NADPH-CYTOCHROME c REDUCTASE AND ITS ROLE IN MICROSOMAL CYTOCHROME P-450-DEPENDENT REACTIONS

BETTIE SUE SILER MASTERS,1 EDWARD B. NELSON, BRENT A. SCHACTER,2 JEFFREY BARON,3 AND ELIZABETH L. ISAACSON

University of Texas Southwestern Medical School at Dallas

The study of liver microsomal electron transport and drug metabolism activities involving NADPH-cytochrome c reductase has been performed using immunochemical techniques (1–4). This approach has been particularly valuable in establishing the role of this flavoprotein in the reduction of microsomal cytochrome P-450 and, thus, in the metabolism of drugs (2, 3). The existence of NADPH-cytochrome c reductase activity in a variety of tissues including kidney, spleen, adrenal cortex, heart, and lung has led to a closer examination of the role of this enzymic activity in cytochrome P-450-mediated reactions in these tissues.

It has been demonstrated by Masters and Ziegler (5) that the two NADPH-dependent electron transport systems in porcine liver microsomes contain separate and distinct flavoproteins (fig. 1): NADPH-cytochrome c reductase and NADPH-dependent mixed function amine oxidase (N-oxidase). This conclusion was based on the differential purification of the two enzymic activities, the induction of the reductase (but not N-oxidase) activity by phenobarbital pretreatment, the comparison of the physical properties of the purified enzymes, and, finally, the immunochemical nonidentity of the two enzymes, either purified or microsomal. Focusing on the upper scheme in fig. 1 in which NADPH-cytochrome c reductase is involved in the transfer of electrons to cytochrome P-450, it can be seen that inhibition of electron transfer from the reductase should result in inhibition of the overall activity catalyzed by such a system if the initial transfer of electrons is rate limiting. It can be seen in fig. 2 that inhibition of NADPH-cytochrome c reductase activity in porcine liver microsomes by anti-reductase γ-globulin results in the concomitant inhibition of ethylmorphine demethylation catalyzed by these microsomes, showing that the oxidative metabolism of this drug is absolutely dependent upon electron transfer via NADPH-cytochrome c reductase. A similar experiment was performed using human liver microsomes in which NADPH-cytochrome c reductase, NADPH-cytochrome P-450 reductase, and NADPH-dependent oxidative demethylation of aminopyrine were measured as a function of increasing concentrations of antibody to the reductase. In fig. 3 the concomitant inhibition of these three activities is clearly demonstrated. Since there is no evidence for a concentration lag in the inhibition pattern as the antibody titer is increased, these data demonstrate an absolute dependence upon electron flux through the reductase flavoprotein.

For these studies, it was important to establish the species cross-reactivity of the anti-reductase γ-globulin. As shown in table 1, the amount of antibody prepared to porcine liver reductase required to inhibit NADPH-cytochrome c reductase activity 50% is approximately the same in porcine and human liver microsomes. On the other hand, approximately 5

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2 An Established Investigator of the American Heart Association.

3 Present address: Department of Internal Medicine, University of Manitoba Faculty of Medicine, 700 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 0V9.

4 Present address: Department of Pharmacology, University of Iowa College of Medicine, Iowa City, Iowa 52240.

Send reprint requests to: Dr. Bettie Sue Masters, Department of Biochemistry, University of Texas Southwestern Medical School, 5323 Harry Hines Blvd., Dallas, Texas 75235.
Microsomes from phenobarbital-treated pigs were previously incubated with varying amounts of \( \gamma \)-globulin prepared from pooled antisera to purified, lipase-solubilized NADPH-cytochrome \( c \) reductase. Aliquots were removed from previously incubated mixtures containing 61.6 mg of microsomal protein and varying amounts of preimmune and immune \( \gamma \)-globulin and assayed for ethylmorphine demethylase and NADPH-cytochrome \( c \) reductase activities. Formaldehyde formation from ethylmorphine was measured at 38°C and NADPH-cytochrome \( c \) reductase activity was determined at 25°C. The data are presented as percentage of control activity to demonstrate the concomitant inhibition of both activities by anti-reductase \( \gamma \)-globulin. NADPH-cytochrome \( c \) reductase activity was calculated on the basis of controls without \( \gamma \)-globulin (○—○) and with preimmune \( \gamma \)-globulin (▲—▲). Ethylmorphine demethylase was calculated on the basis of controls without \( \gamma \)-globulin (□—□) and with preimmune \( \gamma \)-globulin (△—△). Control activities were: NADPH-cytochrome \( c \) reductase, 147 nmol \( \times \) min\(^{-1} \) \( \times \) mg\(^{-1} \), and HCHO formation, 15.1 nmol \( \times \) min\(^{-1} \) \( \times \) mg\(^{-1} \).
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TABLE 1

<table>
<thead>
<tr>
<th>Liver Microsomes from</th>
<th>Mg γ-Globulin/Unit Reductase Activity Producing 50% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td>2.4</td>
</tr>
<tr>
<td>Human</td>
<td>3.6</td>
</tr>
<tr>
<td>Rat</td>
<td>12.5</td>
</tr>
<tr>
<td>Rabbit</td>
<td>111</td>
</tr>
</tbody>
</table>

Fig. 3. The concomitant inhibition of human microsomal aminopyrine demethylation. NADPH-cytochrome c reductase, and NADPH-cytochrome P-450 reductase by antibody to porcine NADPH-cytochrome c reductase.

The samples were prepared by mixing a known amount of γ-globulin with 20 mg of microsomal protein in 0.05 M potassium phosphate buffer, 10−4 M EDTA, pH 7.7, at 2°C. Aliquots were then withdrawn from this mixture and added to the various assays as described in the text: •—•, aminopyrine demethylation; ▲—▲, NADPH-cytochrome c reductase; □—□, NADPH-cytochrome P-450 reductase. The dashed line indicates preimmune γ-globulin. Control activities were: NADPH-cytochrome c reductase, 33 nmol × min⁻¹ × mg⁻¹; NADPH-cytochrome P-450 reductase, 1.1 nmol × min⁻¹ × mg⁻¹; and HCHO formation, 2.0 nmol × min⁻¹ × mg⁻¹.

In order to differentiate between the electron transport systems of adrenocortical mitochondria and microsomes, antibody to homogeneous preparations of adrenodoxin was prepared. This antibody was added to a soluble fraction prepared from sonicated mitochondria, called S2 (11), containing flavoprotein and adrenodoxin, and was found to inhibit the NADPH-dependent reduction of cytochrome c but not 2,6-dichlorophenolindophenol (12). Table 2 shows the results of an experiment in which sonicated mitochondria were titrated with anti-adrenodoxin γ-globulin; NADPH-cytochrome c reductase, NADPH-cytochrome P-450 reductase, and 11β-hydroxylase activities were measured. The data show that all three activities are inhibited to approximately the same extent, which indicates a requirement for adrenodoxin in the catalysis of these three reactions by adrenocortical mitochondria.

When the antibody to adrenodoxin was tested in adrenocortical microsomes for its effect on the 21-hydroxylation of 17α-hydroxyprogesterone, it was found to have no effect (table 3). On the other hand, if the flavoprotein of adrenocortical microsomes was similar to the NADPH-cytochrome c reductase of liver microsomes, the antibody to this enzyme might be expected to inhibit both cytochrome c reduction and 21-hydroxylation in this tissue. In experiments performed in collaboration with Dr. George T. Bryan of the University of Texas at Galveston, anti-reductase γ-globulin was shown to inhibit the formation of 11-deoxycorticosterone from progesterone (fig. 5) from 70-80% at the highest level of antibody tested. Similar results were obtained when the formation of 11-deoxycortisolate from 17α-hydroxyprogesterone was measured (fig. 5). The antibody used in this experiment was elicited in goats and purified to the γ-globulin fraction. The lack of concomitant inhibition of the NADPH-cytochrome c reductase activity can be explained by contamination with adrenocortical mitochondria in which the anti-reductase γ-globulin has no effect (3). Cytochrome
Masters et al.

Liver and Adrenal Microsomes

\[ \text{NADPH-Cytochrome P-450 Reductase} \]

\[ \text{Adrenal Cortical Mitochondria} \]

FIG. 4. The cytochrome P-450-dependent electron transport systems of liver and adrenal cortical microsomes and adrenal cortical mitochondria.

TABLE 2
Comparison of the inhibitory effects of the antibody to adrenodoxin on NADPH-dependent activities in bovine adrenocortical mitochondria

Bovine adrenocortical mitochondria were frozen and thawed five times prior to the determination of enzymatic activities. The determination of 11β-hydroxylase activity in bovine adrenocortical mitochondria was made using 11-deoxycortisol as substrate by measuring the rate of formation of corticosterone by the fluorometric method of Mattingly (13).

<table>
<thead>
<tr>
<th>γ-Globulin</th>
<th>NADPH-Cytochrome c Reductase</th>
<th>NADPH-Cytochrome P-450 Reductase</th>
<th>11β-Hydroxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount</td>
<td>% control</td>
<td>Amount</td>
<td>% control</td>
</tr>
<tr>
<td>mg/mg mitochondrial protein</td>
<td>nmol/mg/min</td>
<td>nmol/mg/initial 15 sec</td>
<td>mg/min</td>
</tr>
<tr>
<td>0</td>
<td>20.4</td>
<td>0.43</td>
<td>100</td>
</tr>
<tr>
<td>20:1 (immune)</td>
<td>7.1</td>
<td>0.17</td>
<td>37</td>
</tr>
<tr>
<td>20:1 (preimmune)</td>
<td>20.1</td>
<td>0.42</td>
<td>98</td>
</tr>
</tbody>
</table>

TABLE 3
Effect of antibody to adrenodoxin on the C-21 hydroxylation of 17α-hydroxyprogesterone in bovine adrenocortical microsomes

The C-21 hydroxylation of 17α-hydroxyprogesterone in adrenocortical microsomes was determined by measuring the rate of formation of 11-deoxycortisol employing the blue tetrazolium method of Elliott et al. (14).

<table>
<thead>
<tr>
<th>γ-Globulin</th>
<th>11-Deoxycortisol</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/mg mitochondrial protein</td>
<td>nmol/mg microsomal protein/min</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.64</td>
<td>100</td>
</tr>
<tr>
<td>10 (immune)</td>
<td>0.67</td>
<td>100</td>
</tr>
<tr>
<td>10 (preimmune)</td>
<td>0.64</td>
<td>100</td>
</tr>
</tbody>
</table>

P-450 reductase activity was not measured in these experiments but has previously been shown to be inhibited in bovine adrenocortical microsomes by antibody to the porcine liver reductase (3).

These data show that adrenocortical mitochondria contain an iron-sulfur protein, adrenodoxin, which is required for the reduction of cytochrome c and cytochrome P-450, and for the 11β-hydroxylation of steroids. There is no spectral evidence for the existence of an iron-sulfur protein in liver microsomes (15, 16) and, indeed, anti-adrenodoxin γ-globulin has no effect on any of the electron transport activities catalyzed by either liver or adrenal cortex microsomes. In addition, the NADPH-cytochrome c reductase activities from both liver and adrenocortical microsomes are immunochemically similar, so that antibody to the liver reductase inhibits all activities catalyzed by the enzyme in adrenal cortex microsomes. It has thus been shown that NADPH-cytochrome c reductase catalyzes the reduction of cytochrome P-450 in the microsomal fractions of both liver and adrenal cortex (3) and is required in the overall electron transport sequence for
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FIG. 5. The inhibition of 21-hydroxylation in bovine adrenocortical microsomes by antibody to NADPH-cytochrome c reductase.

Assays were performed at 29°C in a water bath shaker. Incubation mixtures consisted of an NADPH-generating system containing NADP+, isocitrate, isocitrate dehydrogenase, and microsomes (0.9 mg of protein per ml) in 0.05 M Tris-0.003 M MgCl₂ buffer, pH 7.4, in a total volume of 1 ml. The microsomes had been preincubated with the various ratios of γ-globulin indicated in the figure. The substrates, either tritiated progesterone or tritiated 17-hydroxyprogesterone, were labeled in the 7α-position and purified chromatographically before use. The final concentrations were 76 µM progesterone and 56 µM 17-hydroxyprogesterone. Samples were removed at 0, 15, and 30 min, added immediately to methylene chloride, extracted twice, and developed on treated silica gel thin layer chromatography plates in a benzene-acetone (3:1) system. Spots were compared with known markers and eluted and counted in a liquid scintillation spectrometer.

The hydroxylation of drugs in liver microsomes and steroids in adrenal cortical microsomes.

To complement studies on the spleen microsomal electron transport system which has been implicated in the catabolism of heme by Tenhunen et al. (17, 18), Schacter et al. (19) extended this experimental approach. The relationship between microsomal heme oxygenase and the NADPH-cytochrome P-450 electron transport sequence is schematically depicted in Fig. 1 of Dr. Schmid's paper in this Symposium. It can be seen that if the mixed function oxidation of heme is dependent upon reducing equivalents from the reductase to cytochrome P-450, then the inhibition of this flavoprotein by antibody would result in inhibition of heme oxygenase activity. This inhibition is shown in Fig. 6, in which NADPH-cytochrome c reductase and heme oxygenase activities in spleen microsomes are titrated with anti-reductase γ-globulin which results in concomitant inhibition of both activities as the titer is increased. In addition, microsomes were prepared from the livers of untreated rats and rats pretreated with methemalbumin, which induces heme oxygenase activity. These data (Fig. 7) show that even under conditions in which the heme oxygenase activity is increased 2-fold, there is an absolute dependence upon reducing equivalents from NADPH-cytochrome c reductase. These data, however, do not bear upon the introduction of the second and third atoms of oxygen into the biliverdin molecule, but the oxidation of the α-methene bridge to carbon monoxide has now been shown to be a mixed function oxidation requiring NADPH-cytochrome c reductase and cytochrome P-450.

In collaboration with Dr. Sten Orrenius of the

* R. Schmid, this Symposium, p. 256.
Karolinska Institute in Stockholm, Sweden, we have begun a series of experiments on the role of NADPH-cytochrome c reductase in the cytochrome P-450-dependent \( \omega \)- and \( \omega \)-1-hydroxylation of lauric acid by kidney cortex microsomes. Since the data of Ellin et al. (20) showed that NADH can support the hydroxylation of laurate to at least 50% of the rate with NADPH, it was of interest to determine the role of NADPH-cytochrome c reductase in the reduction of cytochrome P-450 by both pyridine nucleotides. Our studies show that anti-reductase \( \gamma \)-globulin inhibits cytochrome P-450 reduction in kidney microsomes approximately 70–80% in various experiments when NADPH is the electron donor (Fig. 8). With NADH as electron donor, however, there is little or no inhibition of cytochrome c or cytochrome P-450 reduction by the antibody. In these experiments, some inhibition of both NADPH- and NADH-supported activities was obtained with nonimmune \( \gamma \)-globulin, so that the slight inhibition obtained with immune \( \gamma \)-globulin was considered negligible. These data clearly show, nevertheless, that NADH is not reducing cytochrome P-450 through the NADPH-cytochrome c reductase flavoprotein. The data of Ellin et al. (20) would further suggest that separate systems are involved, since the \( \omega \)-hydroxylation and cytochrome P-450 reductase activities from the two pyridine nucleotides are additive.

In summary, studies with antibody to porcine liver microsomal NADPH-cytochrome c reductase have shown: 1) that this enzymic activity is responsible for cytochrome P-450 reduction in the microsomes of liver, adrenal cortex, spleen, and kidney; and 2) that the microsomal electron transport system of which this flavoprotein...
tein is an integral part is responsible for the specific mixed function oxidation reactions catalyzed by these various tissues.

References

Discussion

Dr. Conney, U. Michigan: How does rate of heme oxygenation compare with that of drug hydroxylation?

Dr. Masters: It occurs at a very low rate. The rates of spleen microsomal heme oxygenase activity that we observed in untreated animals, that is, animals which were not pretreated with methemalbumin, were about 0.23 nmol/min/mg of protein. With methemalbumin treatment, the activity increased approximately 4-fold to about 0.95 nmol/min/mg. Other workers have obtained similar values with various pretreatments, such as red cell antibodies, zymosan, etc., but these never exceed 0.5-1.0 nmol/min/mg in spleen.

Dr. Conney: This rate is approximately 10^-9 of the rate obtained in liver microsomal reactions utilizing optimal substrates.

Dr. Masters: That is correct. In addition, the NADPH-cytochrome c reductase activity as determined in spleen microsomes from untreated rats is 4-7% of that obtained with rat liver microsomal preparations; but the rate of electron flux in the spleen microsomal system is more than sufficient to account for the heme oxygenase measured as the rate of bilirubin formation in the presence of NADPH. In other words, it is not rate limiting, if we assume that cytochrome P-450 reduction proceeds in the same manner as in liver, kidney, and adrenal cortex.

Dr. Conney, Hoffmann-La Roche: Increasing the concentration of NADPH-cytochrome c reductase antibody decreases hydroxylase activity progressively as the concentration of antibody is increased. However, after 50-75% of the hydroxylase activity is inhibited, the antibody loses its effectiveness to inhibit the hydroxylation reaction. Why is a portion of the hydroxylase activity resistant to inhibition by NADPH-cytochrome c reductase antibody? Do these results suggest the presence of two different reductases?

Dr. Masters: There are two possible explanations for this, Dr. Conney: 1) When we do a titration with purified enzyme, of course, we get essentially 100% inhibition (it is inhibited to about 98%, but there may be a 2% residual activity left). In liver microsomes we have obtained 80-90% inhibition. One must remember that we are extending the use of an antibody prepared against porcine liver reductase to other tissues and other species, thus getting further away from identity as far as antigenic determinants are concerned, so we have to invoke that as a possible explanation for the failure to achieve complete inhibition. 2) The second possibility that occurs to me is that some antibodies which are not inhibitory may be present in the preparation and these may bind to the reductase, preventing it from being inhibited by inhibitory anti-
bodies. This would be a blocking antibody-type phenomenon.

Dr. Coon: I have a general question. There is a good deal of uncertainty about the involvement of cytochrome $b_5$ as an essential or even as an alternate electron donor in hydroxylation reactions. What is your information on this from the immunochemical point of view, or, perhaps, from other laboratories which have made similar studies? It is my recollection that the cytochrome $b_5$ antibody does not show an inhibitory effect on hydroxylation.

Dr. Masters: This is correct. The studies by Omura certainly indicated that there was no effect of anti-cytochrome $b_5$ serum on liver microsomal hydroxylation reactions. I believe there will be a report later in this meeting, if my information is correct, about an antibody prepared to cytochrome $b_5$ in Gillette's laboratory. We have not been very successful in making good inhibitory preparation to cytochrome $b_5$. Only recently, we have succeeded in obtaining inhibitory preparations of anti-cytochrome $b_5$ serum from rabbits by using cytochrome $b_5$ antigen coupled to human serum protein to increase its immunogenicity.

Dr. Maines, U. Minnesota: Did you measure the heme oxygenase activity of the spleen? If so, did you measure the cytochrome P-450 content of this organ?

Dr. Masters: Yes, spleen heme oxygenase activity has been measured as I mentioned earlier in reply to Dr. Coon's question. With regard to cytochrome P-450 levels, I wonder if Dr. Schacter would like to comment on these data.

Dr. Schacter, U. Manitoba: We have been able to determine that small amounts of cytochrome P-450 are present in rat spleen microsomes by measuring the difference spectrum obtained when the contents in the sample cuvet are treated with carbon monoxide and sodium dithionite while the contents in the reference cuvet are treated only with carbon monoxide. This minimizes the problem of spectral distortion by carboxyhemoglobin. The levels of cytochrome P-450 measured are very low, around 0.02-0.03 mmol/mg of microsomal protein. Of interest is the fact that rat spleen cytochrome P-450 is not induced by phenobarbital nor by 3-methylcholanthrene pretreatment of these animals in doses which will induce cytochrome P-450 in rat liver. On the other hand, we have found that benzopyrene and pregnenolone-16 $\alpha$-carbonitrile will induce spleen cytochrome P-450. The effect has been more consistent with PCN and levels of cytochrome P-450 as high as 0.16 mmol/mg of microsomal protein have been obtained which is about 7- or 8-fold above our control range.