Cytochrome P-450 from Bovine Adrenocortical Mitochondria

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Antibodies were induced against side chain cleavage P-450 from bovine adrenocortical mitochondria by injecting the enzyme into rabbits. Double diffusion in agarose gels revealed single bands between rabbit anti-P-450 antisera and adrenal cytochrome P-450 side chain cleavage prepared by two different methods and also with P-450 of different molecular weights called protein 4, protein 8, and large P-450. In addition, the same anti-serum showed a line of identity with an ammonium sulfate fraction which is an impure precursor of pure cytochrome P-450 side chain cleavage in two methods used for its preparation. The IgG fraction from antisera to P-450 gives a single band with bovine adrenal P-450 side chain cleavage in immunoelectrophoresis. Antiserum to P-450 side chain cleavage inhibits side chain cleavage enzyme activity but not 11β- or 18-hydroxylation and native P-450 competes with 125I-labeled P-450 (side chain cleavage) in combining with anti-P-450 antibodies so that a radioimmunoassay for side chain cleavage P-450 has been developed. Extremely small and possibly nonspecific competition for the anti-P-450 is seen between different forms of cytochrome P-450 in adrenal mitochondria and as a preliminary step in developing a radioimmunoassay for the side chain cleavage P-450, we have prepared antibodies to this protein. The reactions of various physicochemical criteria can exist in a variety of forms of M, = 210,000, 420,000, 850,000 (8), and larger aggregates that have been less well characterized (6); the larger aggregates are collectively referred to as large P-450. All these forms of the enzyme give rise to a single band on electrophoresis on polyacrylamide gels with SDS; this band corresponds to a subunit of M, = 52,000 to 53,000. The single species of protein subunit is not degraded to smaller forms by reactions which cleave S-S bonds (9), so that the forms referred to above are known as protein 4, protein 8, and protein 16 according to the number of subunits present. However, the best preparations contain variable amounts of phospholipid and the amount of phospholipid influences the behavior of the protein on electrophoresis in SDS gels (9). Moreover, on reconstituting the multienzyme system (adrenodoxin reductase, adrenodoxin, and P-450) in aqueous phases from the pure membrane proteins, reduction of P-450 by TPNH and ancillary proteins or reduction of P-450 alone by dithionite proceeds extremely slowly. This problem makes for difficulty in determining the heme content of the enzyme by CO-difference spectroscopy (1). This difficulty has in turn been misinterpreted as a reflection on the purity of the enzyme (4). It is, therefore, of considerable importance to eliminate the possibility that a heme protein which is not a cytochrome P-450 may be present as a contaminant in sufficient amounts to cause disparity between the heme content determined as the pyridine hemochromogen and the heme content determined from the CO complex. Such a possibility would require that the contaminant and the P-450 were indistinguishable by the physicochemical methods used to establish the purity of the P-450 (1). In order to clarify this point, to determine the physicochemical relationships between different forms of cytochrome P-450 in adrenal mitochondria and as a preliminary step in developing a radioimmunoassay for the side chain cleavage P-450, we have prepared antibodies to this protein. The reactions of various forms of the enzyme with the antibodies constitute the basis of this communication.

A cytochrome P-450 responsible for catalyzing the rate-determining step in steroid synthesis has been prepared from bovine adrenocortical mitochondria (1–5). The reaction catalyzed is called side chain cleavage of cholesterol (cholesterol → pregnenolone); this reaction requires TPNH and two ancillary proteins: adrenodoxin and adrenodoxin reductase (6, 7). At least four methods are available for purifying the enzyme (1–5) but only two of these methods appear to produce proteins with identical properties (1, 5). Moreover, it is clear that the cytochrome purified to homogeneity according to rigorous

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† The abbreviations used are: SDS, sodium dodecyl sulfate; pregnenolone, pregn-4-en-3β-ol-20-one; scc, side chain cleavage.

EXPERIMENTAL PROCEDURES

Preparation of Cytochrome P-450

Side Chain Cleavage Enzyme — The enzyme used in these studies was prepared by affinity chromatography using pregnenolone-Sepharose as described previously (5). Other preparations used include...
large P-450 which is also produced by the same affinity column; this enzyme gives a single band on SDS gels but is heterogeneous in molecular weight (5). In addition, side chain cleavage P-450 was prepared by chromatography on DEAE-cellulose in three forms, protein 16, protein 8, and protein 4, named according to the number of protein subunits present in these forms (1, 8).

**Enzyme Assay**

Side chain cleavage (1), 11β-hydroxylase, and 18-hydroxylase activities (10) were measured as described elsewhere under conditions in which activity was linear with time and proportional to the presence of antiserum or IgG, enzyme and antibodies were incubated for 3 h at 4°C before the assay was performed.

**Preparation of Antibody**

Female New Zealand White rabbits were immunized subcutaneously in the nuchal region with 100 μg of cytochrome P-450 prepared by affinity chromatography or on DEAE-cellulose (protein 16). The protein was emulsified with an equal volume of Freund's complete adjuvant (11). The animals were reimmunized every 3 weeks on opposite sides of the neck with 100 μg of the same protein emulsified in Freund's complete adjuvant for a total of six injections. Serum was collected before immunization and once a week after the 6th week. Animals were bled from the central artery of the ear and sera was boiled for 5 min in SDS at a final concentration of 0.5% (w/v) to potassium phosphate (10 mM, pH 7.21, and 0.02% sodium azide (12).

**Micro-Ouchterlony double diffusion tests were performed on microscope slides coated with 4.0 ml of 1.25% agarose in NaCl (0.15 M), potassium phosphate (10 mM, pH 7.2), and 0.02% sodium azide (12). All samples were incubated from 1 to 5 days at 25°C and at 37°C. In the accompanying data, the total amount of protein in each well of the gels is given. Immunoelectrophoresis was performed on agarose-coated slides in barbital buffer, pH 8.6 (12).

**Double Diffusion and Immunoelectrophoresis**

When antiserum to P-450 was placed in the center well of an agarose slide and increasing concentrations of P-450 were placed in the peripheral wells, a single band was seen in the gels (Fig. 1). As the concentration of P-450 was increased, the single narrow band became broader as expected, since at higher concentrations side chain cleavage P-450 becomes heterogeneous with respect to molecular weight in buffered solutions or during various purification procedures (1, 5, 9). However, no additional bands were seen at higher concentrations.

**Radioimmunossay of Cytochrome P-450**

Cytochrome P-450 (100 μg) was iodinated by means of horseradish peroxidase attached to PA beads (Bio-Gel P-200) as described by Theorell and Larsson (13). Following iodination, the preparation was centrifuged to remove Bio-Gel beads and aqueous potassium iodide (10 μl, 0.1 M) was added. The mixture was diayed against potassium phosphate (100 mM, pH 7.2) containing 0.1 mM EDTA and ion exchange resin AG 1-X2 (200 to 400 mesh; Bio-Rad; 5 g/liter) overnight with five changes of buffer. The protein was then applied to a column of Sephadex G-100 (15 x 4 cm) and eluted with potassium phosphate buffer (100 mM, pH 7.0) containing 0.1 mM EDTA (Fig. 2). A single peak of 125I-bound protein was observed; the peak was eluted in 1.5 ml of eluate. This fraction contained the 125I-bound P-450 used for radioimmunossay (7 x 10^6 cpml/ml). Electrophoresis on SDS gels revealed a single peak of 125I which was associated with a single protein band (P-450 scc subunit).

The procedure for radioimmunossay was based upon the method of Lewis et al. (14). The assay tubes contained the following reagents: potassium phosphate (0.01 M; pH 7.2) containing sodium chloride, 0.9% (w/v), and bovine serum albumin, 1% (w/v), 600 μl; EDTA (0.1 M) 100 μl; 125I-labeled cytochrome P-450 (50 μl; 7,000 cpm; 125I-P-450 IgG (6 μg; 100 μl); samples of P-450 (unlabeled) were added to 100 μl of potassium phosphate buffer (50 mM; pH 7.0). Samples were left overnight at 4°C and normal rabbit serum was added to 2% (v/v). Second antibody (sheep anti-rabbit serum) (150 μl; 1:10 dilution) was then added. After standing at 4°C for 8 h, the tubes were centrifuged at 2,500 x g for 20 min. The supernatant layer was removed and the pellet was examined for determination of 125I in a Nuclear Chicago γ scintillation counter (model 1185).

**Purification of Antiserum**

Serum from rabbits immunized against P-450 was purified by chromatography on DEAE-cellulose using a standard procedure (15). The preparation of goat anti-rabbit IgG has been described previously (11). The animals were reimmunized every 3 weeks on opposite sides of the neck with 100 μg of the same protein emulsified in Freund's complete adjuvant (11). The protein was emulsified with an equal volume of Freund's complete adjuvant (11). The animals were reimmunized every 3 weeks on opposite sides of the neck with 100 μg of the same protein emulsified in Freund's complete adjuvant for a total of six injections. Serum was collected before immunization and once a week after the 6th week. Animals were bled from the central artery of the ear and sera was boiled for 5 min in SDS at a final concentration of 0.5% (w/v) to potassium phosphate (10 mM, pH 7.21, and 0.02% sodium azide (12).

**RESULTS**

**Purification of Anti-P-450 Antiserum**

When anti-P-450 scc antiserum was purified by chromatography on DEAE-cellulose, antibody activity toward P-450 scc was observed only in the IgG fraction. In addition, the purified antibody cross-reacted with goat anti-rabbit IgG on double diffusion but not against other species of antibody and migrated like other rabbit IgG fractions on immunoelectrophoresis (data not shown). The following studies used both the crude antiserum and the IgG fraction prepared in this way.

**Studies of P-450/Anti-P-450 Reaction by Double Diffusion**

Effect of Antigen Concentration—When a certain volume of antiserum to P-450 scc was placed in the center well of an agarose slide and increasing concentrations of P-450 were placed in the peripheral wells, a single band was seen in the gels (Fig. 1). As the concentration of P-450 was increased, the single narrow band became broader as expected, since at higher concentrations side chain cleavage P-450 becomes heterogeneous with respect to molecular weight in buffered solutions or during various purification procedures (1, 5, 9). However, no additional bands were seen at higher concentration.

**Specificity of Anti-P-450 scc Antiserum—**Fig. 2 shows that antiserum to P-450 scc reacts with side chain cleavage P-450 producing a single band, but not with highly purified 11β-hydroxylase P-450 (10). Moreover, demonstrable antibodies do not appear within 1 week (Fig. 2, right gel, Well B) and the antibodies to P-450 scc do not cross-react with antiserum prepared by injecting into another rabbit preparations of subunits of P-450 scc prepared in SDS (Fig. 2, right gel, Well A). Normal rabbit serum and bovine serum albumin do not cross-react against P-450 (data not shown).

**Interaction of Antisera Prepared against Protein 16 with Various Forms of P-450—**Rabbit antiserum to protein 16 was prepared by pregnenolone-Sepharose affinity chromatography (5) reacts with P-450 from DEAE-cellulose (1), with P-450 scc prepared as a by-product during preparation of 11β-hydroxylase (10), with large P-450 from affinity chromatography and with proteins 4, 8, and 16 from DEAE-cellulose (1, 8) (Fig. 3). It is important to notice the halo effect around wells containing large amounts of P-450. This effect is due to aggregation of P-450 in the gel and is not seen at low concentrations of this protein but is seen when P-450 is studied at similar concentrations without antiserum in the center well.

**Reaction of Anti-P-450 Serum with Purified and Crude Preparations of P-450—**The crude ammonium sulfate fraction (30 to 60% saturation) prepared from adrenal mitochondria contains many impurities (5); at least 15 bands are seen on SDS gels (data not shown). This fraction serves as a precursor of P-450 scc prepared by various methods (1, 5, 10). As can be seen in Fig. 4, a single line of precipitation is observed with both the ammonium sulfate fraction and with highly purified P-450. Moreover, the junction of these two lines shows a classical line of identity, indicating that only a single species of antigen can be detected by this method.

**Antiserum to P-450 Prepared on DEAE-cellulose—**Antibodies sources of the chemicals used have been published elsewhere (1) except the following. PA beads (Bio-Gel P-200) were obtained from Bio-Rad Laboratories. Horseradish peroxidase was purchased from Sigma and carrier-free 111I (potassium iodide) from International Chemical Nuclear.
Immunochemistry of Cytochrome P-450

**Fig. 1** (top). Double diffusion in agarose gel of anti-P-450 antibodies and various concentrations of side chain cleavage P-450. The center well contained anti-P-450 antiserum (20 μl) and the surrounding wells in the left gel contained the following amounts of P-450: A, 8 μg; B, 16 μg; C, 26 μg; D, 34 μg; surrounding wells in the right gel contained: A, 26 μg; B, 34 μg; C, 50 μg; D, 100 μg.

**Fig. 2** (middle). Double diffusion in agarose gel of various cytochromes P-450 and anti-P-450 anti-serum. In the left gel, the center well contained antiserum to P-450 XC (15 μl) and the surrounding wells contained: A, lip-hydroxylase P-450 (25 pg); B, P-450 see protein 16 (20 pg); C, lip-hydroxylase P-450 (50 pg); D, P-450 see protein 16 (40 pg). In the right gel, the center well contained P-450 see protein 16 (40 pg) and the surrounding wells contained: A, anti-P-450 subunit antiserum (15 μl); B, anti-P-450 see 1 week after the first injection of antigen (15 μl); C, the same after 12 weeks (15 μl).

**Fig. 3** (bottom). Double diffusion in agarose gel of anti-P-450 (side chain cleavage) antibodies and various forms of P-450 side chain cleavage. The center well contained anti-P-450 (side chain cleavage) (15 μl) in both slides. The surrounding wells in the left gel contained the following: A, P-450 protein 16 prepared as described in Ref. 1 (100 μg); B, P-450 prepared during preparation of 11β-hydroxylase (50 μg); C, large P-450 (50 μg); D, protein 16 prepared by affinity chromatography (50 μg); E, 30 to 60% ammonium sulfate fraction (150 μg).

**Fig. 4**. Double diffusion in agarose gels of anti-P-450 (side chain cleavage) antibodies and crude P-450 (side chain cleavage: ammonium sulfate fraction) (see "Results"). The center well contained anti-P-450 antibodies (15 μl) and the surrounding wells contained the following: A, P-450 protein 16 prepared by affinity chromatography (50 μg); C, 30 to 60% ammonium sulfate fraction (150 μg).

**Fig. 5**. Immunelectrophoresis of side chain cleavage P-450 and of anti-P-450 (side chain cleavage). In this slide, electrophoresis was performed for 6 h.

Inhibition of Side Chain Cleavage Enzyme Activity by Anti-P-450 Serum and by Anti-P-450 IgG

Side chain cleavage of cholesterol is inhibited by antiserum to P-450 see (Fig. 6). Inhibition shows a curvilinear relationship to amount of added antiserum whether the side chain cleavage is measured by radioimmunoassay of pregnenolone (14) or by conversion of [3H]cholesterol to [3H]pregnenolone (1). Normal rabbit serum (preimmunization) (Fig. 6A) and bovine serum albumin (Fig. 6B) stimulated the side chain cleavage reaction. This effect of albumin has been noticed previously (7), although the mechanism concerned is uncertain. Presumably normal rabbit serum acts to increase enzyme activity as the result of a similar effect by rabbit serum protein. When the serum used was taken from rabbits after the second injection of P-450, inhibition was less pronounced (Fig. 6A), in keeping with the fact that this serum produced...
no visible bands with P-450 in double diffusion (data not shown); clearly the inhibition of enzyme activity is a much more sensitive indication of antigen-antibody reaction than double diffusion under the conditions used. Antiserum to P-450 was without obvious effect on 11β- or 18-hydroxylase enzyme activities (data not shown).

When purified IgG prepared from antiserum to P-450 was incubated with the enzyme, inhibition of the enzyme reaction was observed as expected (Fig. 6C). In this case, inhibition by IgG occurs without the complicating effect of those serum proteins which stimulate the enzyme activity (Fig. 6, A and B). It can also be seen that IgG from normal rabbit serum (no immunization) was without effect on the enzyme activity (Fig. 6C).

Reactivity of [125]I-labeled P-450 with Anti-P-450

Fig. 7 shows the result of studies in which iodinated P-450 was added to antiserum and unlabeled P-450. It can be seen that P-450 competes with [125]I-labeled P-450 in combining with anti-P-450 antibodies in a concentration-dependent manner.

The assay procedure is capable of detecting 0.01 μg of cytochrome P-450 sec (p < 0.1) and binding is linear in the range of 0.025 to 0.25 μg using a log scale for amount of P-450 added. Addition of 11β-hydroxylase P-450 causes some decrease in bound [125]I, but within the limits tested, evidence of specific binding was not observed since a parallel displaced curve was not seen. The 11β-hydroxylase contains bound Tween 20 and, although this substance does not affect the binding of P-450 with antibody when added without 11β-hydroxylase in amounts equivalent to those accompanying the enzyme additions shown in Fig. 7, higher concentrations of Tween do modify the combination of P-450 with antibody. For these reasons, addition of very large amounts of the enzyme cannot be made without nonspecific effects.

FIG. 6. The effect of anti-P-450 antiserum and IgG on side chain cleavage of cholesterol by P-450 from bovine adrenal cortex. In A and C, side chain cleavage was measured by radioimmunoassay using cholesterol (50 nmol/flask) as substrate (14). In B the substrate was [7a-3H]cholesterol (106 cpm: 50 nmol/flask); following incubation [3H]pregnenolone was isolated and measured as described previously (1). In both cases, each flask contained 6 pmol of P-450, 0.2 nmol of adrenodoxin, 1.0 units of adrenodoxin reductase (1), and 20 nmol of TPNH with potassium phosphate buffer (50 mM, pH 7.0) to a volume of 2 ml. Incubation was continued for 15 min at 37° after the remaining additions were made and the reaction was stopped by boiling. In A, O-O, preimmunization serum; Δ-Δ, antiserum collected 1 week after immunization; Δ-Δ, anti P-450 sec IgG; O-O, normal rabbit IgG.

FIG. 7. Radioimmunoassay of cytochrome P-450 (Cyt-P450). The ordinate shows radioactivity precipitated (i.e. [125]I-labeled P-450 sec) by first (anti-P-450 antiserum) and second (sheep anti-rabbit antisera) antibodies. The abscissa (log scale) shows amount of unlabeled (competing) P-450 added: either P-450 sec or P-450 11β-, 18-hydroxylase (10) according to the symbols below. The assay procedure is a modification of that described by Lewis et al. (15) (see "Experimental Procedures"). O-O, P-450 sec; O-O, P-450 11β-, 18-hydroxylase.

DISCUSSION

The present studies lend strong support to the conclusion that the side chain cleavage P-450 prepared by chromatography on DEAE-cellulose (1) and that prepared by affinity chromatography on pregnenolone-Sepharose (4) are highly
purified. Each of these preparations at various concentrations shows a single band with rabbit anti-P-450 antiserum in double diffusion. Moreover, only a single band of precipitate (line of identity) was seen in double diffusion with anti P-450 serum and a crude ammonium sulfate precipitate, which represents a precursor of pure P-450 in both methods of purification. The enzyme mixed with purified antibody (IgG fraction) also gives a single band on immunoelectrophoresis. Purification. The enzyme mixed with purified antibody (IgG fraction) also gives a single band on immunoelectrophoresis. In addition, the various forms of the cytochrome P-450 which differ in molecular weight also show a single band with the anti-P-450 antiserum. This statement applies to proteins 4, 8, and 16 prepared by chromatography on DEAE-cellulose and to protein 16 and large P-450 prepared by affinity chromatography. Evidently, these various forms of the enzyme do not result from binding of smaller forms by a contaminating protein. These findings indicate that the various forms of the enzyme reveal the same antigenic determinants. Since several available methods of preparing P-450 scc result in enzymes with significant differences in physicochemical properties (1-5), it was of interest to demonstrate that the two preparations developed in this laboratory (based upon DEAE-cellulose and affinity chromatography, respectively) are immunochemically identical since both preparations have been used extensively in published studies. Purification of anti-P-450 antiserum reveals the presence of antibody activity only in the IgG fraction.

Evidently, the physicochemical evidence (SDS gels, analytical ultracentrifugation of the enzyme as subunit or as native enzyme, various forms of chromatography, and isoelectric focusing) (1, 8) are accurate indications of homogeneity of this cytochrome P-450 and the possibility (4) of significant contamination by other heme proteins can be excluded. Moreover, the various forms of side chain cleavage P-450, which show the same physicochemical properties except for molecular weight, are apparently immunochemically indistinguishable and represent various states of aggregation of the pure protein. Evidently, the cause of difficulty encountered in determining the heme content of the enzyme does not result from the presence of impurities containing heme and must be sought elsewhere.

Although the anti-P-450 scc reacts with all these various forms of the side chain cleavage enzyme, it does not react with 11β-, 18-hydroxylase P-450 in double diffusion; this enzyme is prepared from the same mitochondria. Again, the anti-P-450 scc inhibits side chain cleavage enzyme activity but not 11β- and 18-hydroxylase activities. The 11β-, 18-hydroxylase shows limited competition with 125I labeled P-450 scc for the anti-P-450 antiserum, but the possibility that this competition is nonspecific cannot be eliminated at this time (Fig. 7). Evidently the antigenic determinants of native P-450 are not revealed by the subunits of the enzyme, since the serum of animals treated with the subunits does not cross-react with P-450 scc in double diffusion (Fig. 2).

Finally, the cytochrome P-450 has been successfully iodinated and unlabeled P-450 competes with the iodinated enzyme for binding to the antibody. This reaction has provided the basis for a sensitive radioimmunoassay for P-450 scc which can detect 0.01 µg of the enzyme. It is proposed to increase the sensitivity of the assay by suitable modification of the iodination procedure.

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