Vol. 111, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1983 Pages 512-517

PURIFICATION AND PROPERTIES OF 17α-HYDROXYLASE FROM MICROSOMES OF PIG ADRENAL: A SECOND C_{21} SIDE-CHAIN CLEAVAGE SYSTEM

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Received January 17, 1983

We have purified a cytochrome P-450 from microsomes of pig adrenal glands to homogeneity (11.1 n moles heme/mg protein) as demonstrated by electrophoresis on polyacrylamide gels with sodium dodecyl SO_4 and by line of identity with an antibody using double diffusion. The enzyme shows both 17α-hydroxylase and $C_{17,20}$-lyase activities and therefore constitutes a C_{21} side-chain cleavage system like that previously purified in this laboratory from neonatal pig testis. Antibody to the testicular enzyme cross-reacts (line of identity) with both enzymes. It is concluded that the adrenal enzyme is the same or very similar to the testicular enzyme, that each enzyme possesses two enzymatic activities, and that microsomes provide some regulatory device to limit the lyase activity of the enzyme *in vivo*. No evidence was found for the usually accepted existence of an adrenal steroid 17α-hydroxylase without lyase activity.

The adrenal cortex is known to synthesize predominantly C_{21} steroids with limited production of C_{19} (androgens) and C_{18} (estrogens) steroids. In many species including man and pig, the predominant glucocorticoid is a 17α-hydroxy-C_{21} steroid (cortisol). By contrast the gonads of all vertebrate species examined show 17α-hydroxylase activity. This enzyme prepares the $C_{17,20}$ bond for cleavage by a lyase to give C_{19} androgens (1). When cytochrome P-450 from neonatal pig testis was purified and characterized in this laboratory, we were surprised to discover one enzyme with both 17α-hydroxylase and $C_{17,20}$-lyase activities (2, 3). Moreover, the enzyme contains one

This work was supported by a grant from the Ministry of Education (The Otani Research Grant), and by a grant from the National Institutes of Health, Bethesda, MD 20205 (Grant AM 28113).

0006-291X/83 $1.50

heme group per subunit and possesses a single active site (4). This discovery made the corresponding adrenal enzyme of interest since the adrenal cortex catalyzes 17α-hydroxylation without lyase activity (1). The limited production of sex steroids by the adrenal may result from activity in a different zone (reticularis) (5). We decided to purify 17α-hydroxylase from pig adrenal microsomes only to discover that the pure enzyme also shows lyase acitivity which is largely suppressed in the microsomes. Moreover, antibody (IgG) to the testicular C_{21} side-chain cleavage P-450 cross-reacts with both the adrenal and testicular enzymes.

EXPERIMENTAL PROCEDURE

Purification of the C_{21} Side Chain Cleavage P-450. Microsomes from pig adrenal were prepared and extracted with cholate as described elsewhere (2). The extract was diluted with two volumes of aqueous glycerol (20% v/v) and applied to a column of DEAE-cellulose (31 X 5.8cm) equilibrated with the following buffer: sodium cholate (0.4% w/v), glycerol (20% v/v), EDTA (0.1 mM), dithiothreitol (0.1 mM) in potassium phosphate (20 mM; pH 7.4). Elution was continued with this buffer which removed from the column all P-450 in one fraction. The P-450 showed 21-hydroxylase and 17α-hydroxylase activities. The fractions containing P-450 were pooled and dialyzed against the above buffer except that the concentration of phosphate was reduced to 10 mM. The sample was applied to a column of DEAE-sepharose CL-6B (22 X 2.8 cm) which was equilibrated with the dialysis buffer. The column was eluted with the same buffer except that the concentration of phosphate was raised to 20 mM which resulted in elution of a fraction containing P-450 active in 17α-hydroxylation but free of 21-hydroxylase. The active fractions were pooled and applied to a column of CM Sepharose 6B (15 X 2.1 cm) equilibrated with the following buffer: Emulgen 913 (0.2% v/v), glycerol (20% v/v), EDTA (0.1 mM), dithiothreitol (0.1 mM) in potassium phosphate (20 mM; pH 7.4). The column was eluted with the same buffer using a phosphate concentration gradient of 20 to 200 mM. Fractions containing P-450 were pooled, dialyzed against the eluting buffer containing 10 mM phosphate and applied to a column of hydroxylapatite (10 X 2.1 cm) equilibrated with the dialyzing buffer. Elution was performed with a phosphate concentration gradient of the same composition containing 10 to 200 mM phosphate. The P-450 fraction was dialyzed and applied to CM-sepharose CL-6B exactly as described above. A single peak of P-450 was obtained. This enzyme is the C_{21} side-chain cleavage P-450 that forms the subject of this paper. It is free of 21-hydroxylase activity and was stored in a buffer containing Emulgen 913 (0.2%, v/v) glycerol 20% (v/v) and potassium phosphate (50 mM; pH 7.4). Detergent was removed as described elsewhere (2).

Miscellaneous. Other methods used and the sources of chemicals have been given elsewhere (2).

RESULTS

Purity. The enzyme is pure by two criteria - electrophoresis on polyacrylamide gel with sodium dodecyl SO_4 which reveals a single band

(Figure 1, left) and by line of identity with the analagous testicular enzyme (2) using antibody to the testicular enzyme (2, 3) (Figure 1, right). Notice that bands formed by antibody against the two ligands touch (line of identity). The enzyme does not cross-react with 21-hydroxylase from pig adrenal microsomes. The enzyme shows a molecular weight of 53,000 ± 1,000 on gels prepared by the method of Laemmli (7).

Enzyme Activity. Figure 2 shows the hydroxylase activity of pig adrenal microsomes incubated with [4-^{14}C]progesterone and 17α-hydroxy [4-^{14}C]progesterone. 17α-hydroxylase activity (17α-hydroxyprogesterone plus 11-deoxycortisol) is seen with progesterone and limited $C_{17,20}$-lyase activity is seen with 17α-hydroxyprogesterone (androstenedione).

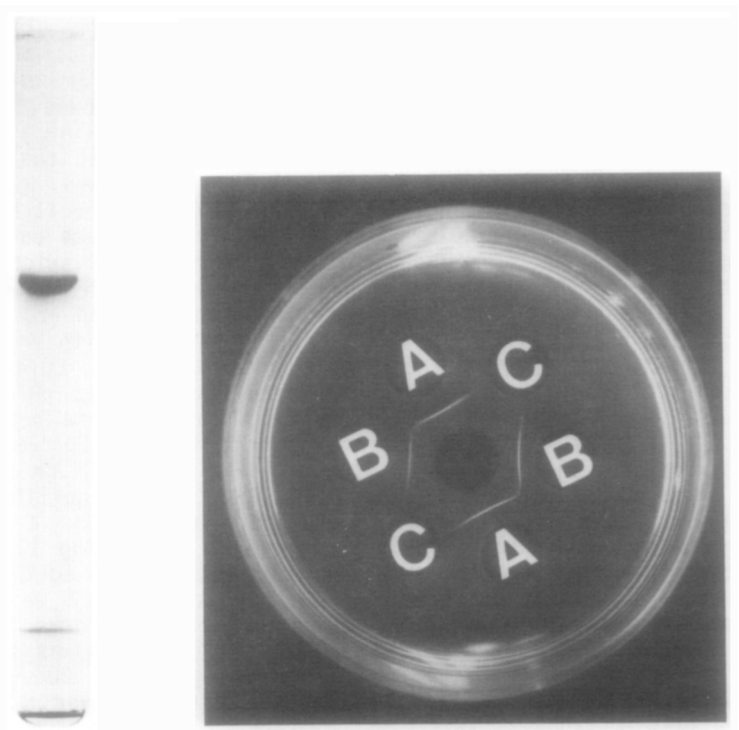

Figure 1. C_{21} Side-chain Cleavage P-450 from Pig Adrenal Microsomes: Evidence of Purity. Left: Electrophoresis on polyacrylamide gel (10) with sodium dodecyl SO_4 (0.1%). Gels were prepared and developed by the method of Laemmli (7). The sample (6μg) was boiled for one minute in 1% sodium dodecyl SO_4 with 0.1% 2-mercaptoethanol. The gel was stained with Coomassie blue. Right: Double diffusion in agar. Center Well: Anti P-450 serum to testicular C_{21} side-chain cleavage P-450 (25μl); Well A: P-450 C_{21} side-chain cleavage from pig testis (7.25μM); Well B: P-450 C_{21} side-chain cleavage from pig adrenal gland (6.8μM); Well C: P-450 21-hydroxylase from pig adrenal gland (6.7μM).

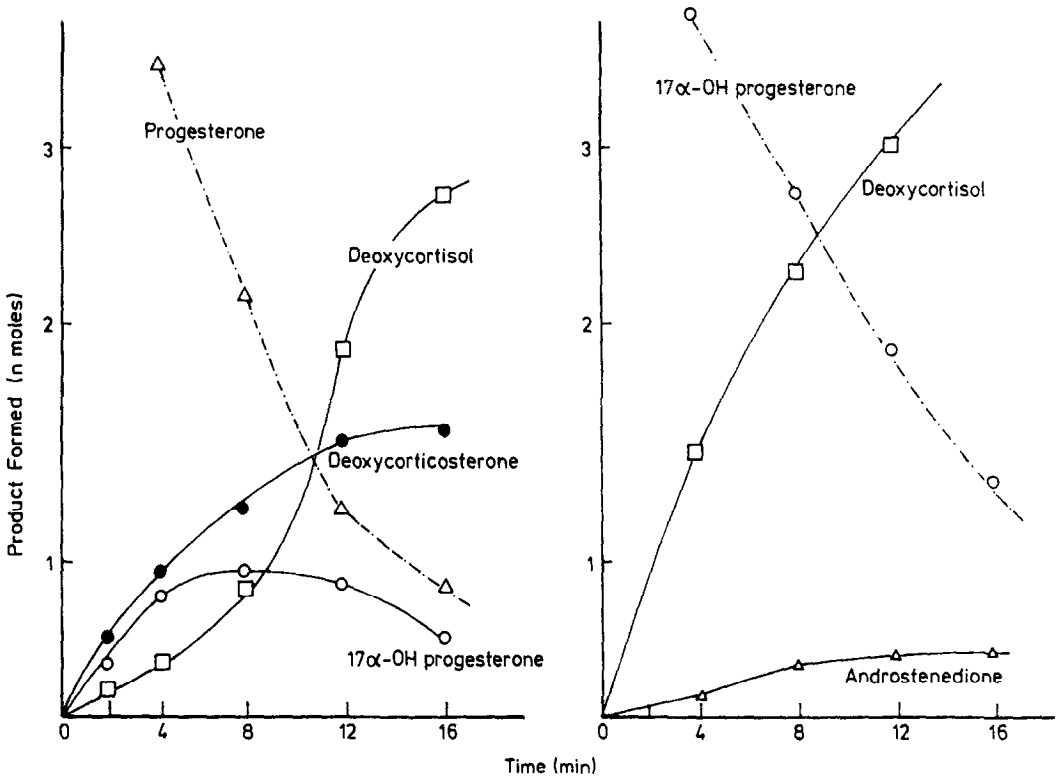

Figure 2. Metabolism of Progesterone and 17α-Hydrodxyprogesterone by Microsomes from Pig Adrenal. Adrenal microsomes (200μg protein per flask) were incubated with [4-^{14}C]progesterone (left) or 17α-hydroxy-[4-^{14}C]progesterone (right), (5 n moles : 4 X 10^4 cpm) with glucose-6-PO_4 5μ moles, glucose-6-PO_4 dehydrogenase 1 unit, $MgCl_2$ 0.5μ moles, NADPH 240 n moles and potassium phosphate 50mM pH 7.4 to a total volume of 1.0ml. Following incubation, steroids were extracted with methylene chloride and applied to thin layer chromatograms in the systems ethylacetate/hexane (3:7, v/v). Steroids were identified and ^{14}C was measured as described elsewhere (2).

Both substrates were hydroxylated at C_{21} (11-deoxycortisol and 11-deoxycorticosterone). In each case disappearance of substrate was accounted for, within the limits of experimental error, by the sum of the products shown. It should be noticed that no lyase activity was seen with progesterone and little of this activity with 17α-hydroxy-progesterone (Figure 2). With purified C_{21} side-chain cleavage enzyme (Table) hydroxylase and lyase activities were seen with progesterone and pregnenolone and lyase activity with 17α-hydroxyprogesterone and 17α-hydroxypregnenolone. The ratio of hydroxylase/lyase is approximately 3, whereas the corresponding ratio for microsomes is >10 (Figure 2).

TABLE I

Reconstitution of Steroid 17α-hydroxylase and $C_{17,20}$lyase Activities Using Homogeneous P-450 from Pig Adrenal Microsomes

Substrates	Enzyme Activities (n mol/min/n mol P-450)		
	17α-hydroxylase	$C_{17,20}$lyase	Hydroxylase/Lyase
Δ^4-substrates	2.66 ± 0.01	0.83 ± 0.07	3.2
Δ^5-substrates	1.43 ± 0.63	0.54 ± 0.08	2.7

Purified C_{21} side-chain cleavage P-450 (40 p moles) from pig adrenal microsomes was incubated with [^{14}C] substrates as described elsewhere (2). The substrates were [4-^{14}C]progesterone, 17α-hydroxy-[4-^{14}C]progesterone (Δ^4) or [4-^{14}C]pregnenolone, 17α-hydroxy-[4-^{14}C]pregnenlone (Δ^5). Activities were measured under conditions giving Vmax.

Again, the products shown account for the disappearance of substrate. The spectral properties of the enzyme will be published in detail elsewhere (6). However as prepared by our method, the enzyme is spectrally a typical low spin cytochrome P-450 with a specific heme content of 11.1 n moles of P-450 per mg protein or 0.6 n moles of heme per n mole of protein (MW 53,000).

DISCUSSION

The C_{21} side-chain cleavage enzyme discussed here is homogeneous according to the two procedures of greatest resolution for membrane-bound proteins, names electrophoresis on polyacrylamide gels with SDS and the immunochemical procedure of double diffusion (Figure 1). The pure enzyme shows no 21-hydroxylase activity and no other activity with progesterone or with 17α-hydroxyprogesterone since disappearance of substrate could be accounted for by production of the appropriate products (Table). It is of interest to notice that adrenal microsomes show limited lyase activity (Figure 2) suggesting that these structures contain some regulatory influence upon the expression of enzyme activity. Since this influence specifically affects lyase activity rather than hydroxylase activity, the underlying mechanism will be of great interest. The limited lyase activity seen with 17α-hydroxyprogesterone may contribute to the low

production of adrenal androgens *in vivo*. However the values observed were obtained with saturating concentrations of substrate and there is no evidence to show that such high concentrations of 17α-hydroxyprogesterone occur in microsomes *in vivo*. During the isolation of the hydroxylase/lyase enzyme, no evidence was found for the existence of a 17α-hydroxylase which remained without lyase activity after release from the microsomal membrane. Presumably 17α-hydroxy-C_{21}-steroids are synthesized by means of the hydroxylase/lyase reported in this communication and microsomes posses some mechanism for the suppression of the lyase activity.

Some comments concerning the purification procedure are important. Firstly the enzyme was prepared from whole adrenal because it is difficult to separate cortex from medulla in the pig, and because we were anxious to use pig adrenal in order to facilitate comparison with other steroidogenic cytochromes P-450 which we are now preparing from this species. It is also important to use fresh tissue because the enzyme is not stable until purified. Some stabilization can be achieved by saturating microsomes with substrate but this causes formation of the high spin form of the P-450 and is difficult to remove.

We are now preparing to determine the amino acid sequence of the enzyme in order to compare this protein with the corresponding testicular enzyme. We are also investigating the mechanism by which microsomes regulate the lyase activity of the enzyme.

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