C₂₁ Steroid Side Chain Cleavage Enzyme from Porcine Adrenal Microsomes

PURIFICATION AND CHARACTERIZATION OF THE 17α-HYDROXYLASE/C₁₇,₂₀-LYASE CYTOCHROME P-450

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The properties and the purity of a cytochrome P-450 (17α-hydroxylase) from porcine adrenal microsomes have been examined following a report that the corresponding enzyme from bovine adrenocortical microsomes is inactive as a 17α-hydroxylase and fails to show a high spin spectrum on addition of substrate, once the enzyme has been purified (Bumpus, J. A., and Dus, K. M. (1982) J. Biol. Chem. 257, 12696–12704). The purity of the porcine enzyme was demonstrated by electrophoresis on polyacrylamide with sodium dodecyl sulfate, immunoelectrophoresis, and NH₂-terminal amino acid sequence (16 residues). The pure enzyme shows Mₐ = 54,000, heme content of >0.8 nmol/nmol of protein, and absorption spectra typical of cytochrome P-450. The enzyme is active with both A₄ (progesterone) and A₅ (pregnenolone) substrates as a 17α-hydroxylase and with the corresponding 17α-hydroxysteroids as a C₁₇,₂₀-lyase. All four substrates produce typical type I spectra with the enzyme (so-called high spin form). We conclude that: 1) porcine adrenal microsomes contain a 17α-hydroxylase/C₁₇,₂₀-lyase which is a single protein molecule readily purified to an enzymatically active form; 2) the C₁₇,₂₀-lyase activity is largely suppressed in the microsomes; and 3) the enzyme closely resembles that found in testicular microsomes. We propose that this enzyme be referred to as the adrenal C₂₁ steroid side chain cleavage enzyme.

The adrenal cortex of many mammalian species synthesizes cortisol which requires 17α-hydroxylation of progesterone. By contrast, the gonads synthesize C₁₉ steroids by 17α-hydroxylation followed by C₁₇,₂₀-lyase activity (Scheme 1). Studies from this laboratory have shown that in pig, the hydroxylase and lyase activities of testicular microsomes result from the action of a single cytochrome P-450 (2–4). This observation has recently been confirmed in the guinea pig (5). In setting out to purify the adrenal 17α-hydroxylase, we expected to find an enzyme without C₁₇,₂₀-lyase activity. The limited production of C₁₈ androgens by the adrenal is usually attributed to the activity of a different zone of the adrenal cortex (6), so that we also anticipated the possibility that a second enzyme, resembling the testicular hydroxylase/lyase, might be found in small quantities, in whole adrenal extracts. We were surprised to find that pig adrenal contains a single 17α-hydroxylase which shows C₁₇,₂₀-lyase activity when purified and which cross-reacts with antibody to the testicular enzyme (7). At the same time, Bumpus and Dus (8) reported the isolation of a 17α-hydroxylase from bovine adrenocortical microsomes that loses 17α-hydroxylase activity during purification (8). This enzyme also fails to show a type I spectral shift on addition of substrate; this spectral shift is usually regarded as the normal and inevitable consequence of substrate binding (4, 9). In addition, the purified bovine enzyme was reported to show side chain cleavage activity with cholesterol as substrate (8). In view of the anomalous behavior of the bovine enzyme, we decided to examine the properties of the purified porcine enzyme in greater detail and to compare the enzyme with the hydroxylase/lyase from porcine testicular microsomes on which NH₂-terminal amino acid sequence data are available (4). We report here that the adrenal enzyme can be readily purified in active form, that it shows a typical type I spectral shift with appropriate substrates, that it does not cleave the side chain of cholesterol, and that it closely resembles the testicular enzyme.

EXPERIMENTAL PROCEDURES

Methods for preparing the adrenal enzyme (7), for measuring enzymatic activities (2, 7), and for examining the properties of the cytochrome P-450 (3, 4) have been published elsewhere. The methods used to determine amino acid composition and sequence have also been published (3). NADPH-cytochrome P-450 reductase was purified from adrenal microsomes by the method of Yasukochi et al., devised for preparation of the liver enzyme (10). Antibodies were raised in rabbits as described previously (11). The purified enzyme remains stable for at least 6 months in 20% glycerol (v/v) and 0.2% Emulgen 913 (v/v) in potassium phosphate (50 mM; pH 7.4) at −70 °C. Measurement of side chain cleavage of cholesterol (12) and the preparation of adrenodoxin and adrenodoxin reductase from pig adrenal were performed as described elsewhere (13). The sources of

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materials used, including standard proteins for electrophoresis, have been given previously (2-4).

RESULTS

Purification of 17α-Hydroxylase—Preparation of the enzyme from 100 pig adrenal glands yield 8–10 mg of homogeneous enzyme which represents between 2 and 4% of the total microsomal P-450 (Table I). The enzymatic activity of the 17α-hydroxylase during purification is also shown in Table I. The enzyme shows both 17α-hydroxylase and C17,20-lyase activities with a ratio of hydroxylase to lyase of 2.1 to 2.4 under conditions of Vmax. When [4-14C]cholesterol was added to the enzyme together with NADPH, adrenodoxin, and adrenodoxin reductase, no evidence of side chain cleavage was observed and the substrate could be completely recovered after 30 min incubation at the limits of experimental error. At no stage during the purification procedure (microsomes to pure enzyme) was side chain cleavage of cholesterol observed.

Although the purification procedure used has been given elsewhere (7), the elution pattern for hydroxylapatite is shown in Fig. 1, because fractions from this column were examined in detail. It can be seen that considerable lyase activity is observed at this stage (Table I and Fig. 1). Enzyme activity was measured in the presence of Emulgen so that absolute values cannot be compared with those for the pure enzyme. The purity of the enzyme is demonstrated by four methods. Firstly, electrophoresis on polyacrylamide with sodium dodecyl sulfate in the Laemmli system, with as much as 20 μg of protein/gel, showed a single band when stained with Coomassie blue (Fig. 2). Secondly, immunoelectrophoresis with antibody (lgG) raised against the analogous testicular enzyme shows a single band (Fig. 3). Antibody (lgG) raised against the adrenal enzyme showed a classical line of identity on double diffusion with the purified adrenal 17α-hydroxylase (Fig. 3). Thirdly, Table II shows the first 16 amino acids at the NH2 terminus of the molecule. Each residue appeared as a single amino acid within the limits of the method (5%). Moreover, this NH2-terminal sequence shows considerable homology (15 identical residues out of 16), with that of the analogous testicular enzyme which we have prepared in homogeneous form (3) (Fig. 4). It is interesting to notice that the adrenal enzyme shows an NH2-terminal methionine like a number of other cytochromes P-450 including three from rat liver (P-450a, P-450b, and P-450c) (14). Fourth, antibody (lgG) to the testicular enzyme inhibits both enzymatic activities in parallel (Fig. 5). When the values for three separate studies like that shown in Fig. 5 were pooled and linearized by log-log transformation, the two lines (hydroxylase and lyase activities) showed no difference in slope (p > 0.7). The same result has already been reported for the testicular enzyme (3) and suggests that the two enzymatic activities are associated with a single protein. Similar inhibition was observed with antibody to the adrenal enzyme (not shown).

Properties of the Enzyme—The purified enzyme shows a molecular weight of 54,000 on sodium dodecyl sulfate gels (Fig. 2). A similar value was observed with column chromatography with buffer containing sodium dodecyl sulfate (not shown). The pure enzyme shows a heme content of 13–14 nmol/mg of protein. No significant difference in heme content was observed when measured by pyridine hemochromogen as opposed to CO difference spectra (not shown). The spectral properties of the enzyme are shown in Fig. 6. The enzyme is prepared in the low spin form with an absorbance maximum at 417 nm and no evidence of a high spin shoulder. When 0.5 mg of enzyme was extracted with methylene chloride, no steroids were found when the extract was examined by high pressure liquid chromatography. The oxidized enzyme also shows peaks at 566, 535, and 650 nm. On reduction, the principal change observed involves the formation of a single peak at 545 nm with a decrease and slight shift in the Soret peak (Fig. 6). The reduced CO spectrum shows a shoulder but no peak at 420 nm and a peak at 448 nm. The ratio of A448:420 is 2.3. No evidence of P-420 has been seen in any of our preparations of this enzyme.

Enzymatic activities as a function of substrate concentration with both Δ4 (progesterone) and Δ5 (pregnenolone) steroid substrates are shown in Fig. 7. Both hydroxylase and lyase activities are seen with Δ4 (progesterone) and Δ5 (pregnenolone) substrates. Binding of these substrates to the enzyme is shown in Figs. 8 and 9. It will be seen that all four substrates bind readily to the enzyme and that binding results in a typical type I difference spectrum. The findings from enzyme catalysis and substrate-induced difference spectra are summarized in Table III with corresponding values for the testicular enzyme (2, 4) for comparison. The two enzymes handle all four substrates in a remarkably similar manner. Table III also shows the greater affinity of both enzymes for the two Δ5 substrates when compared to the Δ4 substrates. Given the minor variations from preparation to preparation, the values for the two enzymes (adrenal and testicular) are very similar. With both enzymes, values for K, (concentration required to give half-maximal spectral shift) are lower for Δ5 than for Δ4 substrates.

The amino acid composition of adrenal 17α-hydroxylase is shown in Table IV together with values for the bovine adrenal enzyme and for the analogous testicular enzyme. It is clear that the amino acid compositions of the two porcine enzymes are extremely similar. The values shown are different from those reported by Bumpus and Dus for the enzyme from beef adrenal cortex (Table IV and Ref. 8) except for Pro, Phe, Lys, His, Arg, Trp, and Cys. The molecular weights of the bovine and porcine enzyme calculated from amino acid compositions

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<th>Stage of Purification</th>
<th>Protein mg</th>
<th>Recovery %</th>
<th>Total nmol</th>
<th>Recovery %</th>
<th>Specific Activity</th>
<th>Hydroxylase (H)</th>
<th>Lyase (L)</th>
<th>Ratio H/L</th>
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<td>9.2</td>
<td>0.89</td>
<td>0.2</td>
<td>4.5</td>
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Adrenal 17α-Hydroxylase

Fig. 1. Chromatography on hydroxylapatite. Fractions from CM-Sepharose containing cytochrome P-450 were pooled and dialyzed as described in Ref. 7. The sample was applied to a column of hydroxylapatite (2.1 × 10 cm) and eluted with potassium phosphate (10 mM; pH 7.4) containing 0.2% Emulgen 913 (v/v), 20% glycerol (v/v), 0.1 mM EDTA, and 0.1 mM dithiothreitol (final concentrations). At a phosphate concentration gradient (with the same additions) was started. Fractions (7 ml) were collected and examined for the various properties shown in the figure.

Fig. 2. Electrophoresis of purified adrenal P-450 (17α-hydroxylase) with sodium dodecyl sulfate on Laemmli disc gels. Lanes A and B, standards and molecular weights as follows: phospho-

Fig. 3. Immunochemical reactions of adrenal P-450 (17α-

TABLE II

Microsequence analysis of porcine adrenal cytochrome P-450 17α-

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<th>Cycle</th>
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<th>Yield</th>
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</table>

Adrenal (17α-Hydroxylase) 5

Met-Trp-Val-Leu-Leu-Val-Phe-Leu-Leu-Thr-Leu-Thr-Tyr-Leu-Phe

Testicular (Hydroxylase/Lyase) 5

Met-Trp-Val-Leu-Leu-Phe-Leu-Leu-Thr-Leu-Leu-Thr-Leu-Leu

Fig. 4. NH₂-terminal sequences of porcine adrenal P-450 (17α-hydroxylase) and that of porcine testicular P-450 (hydroxylase/lyase) (3).

Discussion

The method described here for the purification of adrenal 17α-hydroxylase is based upon conventional chromatography. As with the testicular hydroxylase/lyase (2), ion exchange chromatography with different charges (DEAE and carboxymethyl) proved helpful. Preliminary studies revealed that α-amino-octyl-Sepharose exerts some injurious influence on 17α-hydroxylase which makes it impossible to prepare the enzyme in active form. The enzyme is also unstable in the presence of cholate. The adrenal enzyme required five systems of chromatography before the last contaminants could be removed (see “Results”). The purified enzyme retains enzymatic activity, without detectable loss, for at least 6 months if kept in 0.2% Emulgen 913 (v/v) and 20% glycerol (v/v) at -70 °C. The enzyme shows both 17α-hydroxylase and C₁₇,₂₀-

lyase activities during and after purification, although the ratio hydroxylase/lyase decreases as the enzyme is purified (Table I). In adrenal microsomes, lyase activity was not detected with progesterone as substrate and very little lyase activity was seen with 17α-hydroxyprogesterone (hydroxylase/lyase >10) (7). By contrast, testicular microsomes show greater lyase than hydroxylase activity (hydroxylase/lyase 0.54), and this ratio reverses during purification to 2.5 (2); the purified enzymes from both sources (testis and adrenal) show
than for the corresponding A4 steroids. The values for $K_\text{m}$ are present, we have no knowledge of how such an influence typical of other cytochromes P-450. The heme content of 13-

...form in the presence of substrate (4, 9). The properties of the purified enzyme that are attributable to the heme moiety and its interaction with the protein are typical of other cytochromes P-450. The heme content of 13-

approximately the same ratio of hydroxylase to lyase activities (approximately 2.5; mean of three preparations) (this paper and Ref. 2). Since the two purified enzymes are so similar, it seems likely that the difference between the microsomes from the two organs must indicate some difference associated with other components of the microsomal membrane, although, at present, we have no knowledge of how such an influence might be exerted.

The enzymatic activities with $\Delta^4$ and $\Delta^5$ substrates are similar to those observed with the testicular enzyme (Table III and Ref. 2), revealing greater affinity for $\Delta^5$ substrates than for the corresponding $\Delta^4$ steroids. The values for $K_\text{m}$ are consistent with values for $K_\text{c}$ derived from substrate-induced difference spectra (Table III). Moreover, all four substrates induce typical type I spectra (Figs. 8 and 9) so that the cytochrome shows the expected transition to the high spin form in the presence of substrate (4, 9).

The properties of the purified enzyme that are attributable to the heme moiety and its interaction with the protein are typical of other cytochromes P-450. The heme content of 13-

14 nmol/mg of protein corresponds to a specific heme content of $>0.8$ nmol of heme/nmol of protein which is higher than other steroidogenic cytochromes P-450 which lose heme during purification (2, 16, 17). The spectral properties of the adrenal 17$a$-hydroxylase are unremarkable.

The properties of the pure enzyme are of interest in relation to its role in steroid synthesis. In the first place, the preference of the enzyme for the $\Delta^5$ substrates is consistent with the use of the so-called $\Delta^5$ pathway by pig adrenal in *vivo* (18); that is, cleavage of the C17,20 bond precedes, to a large extent, conversion of the $3\beta$-hydroxysteroid to the $\Delta^3$ 3-ketone form (1). The findings with the pure enzyme suggest that the preference for the $\Delta^5$ pathway by the pig may be, at least partly, attributable to the higher affinity of the enzyme for pregnenolone as opposed to progesterone (Table III). The same preference was also seen with the testicular enzyme (4).

In the second place, the low level of lyase activity in the adrenal microsome raises the question of where and how androgens are synthesized in the adrenal. Circumstantial evidence suggests that androgen synthesis may be confined to the zona reticularis (6). According to this view the zona fasiculata would produce 17$a$-hydroxy-C21 steroids by possessing a 17$a$-hydroxylase without lyase activity. By contrast the cells of the reticularis would resemble the steroidogenic (Leydig) cells of the testis in having both hydroxylase and lyase activities; this would lead to the production of C19 androgens. The properties of the pure enzyme suggest that the adrenal 17$a$-hydroxylase is also associated with lyase activity so that the fasiculata has the potential for making androgens. The site and extent of androgen synthesis may therefore be determined by local factors in the endoplasmic reticulum capable of regulating the expression of lyase activity. The important point is that in the present studies we have exhaustively examined discarded fractions of adrenal extracts (i.e. fractions not on the purification pathway), without finding any additional 17$a$-hydroxylase activity unaccompanied by lyase activity. We are forced to conclude that the bulk of adrenal 17$a$-hydroxylase activity is attributable to an enzyme that also possesses lyase activity. If traces of a pure 17$a$-hydroxylase do exist, it is doubtful whether such traces could account for the high production of 17$a$-hydroxy-C21 steroids by porcine adrenal.

**FIG. 5.** Effect of anti-P-450 (IgG) on enzymatic activities of adrenal P-450. Enzymatic activities were measured as described elsewhere (7) and without the concentrations of antibody shown. The antibody was raised in rabbits against testicular microsomal P-450 (2). O, 17$a$-hydroxylase; $\Delta$, C17,20-lyase; ---, anti-P-450 (IgG); ---, preimmune IgG.

**FIG. 6.** Spectral properties of adrenal P-450 (17$a$-hydroxylase). The cuvette contained cytochrome P-450 (1.1 nmol, 84 $\mu$g of protein/ml of buffer). The buffer consisted of potassium phosphate (100 mM; pH 7.4) containing glycerol (20% v/v), EDTA (0.1 mM), and dithiothreitol (0.1 mM). Spectra were taken in a Hitachi 228 dual beam spectrophotometer.

**FIG. 7.** Enzymatic activities of adrenal P-450 (17$a$-hydroxylase). $^{4}$C-steroids (5 nmol; 20,000 cpm) were incubated with adrenal P-450 (17$a$-hydroxylase) (42 pmol), adrenal NADPH-P-450 reductase (0.3 unit as defined in Ref. 10), NADPH (240 nmol) in potassium phosphate (50 mM; pH 7.4) containing Emulgen 913 (0.002% w/v) for 7 min at 37 $^\circ$C. The details of the incubation procedure (2) and the method of separating (7) and measuring (2) the products of the reaction have been given elsewhere.
Adrenal 17α-Hydroxylase

FIG. 8. Substrate-induced difference spectra of adrenal microsomal P-450 (17α-hydroxylase) with Δ⁴ substrates. Each cuvette contained 1.3 nmol of P-450 (0.1 mg of protein) in potassium phosphate (100 mM; pH 7.4) containing 20% glycerol (v/v), EDTA (0.1 mM), and dithiothreitol (0.1 mM). The sample cuvette contained the concentrations of steroid shown in C. The reference cuvette contained ethanol without steroid. C shows double reciprocal plots of mean values for two experiments including those shown in A and B.

FIG. 9. Substrate-induced difference spectra of adrenal microsomal P-450 (17α-hydroxylase) with Δ⁴ substrates. The conditions used are described in the legend to Fig. 8.

Table IV

Amino acid compositions of porcine and bovine adrenal cytochrome P-450 (P-450 (17α-hydroxylase (P-450,OH) and porcine testicular cytochrome P-450 (P-450,OH).

Data for porcine adrenal enzyme from this paper, for bovine adrenal from Bumpus and Dus (8), and for porcine testes from Nakajin et al. (3). The data of Bumpus and Dus refer to their preparation of 17α-hydroxylase.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Porcine adrenal P-450,OH</th>
<th>Bovine adrenal P-450,OH</th>
<th>Porcine testicular P-450,OH</th>
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<td>Asx</td>
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The results reported by Bumpus and Dus (8) for the bovine enzyme are interesting but difficult to interpret. The enzyme shows no 17α-hydroxylase activity and no high spin transition with progesterone. It is therefore either denatured or it represents another enzyme. No evidence has so far been found for any P-450 that is denatured without being converted to P-420. Since the enzyme reported by these authors shows no evidence of P-420, but does show side chain cleavage of cholesterol (8), it may be that the enzyme represents a mitochondrial contaminant, efforts to exclude this possibility not-
withstanding (8). We have made two observations concerning
the preparation of porcine adrenal cytochrome P-450 that
may prove relevant. Firstly, as stated above, ω-amino-octyl-
Sepharose renders the 17α-hydroxylase inactive and the en-
zyme is very unstable in the presence of cholate. Secondly,
the use of a blender to prepare adrenal hemogenate is associ-
ated with considerable release of mitochondrial P-450 and
adrenodoxin.1 The form of the C27 side chain cleavage enzyme
released during homogenization may differ from that of the
same enzyme remaining in the mitochondrial membrane so
that, for example, binding of the two forms of the enzyme to
adrenodoxin may be different (8). Finally, when our procedure
is applied to bovine adrenocortical microsomes, a 17α-hydrox-
ylase is isolated which is enzymatically active and which
shows spectral properties (including substrate-induced differ-
ence spectra) like those reported here (data not shown).
The adrenal enzyme appears closely to resemble the testic-
ular enzyme. The amino acid compositions are very similar
and NH2-terminal amino acid sequence show one difference
in the first 16 residues. Molecular weights, enzymatic activi-
ties, and kinetic and binding constants are all similar (this
paper and Refs. 2–4). However, the two proteins are not
identical as revealed by significant differences in amino acid
composition (Table IV). Determination of complete amino
acid sequence will demonstrate the extent and nature of the
differences between the two enzymes.
Throughout this manuscript, we have referred to this en-
zyme as adrenal 17α-hydroxylase. In view of the findings
presented here and in a preliminary paper (7), we now propose
that the enzyme be referred to as adrenal C21-steroid side
chain cleavage enzyme (17α-hydroxylase/C17,20-lyase), as in
the case of the testicular enzyme. This name can be conve-
niently abbreviated to C21SCC.

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   12704
   Sci. 4, 2101–2107
    Biol. Chem. 253, 2907–2931
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    Biophys. Acta 483 236–247
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