Inhibition of Cholesterol Side Chain Cleavage by Active Site Directed Antibody to Corpus Luteum Cytochrome P-450*

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Goat antibody IgG produced against bovine corpus luteum mitochondrial cytochrome P-450 (P-450c) associated with cholesterol side chain cleavage (CSCC) was used to compare immunological characteristics of mitochondrial cytochrome P-450s from the bovine adrenal cortex (BAM), bovine corpus luteum (BCLM), and human placenta (HPM). In Ouchterlony double diffusion, anti-P-450c produced a single band with BAM and BCLM P-450c, but not with HPM P-450c, or BAM P-45016p. Appropriate concentrations of this anti-P-450c IgG inhibited the conversion of cholesterol to pregnenolone in BCLM and BAM preparations equivalently, but inhibition of placental P-450c was considerably less. The addition of BCLM iron sulfur protein and iron sulfur protein reductase to HPM P-450c increased CSCC approximately 5-fold. Under these conditions, anti-P-450c inhibited CSCC in HPM. Solubilized and sonicated BCLM preparations were inhibited equivalently but more than whole mitochondrial preparations. Addition of anti-P-450c IgG to BAM increased 11β-hydroxylation activity in concentration-dependent fashion. It appears that the cytochrome P-450c from BAM and BCLM are very similar if not identical, but immunologically different from HPM P-450c. The BAM P-450c is immunologically distinct from BCLM P-450c. The CSCC and 11β-hydroxylation systems of the adrenal are intimately linked because inhibition of P-450c markedly stimulated 11β-hydroxylation. Finally, the inhibition of CSCC activity of BAM, BCLM, and HPM P-450c indicates that the antigenic effect is directed toward the active site.

Cholesterol side chain cleavage activity in the corpus luteum, adrenal, and placenta is dependent upon a specific cytochrome P-450 (P-450c). The physicochemical and catalytic properties of purified P-450c's from these steroidogenic tissues have been compared but no significant difference has been identified (1). Because these criteria are insufficient to establish differences, a study of immunological characteristics of P-450c from these sources was undertaken.

In adrenocortical mitochondria, the two cytochrome P-450s which catalyze 11β-hydroxylation (P-45016p) and cholesterol side chain cleavage are immunochemically different (2), even though many physical and optical properties of P-450c and P-45016p are identical (1). Moreover, it has generally been assumed that the mechanisms of electron transport and hydroxylation are similar, and the ferredoxins in the electron transport system for P-45016p and P-450c in the adrenal mitochondria appear to be immunologically identical and functionally interchangeable (3). However, the ferredoxin associated with CSCC in placental mitochondria was not inhibited by the anti-adrenodoxin antibody leading to the interpretation that the human placental ferredoxin may be different from that of the bovine corpus luteum and adrenal cortex (3, 4).

Recently, Dus et al. raised antibodies in rabbits against cytochrome P-450c from Pseudomonas putida and also cytochrome P-450c from bovine adrenal cortical mitochondria (5). By enzymatic assay, the anti-P-450c inhibited cholesterol side chain cleavage and the anti-P-450c inhibited camphor hydroxylation and partially inhibited CSCC. In addition, cross-reactivity of various degrees was shown to occur between these antibodies and various P-450s and P-450 fragments by means of a competitive binding assay utilizing 125I-labeled antigen.

In this study, antibody to purified P-450c from bovine corpus luteum mitochondria (1) was produced in a goat and was used for immunochemical and functional comparison of the P-450c's from mitochondria of bovine adrenal cortex, bovine corpus luteum, and human placenta. Bovine corpus luteum mitochondrial P-450c was selected because the mitochondria and microsomes of the bovine corpus luteum are thought to have only one steroid hydroxylating P-450, thereby avoiding contamination from other P-450s. In addition, the role of the concentration of ISP in the analysis of the antibody inhibition of the CSCC system is demonstrated. The nature of the antigenic sites is considered.

EXPERIMENTAL PROCEDURES

Materials—[26,14C]Cholesterol (64 mCi/mmol), and [3H]H2O (100 μCi/mmol) were purchased from New England Nuclear. Deoxy corticosterone (4-pregnen-21-ol-3,20-dione), NADPH, and glucose-6-phosphate dehydrogenase were purchased from Sigma. Preparation of Immunoglobulin G from Goat Antibody to Bovine Corpus Luteum Cytochrome P-450 Catalyzing CSCC Activity (P-450c) was purified to homogeneity from bovine corpus luteum mitochondria as described previously (1). The specific content of the purified P-450 was 16.3 nmol/mg of protein.
The purified cytochrome P-450ec (100 µg/ml in borate/saline buffer composed of 0.01 M borate and 0.154 M NaCl at pH 8), was mixed with an equal volume of Freund's complete adjuvant and was injected subcutaneously into a mature male goat. The animal was immunized intravenously 3 weeks later with an additional 100 µg of cytochrome P-450. Blood was collected from the jugular vein prior to immunization and 10 days after the second immunization. The serum was stored in aliquots of about 60 µl at -20°C. Globulin "G" fractions were prepared from both the preimmune and immune sera by the methods of Kelkwich (6) and Levy and Sober (7). The IgG fraction was concentrated by ultrafiltration (Amicon, PM 30) and dialyzed against 0.01 M borate buffer, pH 8.0.

Preparation of Mitochondria and Cytochrome P-450 Fractions—Mitochondria were prepared from bovine corpus luteum, bovine adrenals, and human placentas as described previously (8, 9). For studies with unsonicated mitochondria, the washed mitochondria were suspended in 0.25 M sucrose and 0.1 mM EDTA solution. To prepare sonicated mitochondria, the mitochondrial pellet was suspended in Buffer A (a solution composed of 0.02 M potassium phosphate buffer, pH 7.4, 20% glycerol (v/v), 0.1 mM EDTA, and 0.1 mM dithioerythritol) at a protein concentration of about 25 mg/ml and 50-ml aliquots were sonicated in an ice bath at 20 kc, 85 watts intermittently for a total of 5 min with a Model W 185D Sonifier Cell Disruptor (Heat Systems-Ultrasonic, Inc., Plainview, NY) (1). To prepare solubilized mitochondria, the suspension of sonicated mitochondria was adjusted to a protein concentration of about 25 mg/ml and neutral 10% sodium cholate was added to bring the final concentration to 0.9%. For immunodiffusion tests, the cholate was removed and ammonium sulfate. The precipitate was dissolved in Buffer A containing 0.3% sodium cholate and was reprecipitated with ammonium sulfate at 35% saturation precipitated most of the P-450sec, leaving essentially P-450ec in the supernatant fraction. To prepare the "solubilized fraction," the supernatant fraction was dialyzed against Buffer A to remove the cholate and ammonium sulfate. The precipitate was dissolved in Buffer A containing 0.3% sodium cholate and was reprecipitated with ammonium sulfate at 35% saturation to prepare the P-450sec fraction. The P-450sec was recovered from the supernatant fraction by precipitation with 60% saturated ammonium sulfate (P-450sec preparation).

Determination of CSCC Activity—Enzymatic activity was determined by the method of Hochberg et al. (10) as modified for whole mitochondria by Uzgiris et al. (11). Each incubation vessel contained 0.25 nmol of P-450 (either in mitochondria or solubilized form from placenta, adrenal, or corpus luteum), 4 mM glucose 6-phosphatase, 0.5 unit of glucose-6-phosphate dehydrogenase, [3H]H2O (5 x 10^6 dpm), [26,4C]cholesterol (4.5 x 10^6 dpm, suspended with 0.5 mg of Tween 80), 1.1 mM MgCl2, 11 mM CaCl2, 1 mM NADPH in a total volume of 1.5 ml of Buffer A. Incubations were carried out at 37°C for 60 min. For studies of inhibition with IgG fractions, specified amounts of immune or preimmune IgG fractions were added to the P-450 preparation and an identical volume of 0.01 M bovine serum was added to the control incubation mixture. Complete assay mixtures with added IgG fractions were preincubated for 10 min at 37°C prior to initiation of the reaction by the addition of 1 mM NADPH. The biosynthesis was terminated with 0.2 ml of 1 M HgCl2.

The [14C]isocaproate formed was separated by filtration through an alumina column (G type F Merck; Woelm neutral (TCN); 2:1 (w/w)). H2O was used to correct for technical error. When the radioactivity recovered as isocaproate was plotted as a function of time, CSCC activity increased linearly for 30 min (Fig. 1). Reaction rates presented in Table I were determined from the linear portion of this curve. About one-third of the cholesterol added into the flask was cleaved to isocaproate by the adrenal mitochondria within 60 min. In certain experiments, iron sulfur protein and ISP reductase isolated from bovine corpus lutea were added to the placental mitochondria. These preparations were shown to be devoid of P-450sec, by spectros- copy and devoid of enzymatic activity. Inhibition of CSCC activity by the IgG was determined by comparison with the control flask after incubation for 60 min. Inhibitory effect is expressed as per cent conversion relative to that of the control which did not contain the IgG fraction or which contained IgG from preimmune serum.

Determination of 11β-Hydroxylase Activity—The 11β-hydroxylase activity catalyzed by bovine adrenocortical mitochondria was determined by measuring the rate of conversion of deoxycorticosterone to corticosterone at 37°C over a period of 1 h. One nmol of P-450 in Buffer A was incubated with 0.1 ml of 2 nmol deoxycorticosterone in propylene glycol, 4 mM glucose 6-phosphatase, 1 unit of glucose-6-phosphate dehydrogenase, 1.1 mM MgCl2, 11 mM CaCl2, and the specified volume of the preimmune or immune IgG fraction in a total volume of 2 ml. The reaction was initiated by the addition of 0.2 ml of 10 mM NADPH and terminated by the addition of 0.2 ml of 1 N H2SO4. After incubation, steroids were extracted, dried, and chromatographed on silica gel (60F-254, Merck) thin layer plates using chloroform/methanol (97:3, v/v) for development (12). The amount of corticosterone formed was estimated by the fluorometric method of Mattingly (13) using an Aminco Bowman Spectrophotofluorometer (400 nm excitation, 520 nm emission wavelength).

Inhibition of P-450sec by Anti-P-450sec IgG

<table>
<thead>
<tr>
<th>Source of mitochondria</th>
<th>Specific content of P-450 (mean ± S.D.)</th>
<th>CSCC rate (mean ± S.D.)</th>
<th>Half-maximum inhibition (I50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal cortex</td>
<td>5  0.92 ± 0.09</td>
<td>14.9 ± 3.4</td>
<td>4.2</td>
</tr>
<tr>
<td>Corpus luteum</td>
<td>8  0.22 ± 0.04</td>
<td>16.5 ± 4.2</td>
<td>12.9</td>
</tr>
<tr>
<td>Placenta</td>
<td>6  0.12 ± 0.04</td>
<td>4.0 ± 1.4</td>
<td>NI*</td>
</tr>
</tbody>
</table>

* Determined with whole mitochondria from cited source. ICP, [26-4C]isocaproate. NI, not inhibited.

Fig. 1. Time course of CSCC of sonicated mitochondria. Conditions of assay and isocaproate measurements are described under "Experimental Procedures."
Inhibition of P-450<sub>sec</sub> by Anti-P-450<sub>sec</sub> IgG

Fig. 3. Effect of anti-P-450<sub>sec</sub> on CSCC catalyzed by solubilized P-450<sub>sec</sub> from mitochondria from bovine adrenal cortex, bovine corpus luteum, and human placenta. Solubilized mitochondria from bovine adrenal cortex (△-△), bovine corpus luteum (○-○), and human placenta (■-■) were incubated with various amounts of anti-P-450<sub>sec</sub> as described under "Experimental Procedures." Preimmune IgG was added to the control incubation mixture with solubilized bovine corpus luteum mitochondria (∗-∗).

Table II

<table>
<thead>
<tr>
<th>IgG added (mg protein)</th>
<th>Corticosterone formed (nmol/min/nmol P-450)</th>
<th>Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25.7 ± 2.2</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>27.5 ± 2.3</td>
<td>107</td>
</tr>
<tr>
<td>10</td>
<td>54.0 ± 6.2</td>
<td>210</td>
</tr>
<tr>
<td>20</td>
<td>103.5 ± 13.5</td>
<td>402</td>
</tr>
</tbody>
</table>

Double Diffusion—Ouchterlony double diffusion tests were performed in Petri dishes containing 1.0% agarose in 0.154 M NaCl, 10 mM potassium phosphate, pH 7.4, and 0.05% sodium azide or on commercial Ouchterlony dishes obtained from Cappell Laboratories, Inc. Cochranville, PA. All samples were developed at 4 °C. Preparations of BCLM P-450<sub>sec</sub>, BAM P-450<sub>sec</sub>, BAM P-450<sub>10p</sub>, and HPM P-450<sub>sec</sub> were tested against 750 μg of anti-BCLM-P-450<sub>sec</sub> IgG in the center well. The test wells contained 200 μg of the preparations which had specific contents of 0.6, 1.8, 1.8, and 0.4 nmol/mg of protein, respectively. In other experiments, increased concentrations of the HPM P-450<sub>sec</sub> were tested as well as preparations of cholate extracts of HPM and HPM P-450<sub>sec</sub> from bovine corpus luteum. Certain of the HPM fractions were augmented with BCLM-P-450<sub>sec</sub>, had specific contents of 0.6, 1.8, 1.8, and 0.4 nmol/mg of protein, respectively. In other experiments, increased concentrations of the HPM P-450<sub>sec</sub> were tested as well as preparations of cholate extracts of HPM and HPM P-450<sub>sec</sub> from bovine corpus luteum.

RESULTS

Ouchterlony Double Diffusion—Double diffusion analysis of the solubilized preparations of cytochrome P-450<sub>sec</sub> from BCLM and BAM with the anti-BCL-P-450<sub>sec</sub> demonstrated single clean precipitin lines without spurs. No precipitin line was obtained between the anti-BCL-P-450<sub>sec</sub> and the BAM-P-450<sub>10p</sub> or the HPM P-450 preparations at the available concentrations. Further concentration of the HPM P-450 was technically not possible, but it could be demonstrated that the HPM preparations did not interfere with the precipitin line obtained with BCL-P-450<sub>sec</sub>. In view of the enzymatic inhibition demonstrated with the HPM P-450 preparations, it was concluded that the concentration of P-450<sub>sec</sub> was too low or that there are antigenic differences in the P-450<sub>sec</sub> from the human and bovine sources. Of interest, the BCLM-P-420 did not cause a precipitin line.

Inhibition of CSCC in Intact and Sonicated Mitochondria and in Solubilized P-450<sub>sec</sub> by Anti-P-450<sub>sec</sub>—In the presence of 30 mg of IgG/nmol BAM-P-450, catalytic activity was inhibited about 55% in unsolubilized mitochondria, 80% in solubilized mitochondria, and about 90% in a comparable solubilized P-450<sub>sec</sub> preparation (Figs. 2 and 3). These data add to the evidence that P-450<sub>sec</sub> is located on the inner mitochondrial membrane and is, therefore, less accessible to the antibody (18, 19).

Anti-P-450<sub>sec</sub> Inhibition of P-450<sub>sec</sub> from Various Organs and Species—The catalytic activities of sonicated mitochondria and solubilized P-450<sub>sec</sub> from BCLM, BAM, and HPM were compared at various concentrations of antibody (Table I, Figs. 2 and 3). Preimmune serum IgG was used for control studies.

From Table I, it is evident that the native preparations from the three sources are not comparable in specific CSCC activity. The adrenal concentration of P-450 is high because of the content of P-450<sub>sec</sub> other than P-450<sub>sec</sub> and the enzymatic activities of the three preparations reflect the different stoichiometric relationships of iron sulfur protein, ISP reductase and P-450. Nevertheless, both the sonicated and solubilized preparations of the enzyme from the BCLM and BAM were inhibited by the antibody in a concentration-dependent fashion (Figs. 2 and 3). HPM preparations were not inhibited under these conditions. On the other hand, comparable preparations were not inhibited by IgG obtained from preimmune serum. The half-maximum inhibitory concentration of the IgG (Io) was somewhat greater for the BCLM preparations than for those of adrenal origin (Table I). While the adrenal P-450<sub>sec</sub> appears to be more sensitive to the antibody at low concentrations, nevertheless, the inhibition curves are confluent at higher concentrations reaching 90 to 90% at 30 mg.

Fig. 4. Effect of ISP on CSCC by sonicated human placental mitochondria. Each vessel contained 5 DCPIP units of ISP reductase, 0.25 nmol of human placental mitochondrial P-450<sub>sec</sub>, and 0, 5, 10, 15, or 30 nmol of ISP. Other incubation conditions are described under "Experimental Procedures."
Inhibition of P-450<sub>sec</sub> by Anti-P-450<sub>sec</sub> IgG

(Figs. 2 and 3). Thus, the CSCC enzymes from both endocrine organs appear to be immunochemically similar.

**Effect of P-450<sub>sec</sub> on 11β-Hydroxylation**—The anti-P-450<sub>sec</sub> IgG was added in graduated amounts to a sonicated preparation of bovine adrenocortical mitochondria containing both the CSCC and 11β-hydroxylation systems (Table II). No effect on 11β-hydroxylation was obtained at IgG concentrations of 5 mg/nmol of P-450, but this amount was sufficient to inhibit over 50% of CSCC activity (Fig. 2). At higher concentrations, there was a linearly progressive stimulation of corticosterone formation until at the level of 20 mg of IgG, which inhibits 70 to 75% of CSCC, the increase in 11β-hydroxylation was 4-fold (Fig. 2, Table II). Although the rate of decrease in CSCC appears to be less than the rate of increase in 11β-hydroxylation, this difference can be explained by the requirement of 3 mol of NADPH and oxygen for CSCC compared to 1 mol required for 11β-hydroxylation. No significant changes were observed when IgG from preimmune serum was substituted for the anti-P-450<sub>sec</sub> IgG.

**Effect of ISP and ISP Reductase on CSCC in Human Placental Mitochondria**—The lack of inhibition of CSCC of HPM by anti-BCLM-P-450<sub>sec</sub> might indicate lack of common antigenic determinants because of either the difference in source or the differences in species. An alternative explanation is that the available amounts of electron carriers, ISP and ISP reductase, might be markedly less than the available cytochrome P-450<sub>sec</sub>. If this were the case, the system would not fully utilize the cytochrome P-450<sub>sec</sub> and the pool of unutilized P-450<sub>sec</sub> would bind to the antibody without apparent inhibition. In other words, to test the CSCC system properly, the limiting component should be the antigen, P-450<sub>sec</sub>. Alternatively, a lack of inhibition might result from the presence of an enzymatically inactive P-450<sub>sec</sub> fragment or other antigen, which would bind the antibody.

The effects of adding ISP reductase, ISP, and both at optimal concentrations were examined in sonicated placental mitochondria. First, ISP reductase (20 DCPIP units/nmol of P-450) was added to establish a control level of side chain cleavage which was 4 pmol of cholesterol/min/nmol of P-450 (Fig. 4). This is approximately the rate of CSCC obtained with native placental mitochondria (Table I). The addition of increasing amounts of ISP progressively increased the amount of cleavage to about 6-fold. While Fig. 4 presents data from one experiment, in replicate experiments with different placental preparations, the maximum turnover rate ranged from 14 to 30 (22.7 ± 6.5, mean ± S.D.) pmol/min/nmol of P-450 after the addition of 10 nmol of ISP and 20 DCPIP units.

![Fig. 5. Effect of ISP reductase on CSCC by sonicated human placental mitochondria with optimal amounts of ISP. Each vessel contained 10 nmol of ISP, 0.25 nmol of human placental mitochondrial cytochrome P-450<sub>sec</sub>, and 0, 2.5, 5, 10, 20, 30, or 40 DCPIP units of ISP reductase. Other incubation conditions are described under “Experimental Procedures.”](http://www.jbc.org/)

In a sequential experiment, increasing amounts of ISP reductase were added to incubation mixtures containing 40 nmol of ISP/nmol of P-450 (Fig. 5). Under these conditions, CSCC was increased from about 12 pmol/min/nmol of P-450 in the absence of ISP reductase to about 25 pmol at maximum activity. This rate of CSCC of the placental mitochondria exceeds even that of native adrenocortical mitochondria. Thus, it is clear that isolated placental mitochondria are functioning at low efficiency and the limiting enzymes are the electron carriers especially ISP. Conversely, these data indicate that, at least under the conditions of these experiments, P-450<sub>sec</sub> is present in placental mitochondria in amounts which are many times greater than is necessary for maximal catalytic activity.

The effects of anti-P-450<sub>sec</sub> were assessed in a system which contained 80 DCPIP units of ISP reductase and 40 nmol of IPS/nmol of P-450 in order to make the concentration of P-450<sub>sec</sub> rate-limiting (Fig. 6). The control preparation of native sonicated mitochondria again was not inhibited but the supplemented system was inhibited in a concentration-dependent fashion between the range of 10 and 50 mg of IgG. However, the IC<sub>50</sub> for this system was 29 mg of IgG as compared to IC<sub>50</sub> values of 4.2 and 12.23 for native adrenocortical and corpus luteal preparations, respectively. Furthermore, the maximal amount of inhibition even in the presence of as much as 50 mg of IgG/nmol of P-450 was only about 60%.

Repetition of these experiments with solubilized HPM P-450<sub>sec</sub> yielded identical results. This finding contrasts with those of BCLM and BAM sonicated and solubilized preparations in which the P-450<sub>sec</sub> of the solubilized preparations appeared to be more accessible to the antibody.

**DISCUSSION**

The administration of homogeneously pure cytochrome P-450<sub>sec</sub> from bovine corpus luteum mitochondria into goats produced an antibody which effectively inhibited CSCC in BCLM and P-450 preparations and yielded a single, non-serrated line with BCLM-P-450<sub>sec</sub> by Ouchterlony double diffusion tests. This antibody was used to compare organ and species specificities of cytochrome P-450<sub>sec</sub>, to determine the accessibility of membrane-bound cytochrome P-450<sub>sec</sub> to the antibody, and to study the stoichiometric relationships of the electron transport proteins to the antigen-antibody reaction.
Finally, this preparation was used to demonstrate the interdependence of the various mitochondrial P-450-linked enzyme systems.

In both the corpus luteum and adrenal preparations, the antibody was more effective in inhibiting CSCC in solubilized preparations than in sonicated preparations, and it inhibited the sonicated and solubilized adrenal preparations more than unfragmented adrenal mitochondria (Figs. 2 and 3). Thus, the antigenic sites of the membrane-bound cytochrome are not as readily accessible to the antibody and the most likely reason is that some of those sites are located on the inner membrane of the mitochondrion.

The Ouchterlony test did not distinguish between P-450c from bovine adrenal and corpus luteum sources although the antibody appeared to inhibit CSCC of the BAM-sonicated and -solubilized preparations at somewhat lower concentrations than were necessary for inhibition of comparable BCLM preparations (Table I, Figs. 2 and 3). However, the CSCC activity is expressed in picomoles of isoproterenol per nmol of P-450 and, for the BAM CSCC rate, the denominator includes P-45011β as well as P-450c, because the two cytochromes are spectroscopically indistinguishable.

Adrenal preparations may contain somewhat less P-450c than those from the BCLM; therefore, less anti-P-450 would be required for inhibition. Alternatively, the ratios of ISP reductase, ISP, and cytochrome P-450c are probably not optimal in the native preparations and the limiting component of the transport system may not be the cytochrome P-450c. In spite of these variables, the inhibition by higher concentrations of IgG in adrenocortical preparations than were necessary for inhibition of comparable BCLM preparations (Table I, Figs. 2 and 3). However, the CSCC activity is expressed in picomoles of isoproterenol per nmol of P-450 and, for the BAM CSCC rate, the denominator includes P-45011β as well as P-450c, because the two cytochromes are spectroscopically indistinguishable.

The anti-P-450c did not react with the purified P-45011β in the Ouchterlony double diffusion test and did not inhibit 11β-hydroxylation (Table II). Similar results were obtained by Watanuki et al. (20) and Suhara et al. (2) who used antibody raised in rabbits against BAM-P-450c and BAM-P-45011β and found no cross-reactivity with the alternative antigen. On the other hand, our data differ from that of those investigators because increasing the concentrations of IgG in adrenocortical preparations containing both P-450c and P-45011β markedly increased 11β-hydroxylation (Table II). The reason for this difference is the type of system used. Those investigators used reconstituted systems containing excessive amounts of ISP and ISP reductase. Under optimal conditions, Suhara et al. (2) reported a turnover rate of 110 nmol of corticosterone/min/mmol of P-450. The turnover rate of our native adrenal sonicated mitochondria in the presence of 20 mg of IgG is 105.

Thus, blocking P-450c made sufficient electron transport components available to the 11β-hydroxylation system to achieve maximal efficiency. This demonstrates that the two systems are intimately linked and has important implications for clinical conditions where one system or the other may appear to be either excessively productive or inhibited.

It may be that the apparent lack of stoichiometric equivalence of the components of the transport system in our native systems is artifactual. Adrenodoxin has been shown to undergo rapid autooxidation (21, 22). The ISP/P-450 ratio in separated adrenal and placental mitochondria exceeds or equals 1:1. This is less than optimal for reconstituted systems (23–25) but the ratios obtained in separated mitochondria may differ from those which occur in vivo. Nevertheless, confirmation of the interdependence of the P-450-linked systems in the regulation of adrenal steroidogenesis comes from other experiments involving specific inhibitors of the mitochondrial enzymes. Thus, Graves et al. (26) demonstrated that inhibitors of 11β-hydroxylation such as metyrapone stimulated CSCC, and conversely, Whipple et al. (27) found that inhibition of P-450c with aminoglutethimide resulted in stimulation of 11β-hydroxylation. While the precise stoichiometric conditions and topographical arrangements of the enzymes, electron transport proteins, and cofactors in vivo are not known, these data imply that there is close linkage of the mitochondrial P-450 systems and also that the terminal P-450 may not be the rate-limiting enzyme.

The data obtained with the human placental mitochondria are more complex. By Ouchterlony double diffusion, precipitation of the placental P-450c was not obtained with this antibody. Furthermore, the antibody did not inhibit placental CSCC (Figs. 2 and 3) until large amounts of ISP (of bovine origin) were added to the preparation. One explanation for the lack of enzymatic inhibition, therefore, is that the rate-limiting enzyme in the sequence was the ISP and not the P-450c.

Our experience with placental microsomal and mitochondria P-450s is that these P-450s have considerably less stability than do preparations from any other tissue and that the autooxidizability of ISP clearly results in low levels of that enzyme. This, however, does not explain the negative Ouchterlony test. We attribute this either to the low specific content of our preparations or to the possibility that the placental P-450c is not immunologically identical with the bovine P-450c but does have some cross-reactivity.

These findings with placental proteins are not unique to cytochrome P-450. Barron tested the placental system with antibody to bovine adrenodoxin and found no enzymatic inhibition even though double diffusion produced clear precipitin lines (4). As pointed out by Watanuki et al. the inhibition of enzyme activity is a more sensitive indicator of antigen-antibody reaction than double diffusion (20). Perhaps the reason for Barron’s findings was the presence of relatively large amounts of inactive ferridoxin which would react with the antibody to produce precipitin lines and would bind antibody rendering it unavailable to the active form of ISP.

It is of interest to consider the nature of the antigenic sites, especially in light of the data published by Dus et al. (5). It is clear that both his antibody preparations, and most likely ours also, consist of a mixture of antibodies directed both toward an active site of the enzyme and to other sites. Thus, antibodies which do not interfere with enzymatic function do not interfere with the active site either directly or by altering the conformation of the enzyme. Consequently, P-45011β and liver microsomal P-450 might have one or more antigenic sites to bind to the anti-P-450c prepared by Dus et al. Although Dus et al. did not test for inhibition of 11β-hydroxylation activities, we found that 11β-hydroxylation in the presence of our antibody was actually increased.

One can speculate that there are two binding sites on each P-450 molecule: one being a site for ISP to reduce the P-450 and the other being a site for the substrate to be oxygenated. An antibody preparation might interfere with either site or both.

The ISP site for P-450c and P-45011β are probably similar because the ISPs are interchangeable. Our antibody caused an increase in 11β-hydroxylation indicating that it was not interfering with the ISP site of P-450c and therefore probably not with the ISP site on P-450c. In fact, using the HPM P-450c as a model, the addition of ISP increased CSCC activity even in the presence of the antibody. Thus, it is unlikely that this IgG is inhibiting the system through a significant effect on either the ISP or the ISP site.

On the other hand, the anti-P-450 IgG inhibited CSCC activity by P-450c from diverse sources including the bovine adrenal, bovine corpus luteum, and human placenta. Whatever other differences these P-450s may have, it is likely that
the substrate active sites are similar. Since precipitin lines were not obtained for the human P-450, it appears that other regions of the human protein differ from those of the bovine P-450s. Similarly, P-420 derived from bovine sources did not yield a precipitin line. Thus, we conclude that at least one component of our antibody mixture is directed toward the substrate-associated binding site.

REFERENCES
Inhibition of cholesterol side chain cleavage by active site directed antibody to corpus luteum cytochrome P-450.
K Kashiwagi, A B MacDonald and H A Salhanick


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