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IDENTIFICATION OF RABBIT MICROSOMAL CYTOCHROME P-450 ISOZYME, FORM 1, AS A HEPATIC PROGESTERONE 21-HYDROXYLASE

Hermann H. Dieter[1], Ursula Muller-Eberhard[2], and Eric F. Johnson[3]

Department of Biochemistry
Scripps Clinic and Research Foundation
La Jolla, California 92037

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Six highly purified forms of rabbit microsomal cytochrome P-450, isolated from hepatic microsomes, exhibit differences in the regiospecific metabolism of progesterone. Only one of the isozymes studied, form 1, catalyzes the formation of deoxycorticosterone from progesterone at an appreciable rate. This cytochrome P-450 isozyme may participate in the conversion of progesterone to deoxycorticosterone during pregnancy. All six forms of cytochrome P-450 catalyze 6β- and 16α-hydroxylation at the two concentrations of progesterone tested. Form 3b exhibits a lower apparent Km for 6β-hydroxylation than the other five.

During pregnancy progesterone is secreted into the maternal circulation in gradually increasing amounts. The resultant rise in the plasma concentration of progesterone is accompanied by an increase in the concentration of deoxycorticosterone, a hormone formed by the 21-hydroxylation of progesterone (1). Deoxycorticosterone affects sodium retention and is normally synthesized in the adrenal cortex where it serves as a precursor for the formation of other steroid hormones, eg. cortisol, corticosterone, and aldosterone. However, the elevated plasma levels of deoxycorticosterone observed during pregnancy appear to arise from extra-adrenal conversion of the circulating progesterone to 21-hydroxyprogesterone (1, 2). Dey and Senciall have shown that rabbit liver microsomes catalyze the 21-hydroxylation of progesterone (3) and that this reaction pathway

Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; SDS PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

[1]Present Address: Institute for Toxicology, University of Dusseldorf, Moorenstrasse 5, 4000 Dusseldorf 1, W. Germany.

[2]Present Address: Department of Pediatrics, New York Hospital-Cornell Medical Center, 525 East 68th Street, New York, New York 10021.

[3]To whom correspondence should be addressed.

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contributes significantly to the metabolic fate of exogenously administered progesterone in this species (4).

In the adrenal cortex, the formation of deoxycorticosterone from progesterone is catalyzed by a microsomal form(s) of cytochrome P-450. However, it has been suggested that cytochrome P-450 does not participate in the 21-hydroxylation of progesterone in rabbit liver because of the insensitivity of this reaction to inhibition by carbon monoxide (5). The availability of highly purified preparations of six electrophoretically distinct forms of cytochrome P-450 enabled us to examine directly whether any of these forms catalyzed the 21-hydroxylation of progesterone as well as to characterize differences in the regiospecific metabolism of progesterone among these forms of cytochrome P-450.

METHODS

Cytochrome P-450 forms 2(6), 3b(7), 4(8), and 6(7) as well as cytochrome P-450 reductase(6) were isolated from rabbit liver microsomes as described in the references cited. Form 3c was isolated as a side-product of the preparation of form 2 by a procedure which will be published elsewhere. Form 1 is a side-product of the preparation of form 3b from liver microsomes of untreated rabbits. In this procedure, form 1 is obtained from the DEAE-cellulose chromatography step(7) in which form 3b is retained on the column and form 1 is not bound. The cytochrome eluting from the column in the equilibration buffer is adsorbed to calcium phosphate gel. The gel is subsequently washed with detergent free buffer and the cytochrome is eluted in 0.3M potassium phosphate, pH 7.4, containing 20% glycerol as has been described previously for the preparation of other cytochromes (7). The specific contents of all of the cytochrome preparations used in this study exceeded 15 nmol/mg and each preparation exhibited a single major band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS PAGE) .

Each cytochrome was reconstituted with reductase in the presence of dilauroyl-L-α-lecithin as described in earlier publications (6-8). The reaction mixture contained 0.1 µM cytochrome P-450, 0.17 µM cytochrome P-450 reductase, 30 µg/ml dilauroyl-L-α-lecithin, 500 µM NADPH, and [^{14}C]progesterone in 1 ml of 50 mM potassium phosphate buffer, pH 7.4. Progesterone was added to the reaction mixture in methanol (1% final concentration). The [4-^{14}C]progesterone was obtained from Amersham with a specific activity of 56 mCi/mol, and the radiochemical purity was greater than 99% as determined by thin layer chromatography. The reaction was initiated following a preincubation of 3 minutes at 37° by the addition of NADPH and was terminated following a 1-5 min incubation at 37° by the extraction of the substrate and products. Reaction times were adjusted so that less than 10% of the substrate was utilized.

Progesterone and its metabolites were extracted from the reaction mixture into 9 ml of chloroform, and an aliquot of the organic phase was dried under a stream of nitrogen at 45°. Progesterone and its metabolites were redissolved in 25µl of ethylacetate and separated by thin layer chromatography on silica gel (IB2-F, Bakerflex) using two solvent systems in succession as described by Menard and coworkers(9). The substrate and reaction products were visualized by autoradiography, and those portions of the silica gel identified as containing progesterone or its metabolites were transferred to a scintillation vial containing OCS(Amersham) for quantitation by liquid scintillation counting. The recovery of progesterone and its metabolites routinely exceeded 90%. The amount of each metabolite was corrected for overall yield.

Table 1

Regiospecific Metabolism of Progesterone as Catalyzed by

Six Forms of Rabbit Microsomal Cytochrome P-450

	P-450 Form					
10 μM Progesterone	1	2	3b	3c	4	6
6β-Hydroxylation	0.35	0.07	2.5	0.04	0.1	0.6
16α-Hydroxylation	0.2	0.07	2.5	0.02	0.06	0.2
21-Hydroxylation	5.8	<0.01	<0.01	<0.01	<0.01	0.2
45 μM Progesterone	1	2	3b	3c	4	6
6β-Hydroxylation	0.7	0.2	2.5	--	0.6	1.2
16α-Hydroxylation	0.5	0.2	4.7	--	0.2	0.3
21-Hydroxylation	7.6	<0.01	<0.01	--	<0.01	0.3

The values shown are the average rates for the formation of each product (μM/min/μM P-450) at the initial concentration of progesterone indicated for at least two different preparations of each isozyme. Assay procedures are described in Methods.

Preliminary identification of the metabolites was made by a comparison of their relative mobilities to those of authentic standards. The identification of deoxycorticosterone was corroborated by mass spectral analysis of the metabolite eluted from the silca gel plate. The principal ion was detected at (M/e) 299 (M-31) and other prominent ions were seen at M/e ratios of 271, 253, 229, 161, and 147. Mass spectra were determined using a Finnigan model 4021 GC/EI-CI/MS in the direct injection mode with electron impact ionization (70 eV).

RESULTS AND DISCUSSION

The initial rates of formation of the three principal products of progesterone metabolism by the six isozymes at two initial concentrations of progesterone are shown in Table 1. The six isozymes are: form 2, the principal form induced by phenobarbital in the liver (10, 11) which is also a major form in lung microsomes isolated from untreated rabbits (12, 13); forms 4 and 6, which are differentially induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in a tissue and age specific manner (14, 15); and forms 1, 3b (7, 15, 16) and 3c (17-19) which represent constitutive forms in the liver. The metabolism of progesterone required the reductase, cytochrome P-450, and NADPH.

All six forms catalyze the hydroxylation of progesterone at carbon atoms 6 and 16. Of the six forms, form 3b exhibits the highest turnover number for 16α-

hydroxylation and 6β-hydroxylation. These rates are similar to those reported by Koop and Coon (16) for the 6β- and 16α-hydroxylation of testosterone by form 3b. Saturation is evident at the lower concentrations of progesterone for 6β-hydroxylation as catalyzed by form 3b suggesting that this form exhibits a lower apparent Km for 6β-hydroxylation than the other forms.

Of the six isozymes only form 1 catalyzed the 21-hydroxylation of progesterone to a significant degree. When the dependence of 21-hydroxylation on the concentration of progesterone was examined, a linear double-reciprocal plot was obtained which yielded a maximum velocity of 7.1 μM/min/μM P-450 and an apparent Km of 1.7 μM. This rate is similar to that reported for preparations of cytochrome P-450 purified from bovine adrenal microsomes that catalyze the 21-hydroxylation of either progesterone or 17-hydroxyprogesterone(20, 21). However, the 21-hydroxylation of 17-hydroxylated precursors appears to represent a minor pathway in the adrenal cortex of the rabbit (22), and it is interesting to note that form 1 does not metabolize 17-hydroxyprogesterone to a significant extent (results not shown).

Form 1 was originally isolated from animals pretreated with TCDD as a side-product in the isolation of form 6 and was designated form a (23). More recently, form 1 has been purified from untreated animals as described in Methods. As analyzed by SDS PAGE, this form migrates slightly ahead of form 2 and exhibits a single major electrophoretic band as shown in figure 1. Despite their similar electrophoretic mobilities, antibody to form 2 does not recognize form 1, and the two cytochromes can be distinguished by one-dimensional peptide mapping (14).

Unlike 6β- and 16α-hydroxylation which are considered to be catabolic, the 21-hydroxylation of progesterone results in the conversion of one hormone, progesterone, to another, deoxycorticosterone. These different regiospecific metabolic reactions, therefore, represent divergent pathways. Factors which affect the relative occurrences of the cytochrome P-450 isozymes may, therefore, affect the metabolic fate of progesterone in much the same manner as they affect the balance between activation and detoxification of environmental carcinogens

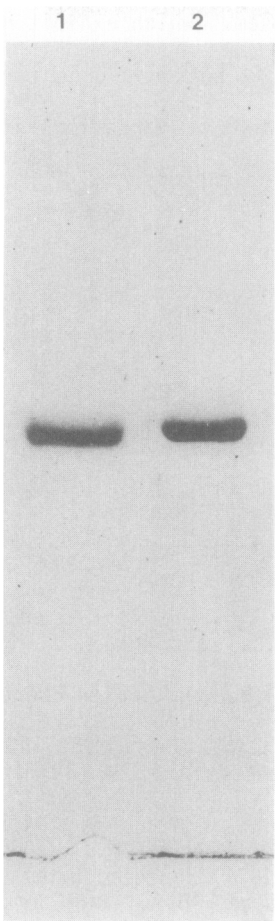

Figure 1. Electrophoretic comparison of cytochrome P-450 forms 1 and 2. Polyacrylamide gel(7.5%) electrophoresis was performed in the presence of sodium dodecyl sulfate as described by Laemmli (24). The nominal dimensions of each track are 1.5 x 10 x 100 mm, and 3 µg of protein were applied to each lane.

and toxins. Winkel et al (2) have determined that significant conversion of progesterone to deoxycorticosterone by extra-adrenal tissues occurs in humans. They have also observed that human individuals differ considerably in this capacity. Many factors such as heredity, diet, hormonal status, and exposure to environmental chemicals are known to alter the relative concentrations of cytochrome P-450 in tissues, and may therefore potentially alter hepatic 21-hydroxylase activity. Recent observations in our laboratory[4] indicate that there is extensive variation among untreated rabbits in hepatic 21-hydroxylase

[4] H.H. Dieter, U. Muller-Eberhard, and E.F. Johnson. Manuscript in preparation.

activity and that liver microsomes which exhibit high rates of progesterone 21-hydroxylase activity serve as a better source for the purification of form 1. These differences are suggestive of variations in the occurrence of form 1 as governed by as yet unidentified factors.

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