about the distribution of haem oxygenase are obtained from the results presented in this paper: (1) the haem oxygenase activity induced by the administration of CoCl_2 is present mainly in smooth-surfaced endoplasmic reticulum; (2) the active site of haem oxygenase is exposed on the outer or cytoplasmic surface of microsomal membranes, and (3) the distribution of the enzyme in the lateral plane of microsomal membranes is different from that of NADPH-cytochrome *c* reductase.

We observed that the haem oxygenase activity in smooth-surfaced microsomal fraction was increased several-fold by the CoCl_2 treatment, whereas that in the rough-surfaced microsomal fraction was hardly increased. When the Golgi fraction was examined, the activity in the fraction was not induced so much by the treatment as that in microsomal fraction. Thus it is reasonable to assume that smooth-surfaced endoplasmic reticulum, the main constituent of smooth-surfaced microsomal fraction, is the site of the induced haem oxygenase. This is in contrast with the basal activity of haem oxygenase of control untreated rats: it was distributed rather uniformly in rough- and smooth-surfaced microsomal fractions. The basal activity in Golgi fraction was about half of that in the microsomal fraction, but we consider at least a part of this activity to be inherent in the Golgi apparatus. The second conclusion, that the active site of haem oxygenase is exposed on the cytoplasmic surface of the microsomal membrane, was obtained from the finding that biliverdin formed by the enzyme system appeared first in the medium and that the enzyme activity was greatly inactivated by trypsin digestion even at low concentration of the proteinase,

which did not affect NADPH-cytochrome *c* reductase.

Finally, we conclude that the distribution of haem oxygenase in the lateral plane of microsomal membranes is different from that of NADPH-cytochrome *c* reductase. Even very little of the reductase in the vicinity of the oxygenase may be sufficient for an efficient provision of reducing equivalents. It has been well documented that microsomal vesicles are biochemically heterogeneous and that the NADH- and NADPH-driven electron-transport chains are partially separated from one another. Extensive subfractionation studies have shown that the components of a given chain are generally distributed in a similar manner (Depierre & Dallner, 1975). For instance, the components of the NADPH-driven electron-transport chain, NADPH-cytochrome *c* reductase and cytochrome *P*-450, are known to be concentrated into the same subfraction. Thus, taken together with the available evidence reported to date (Depierre & Dallner, 1975; Winqvist & Dallner 1976), the distribution of the haem oxygenase system on the microsomal membranes seems to be different from those of other electron-transport systems which involve cytochrome *b*₅ and cytochrome *P*-450.

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