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INTERACTION BETWEEN NADPH-CYTOCHROME *P*-450 REDUCTASE AND HEPATIC MICROSOMES *

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Summary

Solubilized NADPH-cytochrome *P*-450 reductase has been purified from liver microsomes of phenobarbital-treated rats. When added to microsomes, the reductase enhances the monoxygenase, such as aryl hydrocarbon hydroxylase, ethoxycoumarin *O*-dealkylase, and benzphetamine *N*-demethylase, activities. The enhancement can be observed with microsomes prepared from phenobarbital- or 3-methylcholanthrene-treated, or non-treated rats. The added reductase is believed to be incorporated into the microsomal membrane, and the rate of the incorporation can be assayed by measuring the enhancement in ethoxycoumarin dealkylase activity. It requires a 30 min incubation at 37°C for maximal incorporation and the process is much slower at lower temperatures. The temperature affects the rate but not the extent of the incorporation. After the incorporation, the enriched microsomes can be separated from the unbound reductase by gel filtration with a Sepharose 4B column. The relationship among the reductase added, reductase bound and the enhancement in hydroxylase activity has been examined. The relationship between the reductase level and the aryl hydrocarbon hydroxylase activity has also been studied with trypsin-treated microsomes. The trypsin treatment removes the reductase from the microsomes, and the decrease in reductase activity is accompanied by a parallel decrease in aryl hydrocarbon hydroxylase activity. When purified reductase is added, the treated microsomes are able to gain aryl hydrocarbon hydroxylase activity to a level comparable to that which can be obtained with normal microsomes. The present study demonstrates that purified NADPH-cytochrome *P*-450 reductase can be incorporated into the microsomal membrane and the incorporated reductase can interact with the cytochrome *P*-450 molecules in the membrane, possibly in the same mode as

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the endogenous reductase molecules. The result is consistent with a non-rigid model for the organization of cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase in the microsomal membrane.

Introduction

The NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450 are embedded in the endoplasmic reticulum of mammalian cells, being most prevalent in the liver. These enzymes constitute the mixed function oxidase or monoxygenase system. It catalyzes the biotransformation of steroids, fatty acids, drugs, carcinogens and other xenobiotics [1—3]. The reductase * mediates the electron transfer from NADPH to cytochrome *P*-450 which in turn catalyzes the oxygenation of various substrates. In rat liver microsomes, the number of the cytochrome *P*-450 molecules can be 20—30 times greater than that of the reductase molecules [4]. In the absence of a known mobile electron carrier between these two enzymes, it appears that one reductase molecule has to interact with many cytochrome *P*-450 molecules for efficient catalysis. The mode of electron transfer between these two proteins has been investigated by many investigators and has been recently reviewed [5,6]. Since the enzymic oxygenation requires the presence of these two enzymes and phospholipids, the microsomal monoxygenase system has provided an interesting system for studying protein-protein and protein-lipid interactions in the membrane.

Previously, we have demonstrated that solubilized cytochrome *P*-450 can be incorporated into the microsomal membrane. The exogenous cytochrome *P*-450 can be reduced enzymically and can catalyze monoxygenase reactions [7,8]. In the present study, we extend our investigation to the interaction of purified reductase with the microsomal membrane. The rate and extent of the incorporation as well as the effects of the incorporated reductase on the monoxygenase activity were studied. The results are discussed in relationship to the structural organization of the monoxygenase system and the rate-limiting steps of monoxygenase reactions.

Materials and Methods

Chemicals. Trypsin, soybean trypsin inhibitor, and adenosine 2′-monophosphate (2′-AMP) were purchased from Sigma Chemical Co. 3-Methylcholanthrene was from Mann Research Laboratory. Phenobarbital was a gift from Merck and Co. Agarose-hexane-adenosine 2′,5′-diphosphate, Type 2, was obtained from PL Biochemicals, Inc. Ultrogel AcA34 was purchased from LKB Instruments, Inc. Polyethylene glycol 6000 was from Baker Chemical Co. The sources of otherc hemical have been reported previously [8,9].

* NADPH-cytochrome *P*-450 reductase is also known as NADPH-cytochrome *c* reductase; it will be referred to as the reductase to avoid excessive wording. Other abbreviations used are: 2′-AMP, adenosine 2′-monophosphate; MC, PB, or control microsomes, liver microsomes prepared from 3-methylcholanthrene- or phenobarbital-treated, or non-treated rats, respectively.

Preparation of microsomes. Hepatic microsomes were prepared from Long-Evans rats (body weight 70—125 g) which had received a daily intraperitoneal injection of 3-methylcholanthrene (in corn oil, 25 mg/kg body weight) or phenobarbital (in saline, 75 mg/kg body weight) for 4 days, or no treatment. They will be referred to as MC, PB, or control microsomes, respectively. The microsomes were prepared by a conventional differential centrifugation method and stored as described previously [10].

Purification of NADPH-cytochrome P-450 reductase. The enzyme was prepared from PB microsomes by a procedure derived from that of Yasukochi and Masters [11] with substantial modifications. It involved the following steps: (1) Microsomes were solubilized by sodium cholate (3 mg per mg of microsomal protein) at room temperature in a 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 30% glycerol. Usually, 3—4 g of microsomes (protein) were used for one preparation and the final volume of the solubilization mixture was 500—700 ml. (2) The mixture was subjected to polyethylene glycol fractionation and different fractions were used for the preparation of the reductase and cytochrome *P*-450. Most of the cytochrome *P*-450 and many other proteins were precipitated by 16% (w/v) of polyethylene glycol. Approx. 50% of the reductase activity was present in the supernatant which was used as the starting material for the purification of the reductase. (3) The supernatant was applied to a DEAE-cellulose column (4.4 × 15 cm) previously equilibrated with 25 mM Tris · HCl buffer, pH 7.7, containing 0.8% Emulgen 911, 0.1% sodium cholate, 0.05 mM EDTA and 0.05 mM dithiothreitol. Alternatively, the reductase was adsorbed on DEAE-cellulose in a batchwise procedure, and the DEAE-cellulose was packed into a column. The column was washed with 400 ml of equilibration buffer and the reductase was eluted with the same buffer containing 0.35 M KCl. (4) The reductase fractions were pooled and concentrated with a Diaflow (Amicon) system. The sample was subjected to affinity chromatography according to the procedure of Yasukochi and Masters [11], except that an Agarose-hexane-adenosine 2′,5′-diphosphate column (1.5 × 11 cm) was used. The column was previously equilibrated with 10 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1% Emulgen 911, 0.02 mM EDTA and 0.2 mM dithiothreitol. The column was washed with 150 ml of 0.2 M potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1% Emulgen 911, 0.4 mM EDTA, and 0.2 mM dithiothreitol, and then with 20 ml of equilibration buffer. The reductase was eluted with 0.7 mM 2′-AMP in the equilibration buffer. (5) The reductase fractions were concentrated and passed through an Ultrogel AcA 34 column (2.5 × 40 cm) and then through a Sephadex LH-20 column (2.5 × 40 cm) to remove 2′-AMP, protein impurities, and detergents. The elution buffer contained 50 mM potassium phosphate, pH 7.4, and 20% glycerol.

The NADPH-cytochrome *P*-450 reductase activity was assayed using cytochrome *c* as an artificial electron acceptor at ambient temperature (approx. 22°C) by following ΔA_{550nm} with a Cary 17 spectrophotometer. The assay contained 40 nmol cytochrome *c*/0.2 μmol NADPH in a 0.3 M potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA. One unit of reductase activity is defined as that which reduces 1 μmol of cytochrome *c* per min.

Trypsin treatment of microsomes. The hydrophilic portion of the reductase

which contains the catalytic site of the enzyme was cleaved from the microsomal membrane by trypsin digestion. Usually, microsomes corresponding to 50 mg of protein were treated with 0.5 mg of trypsin in 5 ml of Buffer A (0.1 M potassium phosphate buffer (pH 7.4)/5 mM $MgCl_2$/0.1 mM EDTA) at 30°C. The progress of the digestion was assayed by measuring the aryl hydrocarbon hydroxylase activity with a direct fluorometric method [12]. The digestion was terminated by the addition of trypsin inhibitor and the mixture was diluted and centrifuged to sediment the microsomes. The remaining reductase activity and cytochrome *P*-450 content in the microsomes were then assayed.

Binding of the reductase to microsomes. The microsomes were stored frozen at −90°C and freshly thawed samples were used for the study. Usually the microsomes were sonicated briefly to break the aggregates to obtain a suspension of microsomal vesicles. Two types of binding studies were carried out: (1) Microsomes and the reductase were allowed to interact in the reaction flask or cuvette, and the binding was assayed by following the enhancement in catalytic activity. (2) Microsomes and the reductase were incubated at 37°C for 30 min, and the enriched microsomes were separated from the reductase by gel filtration with a Sepharose 4B column (1.5 × 40 cm) according to the precedure described in the legend to Fig. 5. Buffer A was used for elution. The precedure usually took about 2 h and the results were highly reproducible. In all these experiments, the amount of reductase in (or added to) the microsomes is expressed as unit/mg of microsomal protein.

Enzymic reduction of microsomal cytochrome P-450. The NADPH-dependent reduction of cytochrome *P*-450 was measured in an Aminco anaerobic cuvette. Microsomes (0.2—0.3 ml) were incubated with 0.15 ml of reductase solution (or 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol) under N_2 at 37°C for 20 min. The volume was made to 2.4 ml by the addition of 50 mM Tris · HCl buffer, pH 7.4, containing 150 mM KCl and 10 mM $MgCl_2$. The mixture was deoxygenated with N_2 and then gassed with CO for a total of 20 min. A stirring device was used to facilitate the equilibration. Both gases had been deoxygenated previously with an alkaline solution of anthraquinone-2-sulfonate and dithioninte. The reaction was initiated by the addition of 1.0 μmol of NADPH (in 25 μl) to the reaction cuvette. The $\Delta A_{490-450nm}$ was recorded with an Aminco DW-2a spectrophotometer at 25°C.

Determinations of protein and cytochrome P-450. Protein was determined by the method of Lowry et al. [13] with bovine serum albumin as the standard. The cytochrome *P*-450 content was determined by the method of Omura and Sato [14] from the CO difference spectra of the reduced sample, using an extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ for $\Delta A_{490-450nm}$.

Assay of monoxygenase activities. Aryl hydrocarbon hydroxylase activity was usually assayed at 37°C according to the fluorometric method of Nebert and Gelboin [15] as described previously [19]. 3-Hydroxybenzo[a]pyrene was used as a standard [15] and the activity is expressed as nmol of product formed per min per mg of microsomal protein.

Ethoxycoumarin *O*-dealkylase was assayed by the direct fluorometric method of Ullrich and Weber [16]. The amount of 7-hydroxycoumarin

produced was measured by following the fluorescence at 460 nm (excitation at 365 nm) with a Perkin-Elmer double beam recording spectrofluorometer (model 512).

Benzphetamine *N*-demethylase activity was assayed at 37°C by measuring the formaldehyde formed with procedures described previously [9]. The incubation system contained 50 mM Tris · HCl buffer, pH 7.5, 5 mM $MgCl_2$, 150 mM KCl, 5 mM benzphetamine, 10 mM isocitrate, 0.5 unit isocitrate dehydrogenase and microsomes (0.5 or 1 mg of protein) in 2 ml. The reaction was initiated with 1 μmol of NADPH, and the assay was carried out at 37°C for 10 min.

Results

Purification of NADPH-cytochrome P-450 reductase

The reductase prepared by the present method had a specific activity of 30 units per mg of protein when assayed at 22°C. Analysis with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a discontinuous system [17] indicated that almost all the proteins moved as one band corresponding to a polypeptide with a molecular weight of about 79 000. A faint impurity band with a slightly lower molecular weight was also detected and the protein in this band probably accounted for less than 5% of that in the major band. As judged from the absorption spectrum, the enzyme preparation did not contain any detectable amount of heme but still contained some Emulgen 911, which has an absorption maximum at 275 nm. The presence of the detergent, however, did not create any problems for this study. The properties of the present reductase preparations appeared to be similar to those reported by Yasukochi and Masters [11], although the yield (10—15% from microsomes) was lower. This yield was acceptable, since the same microsomal sample also provided starting material for the purification of cytochrome *P*-450.

Elimination of the microsomal reductase and aryl hydrocarbon hydroxylase activities by trypsin digestion

As shown in Fig. 1, the trypsin treatment gradually cleaved the active site portion of the reductase from the microsomal membrane. Within 5 min of incubation, very little destruction of cytochrome *P*-450 was observed and the extent of decrease in microsomal aryl hydrocarbon hydroxylase activity was almost identical to that of the reductase. The experiment demonstrates that the aryl hydrocarbon hydroxylase activity is directly proportional to the reductase activity. It also establishes the validity of using the aryl hydrocarbon hydroxylase activity as an assay for the cleavage of the reductase from the microsomal membrane in the preparation of trypsin-treated microsomes for subsequent studies.

Enhancement of microsomal monoxygenase activities by exogenous reductase

When MC microsomes were preincubated with purified reductase, the aryl hydrocarbon hydroxylase activity increased (Fig. 2). A reductase-dependent enhancement in microsomal benzphetamine *N*-demethylase activity was also observed in PB microsomes. In both cases a 3.5—4-fold enhancement was

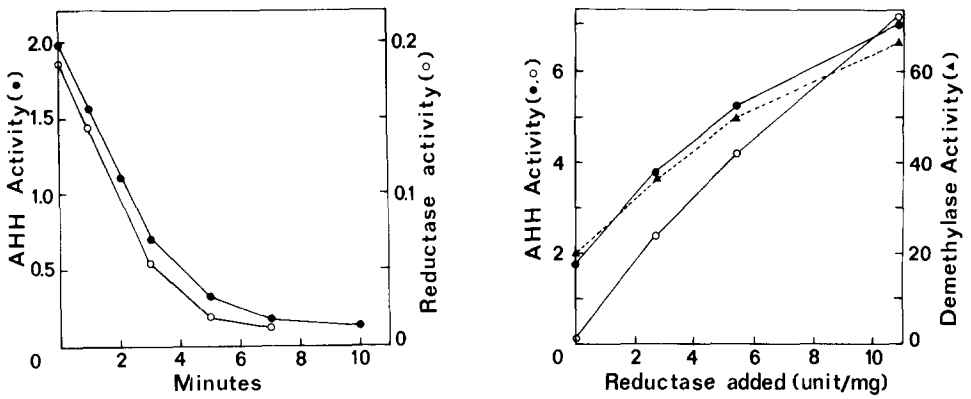

Fig. 1. Effects of trypsin digestion on microsomal reductase and aryl hydrocarbon hydroxylase (AHH) activities. MC microsomes (50 mg of protein) were treated with 0.5 mg of trypsin in 5 ml of Buffer A at 30°C. The digestion was terminated by transferring aliquots of the mixture into an ice-cold trypsin inhibitor solution. The aryl hydrocarbon hydroxylase activity (•———•), expressed in nmol/min per mg of microsomal protein, was assayed with a direct fluorometric method at 37°C. The disappearance of benzo[a]pyrene was assayed by measuring the fluorescence at 407 nm (excitation at 387 nm) with a recording spectrofluorometer [12]. The reductase activity in microsomes (○———○), expressed in units/mg of protein, was assayed after the microsomes had been sedimented by centrifugation at 105 000 × *g* for 60 min.

Fig. 2. Enhancement of microsomal monoxygenase activities by exogenous reductase. MC microsomes (•———•) or trypsin-treated MC microsomes (○———○) corresponding to 0.1 mg of protein were preincubated with purified reductase in 0.25 ml of Buffer A at 37°C for 30 min, and then more buffer and substrates were added for assaying the aryl hydrocarbon hydroxylase (AHH) activity. PB microsomes (▲- - - - -▲) corresponding to 0.5 mg of protein were preincubated with the reductase in a similar manner and then more buffer and substrate were added for assaying the benzphetamine demethylase activity. The enzyme activities are expressed in nmol/min per mg of microsomal protein, and the amounts of reductase added are expressed as unit/mg of microsomal protein.

obtained with 10.8 units of reductase added per mg of microsomal protein. The enhancement in aryl hydrocarbon hydroxylase activity was also observed with trypsin-treated MC microsomes. These microsomes had less than 10% of the original reductase and aryl hydrocarbon hydroxylase activities; but in the presence of added reductase (10.8 units/mg), their aryl hydrocarbon hydroxylase activity was at the same level as that attained by MC microsomes under similar conditions. These results were obtained with the same batch of reductase preparation. The treated and untreated MC microsomes were also of the same origin. When different preparations of enzyme and microsomes were used, the extent of the enhancement tended to vary but the same trend of enhancement was always observed.

Enhancement in the benzphetamine demethylase, aryl hydrocarbon hydroxylase, and ethoxycoumarin dealkylase activities was also observed with control, PB, and MC microsomes (Table I). A larger enhancement in the benzphetamine demethylase activity was observed with PB microsomes than with control microsomes, and the largest enhancement in aryl hydrocarbon hydroxylase or ethoxycoumarin dealkylase activity was obtained with MC microsomes. The results are consistent with the report that the phenobarbital-induced cytochrome *P*-450 is more efficient in catalyzing the *N*-demethylations; whereas the 3-methylcholanthrene-induced enzyme (cytochrome *P*-448) has higher aryl hydrocarbon hydroxylase and ethoxycoumarin dealkylase activities

TABLE I

EFFECTS OF EXOGENOUS REDUCTASE ON MICROSOMAL MONOXYGENASE ACTIVITIES

The assay conditions were similar to those in the legend of Fig. 2, except that the reductase was added at 9.4 units/mg of microsomal protein for the experiments on benzphetamine demethylase and aryl hydrocarbon hydroxylase activities; and was 4.6 units/mg for the study of ethoxycoumarin dealkylase activity. All activities were assayed at 37°C and the activities are expressed in nmol/min per mg.

	Benzphetamine demethylase	Aryl hydrocarbon hydroxylase	Ethoxy-coumarin dealkylase
Control microsomes	6.5	0.51	0.05
Control microsomes + reductase	15.7	1.02	0.08
PB microsomes	19.2	0.92	0.26
PB microsomes + reductase	63.5	1.84	0.30
MC microsomes	—	2.80	2.43
MC microsomes + reductase	—	7.42	6.21

than the cytochrome *P*-450 in control microsomes [8,18]. Since the latter two activities can be readily assayed by sensitive fluorometric methods, they were used in further studies on the interaction between the reductase and microsomes. The interaction can be easily assayed by the enhancement in dealkylase activities, especially when trypsin-treated microsomes are used.

Effects of exogenous reductase on microsomal ethoxycoumarin O-dealkylase activity

In the ethoxycoumarin dealkylase assay, the rate of 7-hydroxycoumarin formation was linear for about 5 min, and then slowed down. Upon addition of purified reductase to the assay mixture, a gradual increase in the reaction rate was seen and a maximum of 4-fold enhancement was observed (Fig. 3). This suggests a time-dependent incorporation of the reductase into the microsomal membrane. The effects of time and temperature on the enhancement are shown in Fig. 4. Trypsin-treated MC microsomes were preincubated with the reductase in a small volume at different temperatures, and the dealkylase activity was assayed at 20°C. At 37°C, it required about 30 min to acquire a maximal enhancement. The enhancement was not observed when the microsomes were incubated with Emulgen, at levels comparable to that introduced from the reductase sample. The result is consistent with the hypothesis that incorporation of the reductase into the microsomal membrane is required for the enhanced dealkylase activity. A much shorter preincubation time is expected, if the enhancement effect of the reductase was produced by mere binding to the surface of the microsomes. The rate of the incorporation was slower at 30°C and much slower at 20°C. At 0°C, little enhancement was observed after 50 min. After an overnight incubation in the cold room (approx. 5°C), however, the dealkylase activity was increased to a level attainable by a 30 min incubation at 37°C (data not shown). When the experiment was repeated with MC microsomes, a similar incorporation pattern was observed, although the extents of enhancement in the dealkylase were lower (data not shown). The results lend further support to the hypothesis that the enhanced dealkylase activity is manifested by the incorporated reductase.

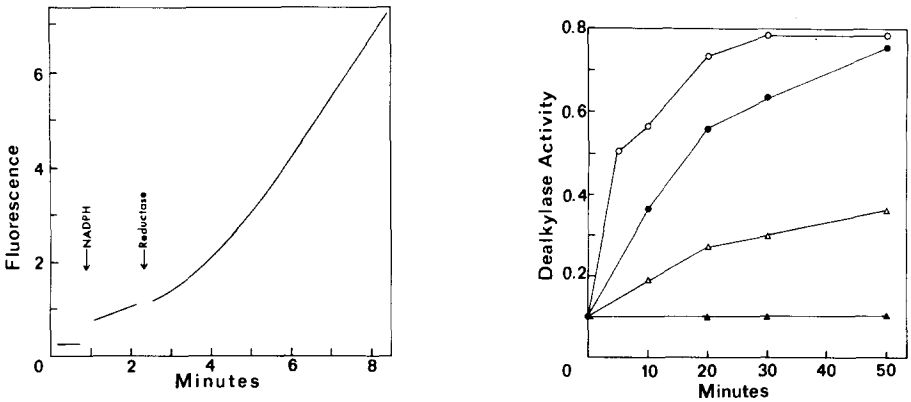

Fig. 3. Increase of ethoxycoumarin dealkylase activity by added reductase. The reaction mixture included trypsin-treated MC microsomes (containing 0.4 mg of protein, 0.84 nmol of cytochrome *P*-450, and 0.01 unit of reductase), 60 nmol of 7-ethoxycoumarin, 10 μmol of isocitrate, 1 unit of isocitrate dehydrogenase, 160 nmol of NADPH and Buffer A in a final volume of 2 ml. The reaction was allowed to proceed at 37°C, and then 3.6 units of reductase (in 0.1 ml) was added to observe the enhancement in dealkylase activity. The fluorescence is in arbitrary units.

Fig. 4. Temperature-dependent incorporation of the reductase as measured by the enhanced dealkylase activity. Trypsin-treated MC microsomes (containing 0.18 mg of protein, 0.29 nmol of cytochrome *P*-450 and 0.003 unit of reductase) were incubated with 0.5 unit of reductase in 0.22 ml of Buffer A in a cuvette at 37°C (○———○), 30°C (●———●), 20°C (△———△), or 0°C (▲———▲) for the periods indicated. Buffer A (at 20°C) and substrates were added, and the cuvette was equilibrated at 20°C for 2—3 min. The reaction was initiated with 80 nmol of NADPH and the rate of ethoxycoumarin dealkylase activity was measured at 20°C.

Binding of purified reductase to microsomes and its effect on catalysis

After incubation with purified reductase, the microsomes (enriched) were separated from the unbound reductase either by gel filtration with a Sepharose 4B column or by centrifugation at 105 000 × *g* for 60 min. The gel filtration method usually gave more reproducible results and was used in this series of experiments. The elution profile is shown in Fig. 5. The enriched microsomes usually eluted in fractions 21—23 and were clearly separated from the unbound reductase eluted in fractions 40—65. Similar elution profiles were also observed when higher amounts of reductase were used in the experiments. When the enriched microsomes were subjected to a second gel filtration with the same column, the enriched microsomes were again eluted in a peak centered around fraction 22.

The relationship between the reductase in the incubation and that which became bound to microsomes is shown in Fig. 6. The quantity of reductase bound depended on the amount of reductase in the incubation mixture, and a 10-fold increase in reductase was observed when 5.5 units of reductase were added per mg of microsomal protein. After passing the enriched microsomes through a second Sepharose 4B column, the reductase activity usually decreased by 5—10%, partially due to the denaturation of this enzyme during the experiment. This suggests that the enzyme is incorporated into or tightly bound to the microsomes and the binding is essentially irreversible at around 5°C. The effect of the bound reductase on microsomal aryl hydrocarbon hydroxylase activity is also shown in Fig. 6. The incorporated reductase can

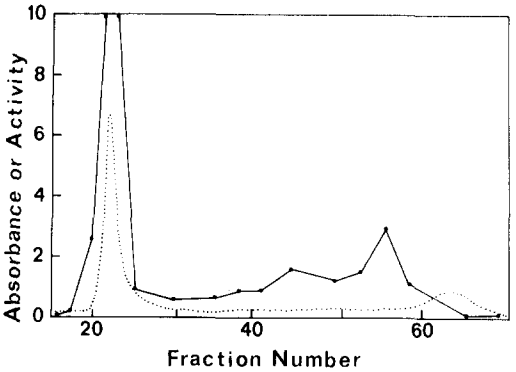

Fig. 5. Elution profiles of the reductase-enriched microsomes. MC microsomes (3 mg of protein) were incubated with 5.4 units of reductase in 0.33 ml at 37°C for 30 min. The sample was chilled, applied to a Sepharose 4B column (1.5 X 40 cm), and eluted with Buffer A. The gel filtration was performed at cold room temperature (approx. 5°C). 1-ml fractions were collected. The traces are: ●———●, reductase activity; and - - - - - -, A_{280nm}, both in arbitrary units.

effectively enhance the aryl hydrocarbon hydroxylase activity when the reductase level was below 1 unit per mg of microsomal protein; above that level the aryl hydrocarbon hydroxylase activity began to level off and reached a plateau at an aryl hydrocarbon hydroxylase level of 7.3 nmol/min per mg of

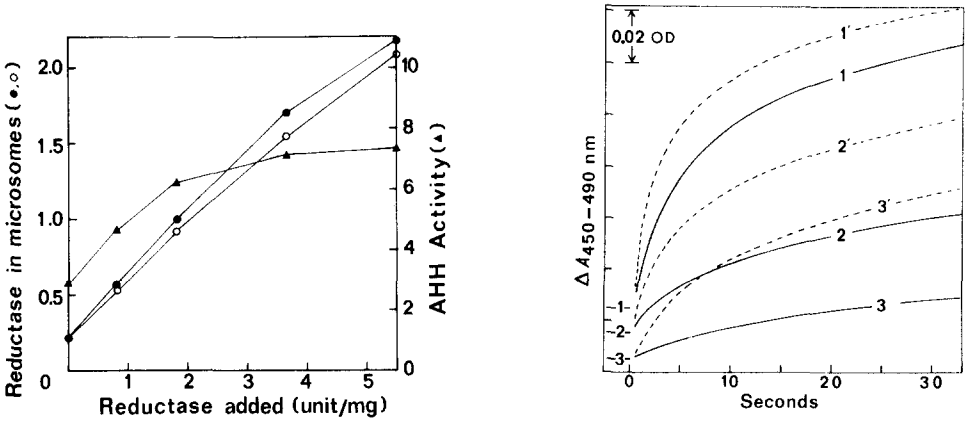

Fig. 6. Binding of reductase to microsomes and its effects on aryl hydrocarbon hydroxylase activity. MC microsomes were incubated with different amounts of reductase and the enriched microsomes were separated from the unbound reductase with a Sepharose 4B column using the conditions described in Fig. 5. The reductase and aryl hydrocarbon hydroxylase activities in the enriched microsomes are shown as ●———● and ▲———▲, respectively. The enriched microsomes were also subjected to a second chromatography with the Sepharose 4B column and the reductase activity is shown as ○———○.

Fig. 7. Enzymic reduction of microsomal cytochrome *P*-450. Three pairs of experiments (traces 1,1′; 2,2′; and 3,3′) are shown. In traces 1, 2, and 3 the reaction mixture contained PB microsomes (2.0 mg of protein and 4.8 nmol of cytochrome *P*-450), MC microsomes (3.0 mg of protein and 4.1 nmol of cytochrome *P*-450), and trypsin-treated MC microsomes (2.3 mg of proteins and 4.5 nmol of cytochrome *P*-450), respectively. In traces 1′, 2′, and 3′, the reaction mixture also contained 8.2 units of reductase in addition to the corresponding microsomes. Before the initiation of the reaction, the traces of each pair are shown as one horizontal line on the lower-left corner of the graph.

microsomal protein. It appears that when the reductase level is very high, the rate of the monoxygenase is limited by the quantity of cytochrome *P*-450.

Reduction of microsomal cytochrome P-450 by NADPH

In order to elucidate the mode of action of the exogenous reductase, the enzymic reduction of cytochrome *P*-450 was studied. In these experiments, an absolute anaerobic condition was not reached and the rate in the initial second of the reaction was not accurately measured, due to inherent problems of the anaerobic cuvettes. However, all the measurements were made under similar conditions and the results demonstrated that the rate of NADPH-dependent cytochrome *P*-450 reduction was accelerated by the added reductase in all three types of microsomes (Fig. 7). This result is consistent with the belief that the reductase enhances the monoxygenase by facilitating the electron transfer from NADPH to cytochrome *P*450.

Discussion

Similar to our previous studies with solubilized cytochrome *P*-450 [7,8], the present work demonstrated that purified NADPH-cytochrome *P*-450 reductase can also be incorporated into the microsomes and enhance monoxygenase activities. In a brief communication, Miwa and Cho [19] have reported the stimulation of microsomal *N*,*N*-dimethylamphetamine *N*-demethylase activity by solubilized reductase. During the preparation of this manuscript, Pokrovsky et al. [20] have also reported the binding of reductase to microsomes and the enhancement in benzo[a]pyrene hydroxylase (aryl hydrocarbon hydroxylase) activity. The present study confirms the binding and enhancement phenomena observed by these workers [19,20], and has characterized the process of incorporation as well as the mechanism of enhancement.

The increase in monoxygenase activities can be observed readily by preincubating the purified reductase with the microsomes before the enzyme assay. The enhancement is a general phenomenon observable with different types of monoxygenase activities and with microsomes that contain different forms of cytochrome *P*-450. The enhancement is even more dramatic with trypsin-treated MC microsomes which contained less than 10% of the original reductase and aryl hydrocarbon hydroxylase activities (Fig. 2). With the treated MC microsomes, the aryl hydrocarbon hydroxylase activity is almost directly proportional to the reductase added and can attain a level similar to that of the enriched MC microsomes. The result suggests that the exogenous reductase molecules function in a similar manner to the endogenous reductase molecules in the microsomes. Although enhancement in monoxygenase activities was observed with different types of microsomes, the extents of the enhancement were different. This indicates that the enhancement process is also dependent upon the catalytic activity of cytochrome *P*-450. It appears that when a sufficient amount of reductase is supplied, the rate of the monoxygenase reaction is limited by the turnover rate of cytochrome *P*-450 in the final step of the oxygenation reaction. Thus, a higher increase in benzphetamine *N*-demethylase activity is observed with PB microsomes than control microsomes, and the highest enhancement in aryl hydrocarbon hydroxylase or ethoxycoumarin

dealkylase activity is observed with MC microsomes. This interpretation is in agreement with our previous results on the effect of incorporated cytochrome *P*-450 on microsomal monoxygenase activities [8]. The enhancement observed in these studies appears to be due to the incorporation of the reductase into the microsomes and not due to the solubilization of the microsomal cytochrome *P*-450 by the detergent in the reductase preparation. The turbidity of the microsomes was not visually different before and after the preincubation, and the estimated amount of Emulgen 911 introduced into the system, less than 0.2 mg per mg of microsomal protein, was insufficient for the solubilization of microsomes. At the concentration used, this detergent is known to inhibit slightly the monoxygenase activity in microsomes [8].

The data in Figs. 3 and 4 suggested that the enhancement in monoxygenase activity is due to the temperature-dependent incorporation of the reductase into the membrane. The temperature affects the rate but not the extent of the incorporation. The results are similar to those observed previously on the incorporation of solubilized cytochrome *P*-450 into microsomes. A direct evidence of the incorporation or the tight binding of the reductase to microsomes came from the gel filtration experiments (Figs. 5 and 6). When a 25-fold excess of reductase is incubated with microsomes, a 10-fold increase in microsomal reductase activity is observed. This is a dramatic increase in terms of the reductase activity but represents only a 6 or 7% increase in protein in the microsomes. This level of reductase binding is higher than those reported by previous workers [19,20], possibly due to the following reasons: (1) The reductase preparation is different; the present reductase preparation is of a much higher purity. (2) The incubation conditions were different; the present work used smaller incubation volumes which favor the incorporation process (unpublished observation). Although the exogenous reductase molecules are catalytically active, it is not known whether they are all embedded in the membrane in a manner similar to the endogenous reductase molecules.

The relationship between the reductase content and the monoxygenase activity of microsomes is also illustrated in Figs. 1 and 2. When the reductase is removed from microsomes by trypsin digestion, a parallel decrease in aryl hydrocarbon hydroxylase activity follows: when purified reductase is added to microsomes, the aryl hydrocarbon hydroxylase activity increases. The data in Fig. 6, however, show that the increase reaches a limit with high levels of exogenous reductase. Under the latter conditions, it is likely that the rate of reaction is limited by the rate of turnover of cytochrome *P*-450 in the final stage of the oxygenation process. It appears that under normal physiological conditions, the rates of the monoxygenase reactions are dependent on the quantities (and catalytic activities) of both cytochrome *P*-450 and the reductase. This suggestion is consistent with the concept that there may be more than one slow step in the monoxygenase reaction and different rate-limiting steps may be involved under different conditions [6]. It also agrees with our previous results with riboflavin-deficient rats that the aryl hydrocarbon hydroxylase activity is dependent on the amounts of both cytochrome *P*-450 and the reductase in the microsomes [21].

The results in Fig. 7 indicate that the added reductase can increase the rate of the NADPH-dependent reduction of cytochrome *P*-450, and this increase

can roughly be correlated with the enhancement in benzphetamine demethylase and aryl hydrocarbon hydroxylase activities (Fig. 2). It appears that the increases in monoxygenase activities are due to the increased electron transfer from NADPH to cytochrome *P*-450. However, more precise kinetic measurements are required before this relationship can be firmly established. It has been proposed that the reductase can serve as an effector in facilitating the regeneration of free cytochrome *P*-450 in the last step of the oxygenation process [22]. Is it possible that the enhancement in monoxygenase activity is not due to the oxidoreductase function but due to its proposed roles as an effector? This is unlikely based on the results with trypsin-treated microsomes (Fig. 2), unless normally only 10% of the reductase is required to function as oxidoreductase and the majority of the enzyme plays the role of an effector.

The present observation that purified reductase can be incorporated into the microsomal membrane and interact with cytochrome *P*-450 in a manner similar to the endogenous reductase molecules is consistent with our previously proposed non-rigid model for the organization of the monoxygenase enzymes in the microsomal membrane [6,23]. In order for the exogenous and endogenous enzyme molecules to interact, lateral mobility of these enzymes in the membrane is required. The rate of this mobility remains to be determined.

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