Evidence for Concerted Kinetic Oxidation of Progesterone by Purified Rat Hepatic Cytochrome P-450g

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Received September 8, 1987; Revised Manuscript Received February 26, 1988

ABSTRACT: Purified cytochrome P-450g, a male-specific rat hepatic isozyme, was observed to metabolize progesterone to two primary metabolites (6β-hydroxyprogesterone and 16α-hydroxyprogesterone), two secondary metabolites (6β,16α-dihydroxyprogesterone and 6-ketoprogesterone), and one tertiary metabolite (6-keto-16α-hydroxyprogesterone). The $K_{m,app}$ for the formation of these products from progesterone was determined to be approximately 0.5 μM, while the $K_{m,app}$ for metabolism of 6β- and 16α-hydroxyprogesterone was found to be 5–10 μM. The ratio of primary to secondary metabolites did not change significantly at progesterone concentrations from 6 to 150 μM, and a lag in formation of secondary metabolites was not observed in 1-min incubations. Concerted oxidation of progesterone to secondary products without the intermediate products leaving the active site was suggested by these results and confirmed by isotopic dilution experiments in which little or no dilution of metabolically formed 6β,16α-dihydroxyprogesterone and 6-keto-16α-hydroxyprogesterone was observed in incubations containing a mixture of radiolabeled progesterone and unlabeled 6β-hydroxyprogesterone or 16α-hydroxyprogesterone. Incubation of 6β-hydroxyprogesterone with a reconstituted system in an atmosphere of $^{18}O_2$ resulted in >90% incorporation of ^{18}O in the 16α-position of 6β,16α-dihydroxyprogesterone but no incorporation of ^{18}O into 6-ketoprogesterone, even though the reaction was dependent upon enzyme and O_2, and not inhibited by mannitol, catalase, or superoxide dismutase. Factors which characterize the metabolism of progesterone by cytochrome P-450g in terms of active-site constraints and the catalytic competence of the enzyme in microsomes were also explored.

The function of some proteins and enzymes in the homeostasis of an organism sometimes remains a mystery long after the molecules are discovered. A group of enzymes for which this is true is the eukaryotic hepatic cytochrome P-450 family. This enzyme family, which consists of a large number of isozymes, has been shown to oxidize a broad spectrum of substrates (Guengerich et al., 1982; Levin et al., 1984). In addition, some substrates are oxidized by individual isozymes with specific but overlapping regioselectivity (Wood et al., 1983; Wilson et al., 1984). Many of the substrates are xenobiotics which, in addition to being substrates for several isozymes, also induce the synthesis of the proteins (Conney, 1967). Thus, it has been postulated that the role of the enzymes is primarily to function in the biodegradation of foreign substances introduced into the organism. However, some endogenous chemicals such as steroids, fatty acids, and prostaglandins are also substrates for these enzymes. It is also well documented that eukaryotic organisms have highly developed nonhepatic cytochrome P-450 systems which are involved in the synthesis of steroid hormones essential to an organism's homeostasis (Hall, 1985). However, the degree, if any, in which the hepatic cytochrome P-450 isozymes function in this role is not known, although they have been shown to oxidize steroids with high regioselectivity [cf. Wood et al. (1983) and Swinney et al. (1987)] to products whose biological activity is also as yet unknown.

We present data which indicate that a rat hepatic isozyme, cytochrome P-450g, metabolizes progesterone through multiple oxidations without the intermediate products leaving the active site. Although concerted mechanisms are known for two nonhepatic steroidogenic cytochromes P-450 (e.g., aromatase and cytochrome $P\text{-}450_{scc}$) (Hall, 1985), this is the first report of such a mechanism of hydroxylation by a hepatic cytochrome P-450.

Experimental Procedures

Instrumentation. Chemical ionization mass spectra were obtained by using a Finnigan 1015 gas chromatograph/mass spectrometer (GC/MS)[1] equipped with a Finnigan 9500 GC and a Finnigan 6000 data system with Revision I software (Finnigan Instrument Corp., Sunnyvale, CA). Electron-impact mass spectra (m/z 10 to m/z 700) were obtained by using a VG ZAB-2F with a VG 2025 data system.

Chemicals. Progesterone and [4-^{14}C]progesterone (57 mCi/mmol) were obtained from Sigma Chemical Co. (St. Louis, MO) and New England Nuclear (Boston, MA), respectively. 15β-Hydroxyprogesterone was synthesized as previously reported (Swinney et al., 1987). 15α-Hydroxyprogesterone was a gift of Dr. Harold Karns (Upjohn, Kalamazoo, MI). 11β-Hydroxytestosterone and 16α- and 17α-hydroxyprogesterone were obtained from Sigma. 6α-Hydroxyprogesterone was a gift of the Steroid Reference Collection, MCR, London, U. K., and 2α-, 6β-, 11β-, and 21-hydroxyprogesterone and 6- and 16-ketoprogesterone were acquired from Steraloids, Inc. (Wilton, NH).

Dilauroylglycero-3-phosphorylcholine, purchased from Calbiochem (San Diego, CA), was prepared in water and sonicated immediately before use. Organic solvents were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI), with the exception of HPLC-grade tetrahydrofuran which was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). All other reagent-grade chemicals were obtained from Sigma. Oxygen gas (98% $^{18}O_2$) and $H_2{}^{18}O$

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[1] Abbreviations: HPLC, high-pressure liquid chromatography; CI, chemical ionization; GC/MS, gas chromatography/mass spectrometry; 6β-OHP, 6β-hydroxyprogesterone; 6=OP, 6-ketoprogesterone; 6β,16α-diOHP, 6β,16α-dihydroxyprogesterone.

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(98 atom %) were obtained from Cambridge Isotope Laboratories, Inc.

Microsomes, Enzymes, and Antibody Preparations. Male Long Evans rats (8-weeks old) were obtained from Blue Spruce Farms (Altamont, NY). Preparation of microsomes was as previously described (Bandiera et al., 1986). Cytochrome P-450g was purified to electrophoretic homogeneity as previously reported (Ryan et al., 1984).

Cytochrome P-450 determinations were by the method of Omura and Sato (1964) in potassium phosphate buffer, pH 7.4 (0.05 M), with 20% glycerol. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

NADPH–cytochrome P-450 reductase was purified from phenobarbital-treated rats to a specific activity of 40 000 units/mg of protein by a combination of the methods of Dignam and Strobel (1975) and Yasukochi and Masters (1976). One unit of reductase catalyzes the reduction of 1 nmol of cytochrome *c* per minute at 22 °C in 0.3 M potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA and 0.1 mM NADPH.

Production, purification, and characterization of monospecific polyclonal antibody against cytochrome P-450g were as previously described (Bandiera et al., 1986).

Enzyme Assays. Typically, incubations with hepatic microsomes contained cytochrome P-450 (0.2 nmol), NADPH (1 μmol), magnesium chloride (3 μmol), sucrose (10 μmol), potassium phosphate buffer, pH 7.4 (50 μmol), and progesterone (250 nmol in 20 μL of methanol) in a total volume of 1 mL and were agitated for 5 min at 37 °C following addition of substrate. In some experiments, 0.5 nmol of microsomal cytochrome P-450 was incubated for 20 min at 37 °C. An NADPH-generating system consisting of glucose 6-phosphate (5 μmol), glucose-6-phosphate dehydrogenase (2 units/mL), EDTA (0.1 μmol), and NADPH (1 μmol) was included in these experiments.

Incubations with purified cytochrome P-450g (0.02–0.10 nmol), unless otherwise stated, contained NADPH–cytochrome P-450 reductase (1500 units, 0.4 nmol), dilauroylphosphatidylcholine (16 μmol), NADPH (0.5 μmol), magnesium chloride (3 μmol), potassium phosphate buffer, pH 7.4 (50 μmol), and substrate (50 nmol in 20 μL of methanol) in a 1-mL final volume. Samples were incubated at 37 °C for 5 min. All reactions were terminated by adding 1 nmol of internal standard (11β-hydroxytestosterone in 50 μL of methanol followed immediately by 6 mL of methylene chloride. After mixing and centrifugation of the samples, the organic phases were evaporated under a stream of nitrogen, and the residues were dissolved in 200 μL of acetonitrile and analyzed by HPLC.

Incubations with $^{18}O_2$ were performed in screw-capped (Teflon-lined rubber septum) 15 × 125 mm tubes equipped with a glass side arm. The main chamber of the tube contained all the incubation components except the solution of NADPH. Buffers were boiled and stored under an atmosphere of argon prior to use. Reaction mixtures were made anaerobic by alternatively evacuating (80 mmHg) and flushing the tubes with argon a total of 7 times. During the final three cycles, the mixtures were frozen under vacuum in a dry ice/acetone bath. After the system was flushed once with $^{18}O_2$, NADPH was added (20 μL, 0.5 mM) through the septum when the system was under slight negative pressure. The incubation was stopped after 20 min by rapid freezing in acetone/dry ice. Substrate and metabolites were extracted quantitatively in 6 volumes of methylene chloride. Following evaporation of solvent under nitrogen, the sample residues were dissolved in acetonitrile and analyzed by HPLC. The eluate fractions corresponding to each metabolite were combined and evaporated to dryness under a stream of dry N_2, the residues were dissolved in 100 μL of Regisil, and the solution was allowed to stand at 70 °C in a capped tube overnight. The percentage of ^{18}O was then determined by CI GC/MS.

HPLC. Analysis of metabolic reactions was performed by using a Perkin-Elmer Series 4 liquid chromatograph equipped with an ISS-100 autosampler and an LC-95 UV spectrophotometer (254 nm), and data were processed with an LCI-100 laboratory computing integration system.

Separation of progesterone, its metabolites, and the internal standard, 11β-hydroxytestosterone, was achieved by using an IBM 5-μm 25-cm, ODS column preceded by a 5-cm guard column handpacked with ODS as previously reported (Swinney et al., 1987). The column was eluted with water/acetonitrile/tetrahydrofuran/methanol under the following conditions (system I): 3-min isocratic at 85/6/4/5; 25 min with a 0.5 convex gradient to 68/11/8/13; 10 min with a linear gradient to 57.5/14.5/11/17; 10 min with a linear gradient to 15/57/11/17 followed by a 5-min ramp to 3/90/4/3. A second system was used as further evidence for comigration of products (system II): 3-min isocratic 85/13/1/1; 15 min with a 0.5 gradient to 68/30/1/1; and 10 min with a linear gradient to 57.5/14.5/11/17 with the above column and LC setup. In instances when greater sensitivity was needed for determination of kinetic constants, a third system (system III) was used as follows: 3-min isocratic H_2O/acetonitrile (60/40); 5 min with a linear gradient to 40/60; and 5 min with a linear gradient to 20/80 with the above column and LC setup. All chromatographic separations were performed at ambient temperature.

Metabolites were quantitated by comparing the peak height of metabolites with internal standard peak height as previously described (Swinney et al., 1987). When [4-^{14}C]progesterone was used as substrate, peaks corresponding to UV absorbance were collected and the radioactive metabolites quantitated by scintillation spectroscopy.

Results and Discussion

Metabolite Formation and Identification. The products of progesterone metabolism in a system reconstituted with limiting cytochrome P-450g, excess NADPH–cytochrome P-450 reductase, and an optimal concentration of dilauroylphosphatidylcholine were analyzed by reverse-phase HPLC (Figure 1A). We have previously shown that this chromatography system is capable of resolving 17 monohydroxyprogesterones (Swinney et al., 1987). Six metabolites were observed with UV absorbance at 254 nm. Three of the metabolites comigrated with the authentic monohydroxy standards, 6β-, 15α-, and 16α-OHP, and one metabolite with the authentic keto standard, 6=OP. The two polar early eluting metabolites did not comigrate with any available standards. The rate of product formation from the metabolism of progesterone by cytochrome P-450g was determined by UV absorption (Table I) and confirmed by scintillation spectroscopy in incubations containing [4-^{14}C]progesterone. The extinction coefficients for the two early eluting products were determined from experiments with radioactive progesterone. The metabolites were collected, and the trimethylsilyl ether derivatives were analyzed by methane CI mass spectrometry coupled to a gas chromatograph. The mass spectrum associated with each HPLC peak collected was consistent with a single chemical entity. The trimethylsilyl ethers of 6β-OHP and 16α-OHP gave the appropriate molecular weight for the parent ions (*m/z*

Table I: Metabolism of Progesterone Derivatives by Purified Cytochrome P-450g[a]

substrate	products [nmol min^{-1} (mol of P-450g)$^{-1}$]						
	6β,16α	6=O,16α	15α	16α	6β	6=O	unknown[b]
progesterone	3.5	7.9	0.4	12.7	9.3	8.9	
16α-OHP	21.9	11.0					
6β-OHP	3.8	5.5				76.5	
6=OP		11.3					
6β,16α-diOHP		1.0					
15α-OHP							11.5

[a] Incubations containing 0.05 μM cytochrome P-450g, 0.4 μM NADPH–cytochrome P-450 reductase, 16 μM phospholipid, 1 μM NADPH, and 50 μM substrate (with the exception of 6β,16α-diOHP in which substrate concentration was 25 μM) were agitated for 5 min at 37 °C. [b] Unknown products consist of at least three metabolites.

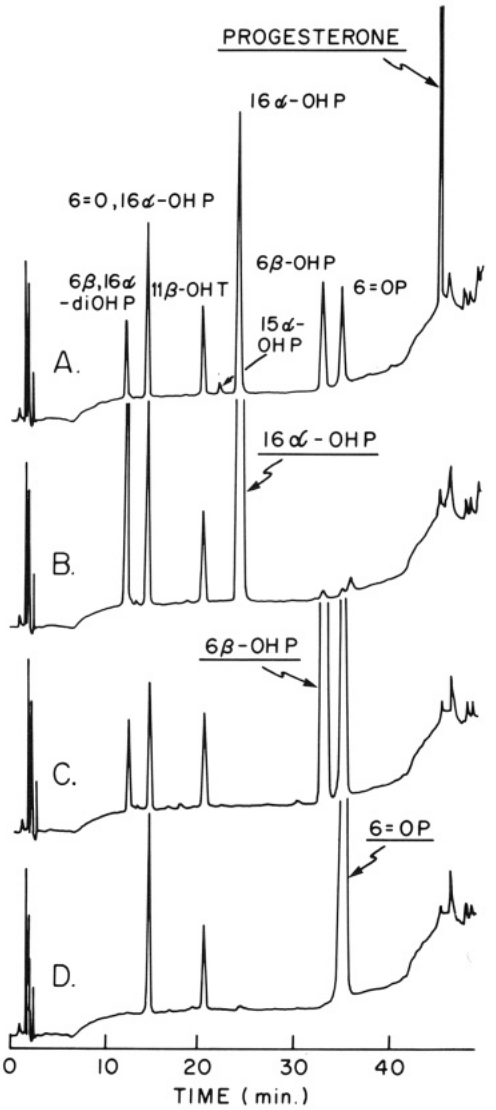

FIGURE 1: Chromatographic profiles of cytochrome P-450g dependent steroid metabolism. Metabolism of (A) Progesterone, (B) 16α-hydroxyprogesterone, (C) 6β-hydroxyprogesterone, and (D) 6-keto-progesterone. Incubations containing 0.05 μM cytochrome P-450g, 0.4 μM NADPH–cytochrome P-450 reductase, 16 μM dilauroyl-phosphatidylcholine, 1 mM NADPH, and 50 μM substrate were agitated for 5 min at 37 °C. One nanomole of 11β-hydroxytestosterone was added as internal standard and the UV absorbance at 254 nm monitored after reverse-phase HPLC separation of metabolites (system I).

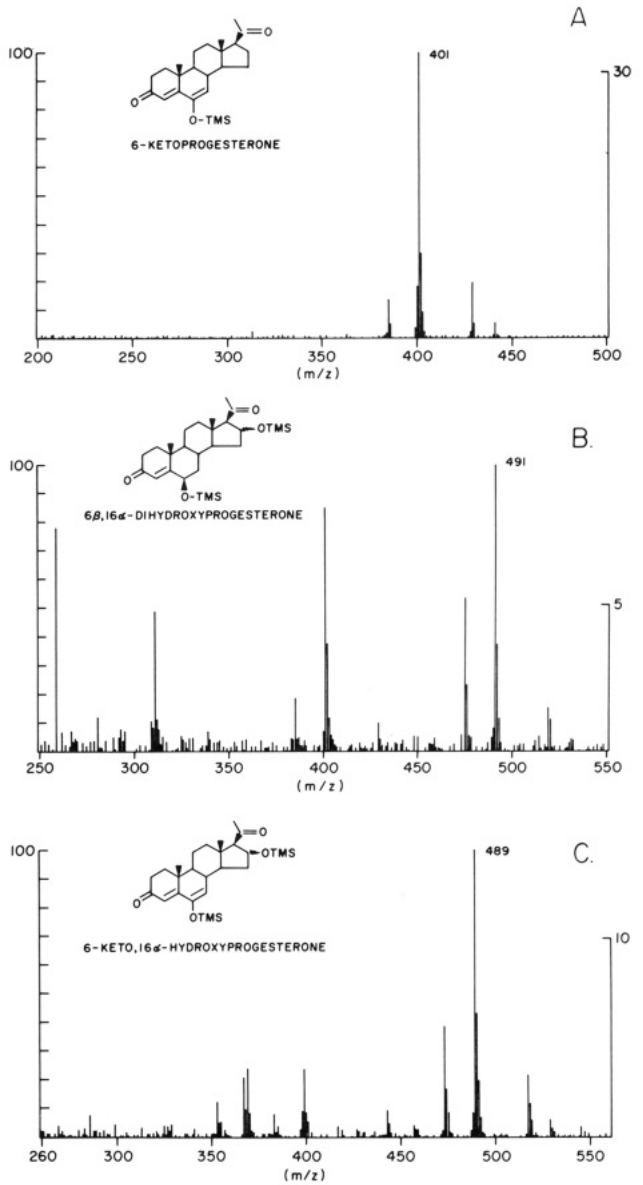

FIGURE 2: Mass spectra of metabolites. Methane chemical ionization mass spectra of the trimethylsilyl ethers of the cytochrome P-450g generated products of progesterone metabolism. (A) 6-Ketoprogesterone; (B) 6β,16α-dihydroxyprogesterone; (C) 6-keto-16α-hydroxyprogesterone.

403), authentic 6=OP and the 6=OP formed by cytochrome P-450g dependent metabolism (Figure 2A) both showed a parent ion of m/z 401, data consistent with the formation of a trimethylsilyl ether of the conjugated enolate of 6=OP. The first peak to elute had a parent ion of m/z 491, indicating the formation of a dihydroxy product of progesterone (Figure 2B), while the second polar peak showed a parent ion of m/z 489, data consistent with the formation of a ketohydroxy product (Figure 2C). The ketohydroxy compound was tentatively identified as a 6-ketohydroxyprogesterone due to the presence of an enolizable keto functionality which was readily silylated.

Electron-impact mass spectrometry again confirmed the identity of the 6-keto product as the mass fragmentation patterns for the nonderivatized metabolite and authentic

compound were identical (data not shown). The first eluting product showed a parent ion at m/z 346 (M^+, 8% relative abundance) and the following daughter ions: 328 ($M^+ - H_2O$, 19%), 313 ($M^+ - CH_3 - H_2O$, 13%), 285 ($M^+ - H_2O - COCH_3$, 18%), 247 ($M^+ - C_5O_2H_7$, 30%), 100 ($C_5O_2H_8^+$, 28%), and 43 ($COCH_3^+$, 100%). The molecular weight of the parent ion is consistent with a dihydroxy metabolite; the daughter ion at m/z 100 was reported by Zaretskii et al. (1966) to be formed from a fragment ion which includes carbons 15, 16, and 17 of the steroid D ring and the substituents bound to these atoms when a hydroxy group is bound to the 15β- or 16α-positions. The daughter ion at m/z 247 indicates the other hydroxyl group is located on the A, B, or C rings of the steroid. The second eluting polar metabolite showed a parent ion at m/z 344 (M^+, 4% relative abundance) and the following daughter ions: 326 ($M^+ - H_2O$, 13%), 311 ($M^+ - CH_3 - H_2O$, 12%), 283 ($M^+ - H_2O - COCH_3$, 14%), 245 ($M^+ - C_5O_2H_7$, 21%), 100 ($C_5O_2H_8^+$, 38%), and 43 ($COCH_3^+$, 100%). All ions except the ones at m/z 100 and 43 were two mass units less than the dihydroxy compound. These data indicate that the ketohydroxy also has a D-ring hydroxy group in the 15β- or 16α-positions as well as a keto group located somewhere else in the molecule. As the CI data suggest, the keto group is at the 6-position; the second eluting compound can be tentatively identified as either 6=O,16α-OHP or 6=O,15β-OHP. However, metabolism of authentic 15β-OHP by cytochrome P-450g did not produce metabolites that were cochromatographic with the dihydroxy or ketohydroxy metabolites formed from progesterone. Hence, the hydroxyl groups in these metabolites are not at the 15β-position.

The formation of all products of cytochrome P-450g dependent oxidation of progesterone was (a) dependent on cytochrome P-450g, O_2, and NADPH, (b) independent of hydrogen peroxide, hydroxy radical, and superoxide anion because catalase (10 μg/mL), mannitol (50 mM), and superoxide dismutase (100 μg/mL) did not inhibit progesterone metabolism, and (c) inhibited by monospecific antibody against cytochrome P-450g (data not shown). Thus, the formation of the dihydroxy metabolite must result from two consecutive oxidations by cytochrome P-450g and the formation of the ketohydroxy metabolite from three consecutive oxidations. Therefore, it is reasonable to assume that these products result from further metabolism of 6β-OHP, 15α-OHP, 16α-OHP, and/or 6=OP. Investigation of the products formed upon oxidation of these compounds gave the following results. The above metabolites were all efficiently oxidized by cytochrome P-450g (Table I). 16α-OHP was oxidized to two products, one which comigrated with the dihydroxy and one which comigrated with the ketohydroxy (Figure 1B). The products were collected and analyzed by GC/MS and found to have the same mass spectral properties as the products formed from progesterone metabolism. Three products were formed from 6β-OHP: one which comigrated with 6=OP, one which comigrated with the dihydroxy metabolite, and one which comigrated with the ketohydroxy metabolite (Figure 1C). The mass spectral properties of these products were also identical with those formed from progesterone. 6=OP was metabolized to one product which comigrated with the ketohydroxy metabolite (Figure 1D). The dihydroxy product, when isolated from products of progesterone metabolism and incubated in the reconstituted system, was observed to form only one product which comigrated with the ketohydroxy compound. 15α-OHP was metabolized to at least three polar metabolites by cytochrome P-450g, but none of these products comigrated

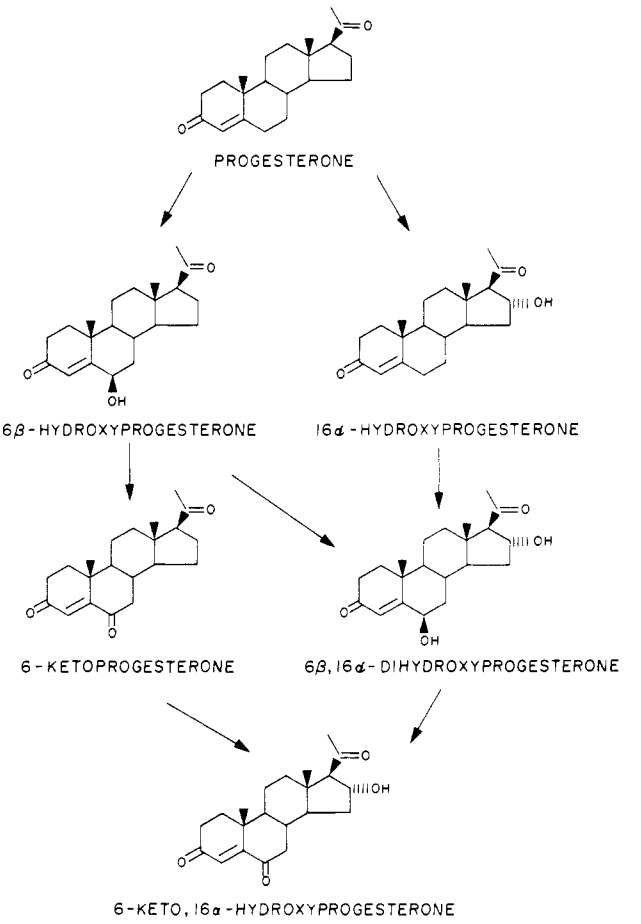

FIGURE 3: Cytochrome P-450g dependent metabolism of progesterone.

with products formed from progesterone metabolism. The metabolites of 6=OP and 6β- and 16α-OHP and the dihydroxy product comigrated with the products of progesterone metabolism in two different reverse-phase HPLC systems (systems I and II) as well as by gas chromatography. In addition, the peaks were always symmetrical in shape, an observation which, together with the integrity of the individual mass spectra, indicates that only one chemical entity eluted in each chromatographic peak.

From the results of the experiments described above, the first polar product was identified as 6β,16α-diOHP while the second eluting polar compound was identified as 6=O,16α-OHP. It is concluded that cytochrome P-450g metabolizes progesterone to two primary hydroxylated products, 6β-OHP and 16α-OHP, which are in turn further metabolized to two secondary oxidation products, 6=OP and 6β,16α-diOHP. 6=OP is formed from 6β-OHP whereas 6β,16α-diOHP can be formed from either 6β-OHP or 16α-OHP. One or both of the two secondary metabolites are oxidized once again to a tertiary metabolite, 6=O,16α-OHP (Figure 3).

Time Course and Kinetics of Product Formation. The time-dependent metabolism of progesterone in a reconstituted system containing cytochrome P-450g was investigated, and the rate of formation of each product was observed to be linear with respect to time for up to 5 min (Figure 4). In addition, no lag in the formation of the secondary and tertiary oxidation products was observed, and the ratio of primary to secondary products did not vary from 2.2% to 56% substrate conversion.

The kinetic constants ($K_{m,app}$ and V_{max}) were determined for the metabolism of progesterone, 6β-OHP, 16α-OHP, and 6=OP under conditions of less than 15% substrate conversion. The Michaelis constants associated with the oxidation of

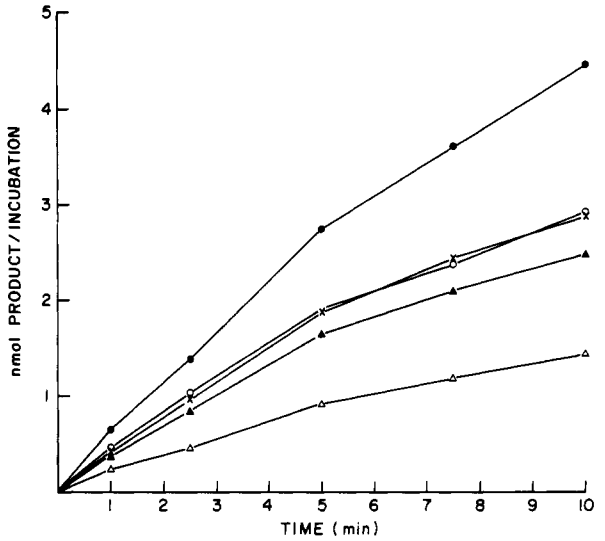

FIGURE 4: Time-dependent metabolism of progesterone by cytochrome P-450g. Incubations contained 0.05 μM cytochrome P-450g, 0.4 μM NADPH–cytochrome P-450 reductase, 16 μM dilauroylphosphatidylcholine, 1 mM NADPH, and 50 μM progesterone. Data are presented as the mean of triplicate incubations. Products of metabolism are 16α-OHP (●), 6=O,16α-OHP (○), 6β-OHP (×), 6=OHP (▲), and 6β,16α-diOHP (△).

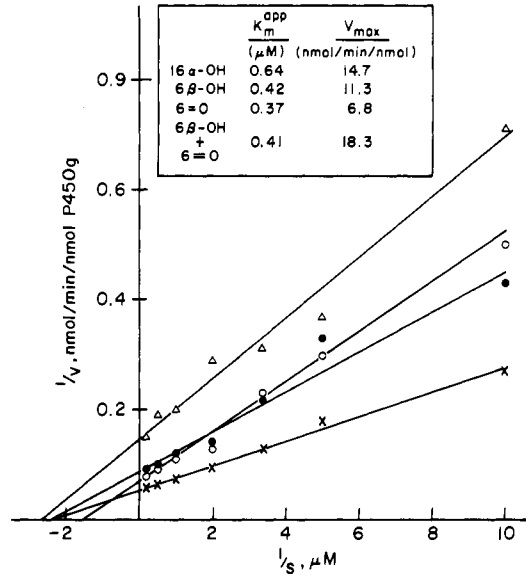

FIGURE 5: Lineweaver–Burk plot of progesterone metabolism by cytochrome P-450g. Incubations containing 1 pM cytochrome P-450g, 0.4 μM NADPH–cytochrome P-450 reductase, 16 μM dilauroylphosphatidylcholine, 1 mM NADPH, and progesterone were agitated 2 min at 37 °C. Conditions were such that metabolism was less than 15% of added substrate. Curve fitting was by an unweighted least-squares linear regression analysis, and r^2 values were >0.95. Products of metabolism are 6=OP (△), 16α-OHP (○), 6β-OHP (●), and 6β-OHP + 6 =OP (×).

progesterone to 6β-OHP, 16α-OHP, and 6=OP were identical within experimental error (0.37–0.64 μM; linear regression analysis, $r^2 > 0.95$) (Figure 5). In contrast, the $K_{m,app}$ for metabolism of 6β-OHP to 6=OP was 11.0 μM ($r^2 > 0.99$) and of similar magnitude to the Michaelis constants associated with the oxidation of 16α-OHP and 6=OP (5.6–11.0 μM, $r^2 > 0.97$) (Figure 6). Thus, the $K_{m,app}$ for metabolism of progesterone by cytochrome P-450g is 10–20 times lower than the $K_{m,app}$ for metabolism of 6β- or 16α-OHP.

Multiple oxidations of a substrate by one enzyme can occur in one of two ways: (1) the first intermediate leaves the active site as product and then competes with the remaining substrate

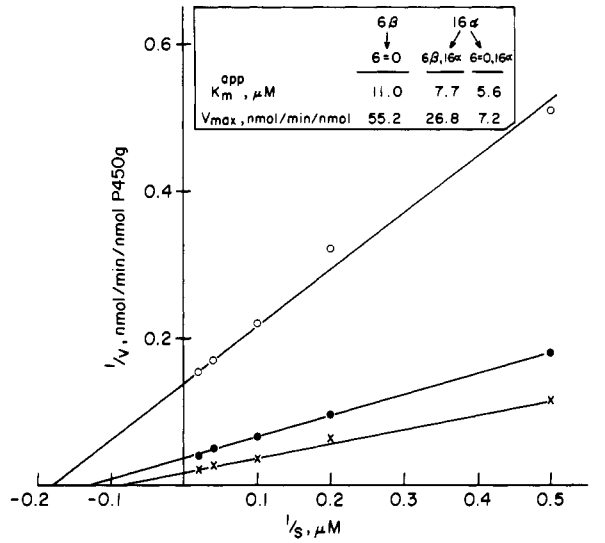

FIGURE 6: Lineweaver–Burk plot of the metabolism of 6β- and 16α-hydroxyprogesterone by cytochrome P-450g. Incubations containing 0.01 μM cytochrome P-450g, 0.4 μM NADPH–cytochrome P-450 reductase, 16 μM phospholipid, 1 mM NADPH, and 6β- or 16α-hydroxyprogesterone were agitated 2.5 min at 37 °C. Conditions were such that metabolism was less than 15% of added substrate. Curve fitting was by an unweighted least-squares linear regression analysis, and r^2 values were >0.98. The product of 6β-OHP metabolism is 6=OP (×), and the products of 16α-OHP metabolism are 6β,16α-diOHP (●) and 6=O,16α-OHP (○).

for further oxidation (sequential mechanism), or (2) the first product or intermediate[2] remains in the enzyme active site following the first enzymatic conversion and is further oxidized before the product vacates the enzyme active site (concerted kinetic mechanism). From the rate constants associated with progesterone metabolism by cytochrome P-450g, a lag in secondary product formation would be expected since the $V_{max}/K_{m,app}$ for the first oxidation (approximately 20 for 16α-OHP and 25 for 6β-OHP) is much greater than for the second oxidation (approximately 3 for 16α,6β-diOHP and 7 for 6=OP). If the first product (intermediate) never leaves the active site, as defined by a converted kinetic mechanism, no observable lag in secondary product formation would be expected, and the $K_{m,app}$ for formation of the secondary product would depend primarily on the initial substrate concentration and not the concentration of the primary product. Thus, the absence of a lag in secondary product formation, together with the observed kinetic constants, suggests a concerted kinetic mechanism for the oxidation of progesterone by cytochrome P-450g whereby progesterone binds tightly to the enzyme and is oxidized to monohydroxy products (intermediates) which in turn can be further oxidized without leaving the active site of the enzyme.

Isotope Dilution Experiments. The above data, while suggestive of a concerted kinetic mechanism as the mode of multiple oxidations of progesterone by cytochrome P-450g, are not conclusive. Therefore, isotope dilution experiments were undertaken to delineate the mode of multiple oxidations by this enzyme. [4-^{14}C]Progesterone was incubated with the enzyme in the presence of equal molar (10 μM) concentrations of either 6β-OHP or 16α-OHP, and the nanomoles of each

[2] It is not within the realm of this investigation to distinguish if intermediate products exist as either the true products (6β-OHP, 16α-OHP, etc.) which never vacate the active site prior to further oxidation, true intermediates which might be short-lived, reactive complexes of oxygenated steroid species and metalloenzyme which lead to products when released from the enzyme, or a species compromising the above extremes.

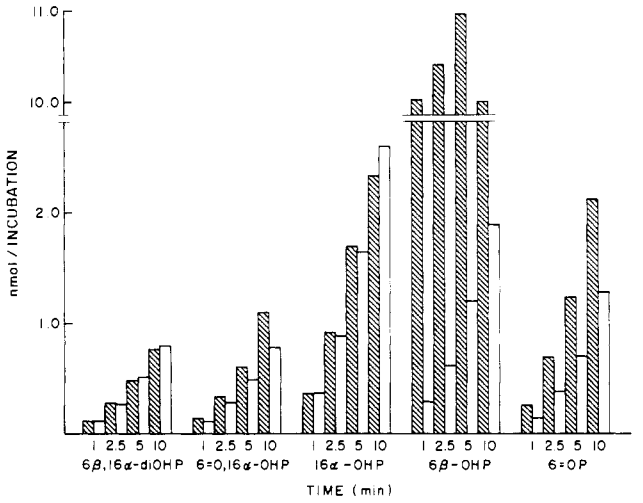

FIGURE 7: Isotopic dilution of cytochrome P-450g dependent [^{14}C]progesterone metabolism with unlabeled 6β-hydroxyprogesterone. Incubations contained 10 μM each substrate, 0.02 μM cytochrome P-450g, 0.4 μM NADPH–cytochrome P-450 reductase, 1 mM NADPH, and 16 μM dilauroylphosphatidylcholine in a final volume of 1 mL. Product formation was determined by UV absorbance at 254 nm (hatched bars) and scintillation spectroscopy (open bars).

product formed from radiolabeled progesterone was determined by scinillation spectroscopy, and the nanomoles of total product formed was determined by UV absorbance.

Figure 7 shows graphically the results obtained when isotopically labeled progesterone is incubated simultaneously with unlabeled 6β-OHP in a reconstituted system containing cytochrome P-450g. At all time points, the [4-^{14}C]-6β-OHP formed was diluted by the unlabeled 6β-OHP initially added to the reaction (35-fold at 1 min, 17-fold at 2.5, 9-fold at 5 min, and 5-fold at 10 min). Thus, the secondary products formed from 6β-OHP should be diluted to the same extent if the reactions were proceeding through a sequential mechanism. No significant dilution of the isotope was observed ($\leq$20%) in the formation of 6β,16α-diOHP or 6=O,16-OHP as well as for 16α-OHP (which serves as an internal control for this experiment). However, a 2-fold dilution was observed for formation of 6=OP at all time points. As 6=OP can only be formed by enzyme-dependent oxidation of 6β-OHP, this product must be coming directly from progesterone through two concerted oxidations. The small dilution of this product indicates that some of the unlabeled 6β-OHP can compete for the active site (as would be expected from the $V_{max}/K_{m,app}$ values, i.e., 25 for formation of 6β-OHP and 7 for formation of 6β=OHP), though it is interesting to note that the dilution of this product does not change from 1 to 10 min (Figure 7) while the isotopic dilution of 6β-OHP changes from 35- to 5-fold, respectively. The absence of significant isotopic dilution of the dihydroxy and ketohydroxy metabolites may result from their being formed exclusively by way of 6β-OHP in a totally concerted manner or their being formed exclusively by way of 16α-OHP or a combination of these two possibilities. Incubation of 16α-OHP as cosubstrate with [4-^{14}C]progesterone led to results which are consistent with a concerted kinetic mechanism for the formation of all products (Table II). The isotopic dilution experiments, in addition to proving that the mechanism of oxidation is concerted, also establish that only one enzyme is catalyzing all the reactions (if more than one enzyme was involved, the 6=O, 6β,16α-diOH, and/or 6=O,16α-OHP would be diluted with unlabeled product).

Dissociation Constants. Progesterone, 6β-OHP, and 16α-OHP all bind to cytochrome P-450g (1 μM) in the presence of dilauroylphosphatidylcholine (16 μM) to give a type I

Table II: Isotope Dilution of Cytochrome P-450g Dependent [^{14}C]Progesterone Metabolism with Unlabeled 6β- and 16α-Hydroxyprogesterone[a]

substrate	compound (nmol/incubation)				
	6β,16α	6=O,16α	16α	6β	6=O
[^{14}C]progesterone	0.39	0.49	1.43	1.12	0.70
[^{14}C]progesterone + 16α-hydroxyprogesterone					
from UV	0.44	0.54	8.79	0.96	0.62
from [^{14}C]P	0.38	0.51	1.24	0.95	0.65
nmol(UV)/nmol([^{14}C]P) ratio	1.2	1.1	7.1	1.0	0.95
[^{14}C]progesterone	0.53	0.61	1.80	1.21	0.88
[^{14}C]progesterone + 6β-hydroxyprogesterone					
from UV	0.48	0.59	1.68	10.86	1.37
from [^{14}C]P	0.51	0.49	1.64	1.20	0.69
nmol(UV)/nmol([^{14}C]P) ratio	0.95	1.22	1.03	9.08	1.99

[a] Five-minute incubations contained 10 μM each substrate, 0.02 μM P-450g, 0.4 μM NADPH–cytochrome P-450 reductase, 16 μM dilauroylphosphatidylcholine, and 1 mM NADPH.

binding spectrum (peak 386 nm and trough 419 nm in the difference spectrum). The dissociation constants (K_s), as monitored by $\Delta OD_{360-660nm}$ upon addition of 0.4–100 μM substrate in 20 μL of methanol, were determined to be 1.2, 3.7, and 9.3 μM for progesterone, 6β-OHP, and 16α-OHP, respectively (linear regression analysis, $r^2 > 0.97$).

For enzymes which produce discrete enzyme-bound chemical intermediates, two general types of mechanisms can function to retain the intermediates at the enzyme active site and thus prevent accumulation of these chemical species in the medium: (1) the intermediates may be stabilized kinetically at the active site, or (2) the intermediate complexes may be bound considerably more tightly than substrate or product, thus preventing competition by substrate or product (Lambeth et al., 1982). The above binding constants indicate that the first mechanism operates for cytochrome P-450g. The intermediate complexes formed with 6β-OHP and 16α-OHP bind with a lesser affinity than does progesterone; therefore, the kinetics associated with oxidation of the intermediate products must be rapid with respect to their ability to dissociate from the enzyme active site. This analysis assumes that the direct binding of the products, 6β-OHP and 16α-OHP, adequately represents the binding of these products when they are formed in the active site from progesterone metabolism.

Oxidation of 6β-OHP in the Presence of $^{18}O_2$. Cytochrome P-450 dependent monooxygenase oxidations involve the activation of molecular oxygen to generate a highly electrophilic molecule of the oxygen termed "oxene". This occurs by an initial two-electron reduction of molecular oxygen followed by loss of a molecule of water, leaving the residual oxygen atom (oxene) bound to the heme iron. In aliphatic hydroxylations, evidence suggests that the bound oxygen atom, which is postulated to be tripletlike in nature, first abstracts a hydrogen atom from the substrate to form an iron-bound hydroxy radical and a substrate radical. Through oxygen rebound, the hydroxy radical combines with the substrate radical to form the hydroxylated product (Guengerich & McDonald, 1984; White & Coon, 1980).

To investigate the mechanism by which 6β-OHP is oxidized by cytochrome P-450g to 6=OP, we incubated 6β-OHP in an $^{18}O_2$ environment. The products of the incubations were collected from HPLC and the trimethylsilyl ethers monitored by GCMS (methane CI) to determine the incorporation of ^{18}O into the products. The percentage of $^{18}O_2$ in the reaction environment was found by determining the incorporation of ^{18}O into the 16α-position of 6β,16α-diOHP. This was observed

to be 100% within experimental error (10%). The amount of ^{18}O incorporated into 6=OP was found to be less than 1%. To investigate the possibility that the keto oxygen in metabolically formed 6=OP was exchanging with solvent water oxygen, the reconstituted system was incubated in 85% $H_2{}^{18}O$ with either progesterone or 6β-OHP as substrate. Isolation of 6=OP and evaluation of ^{18}O incorporation by GC/MS (methane CI) of the trimethylsilyl ether derivatives indicated that no exchange of the keto oxygen had occurred with solvent water oxygen. Incubation of 6=OP in 90% $H_2{}^{18}O$ (and other components of the incubation system minus enzymes) for 3 days followed by solvent extraction and analysis by GC/MS revealed less than 10% ^{18}O incorporation into 6=OP.

We were not surprised by the incorporation of ^{18}O into the 16α-position of 6β,16α-diOHP since this result is consistent with the established mechanism for aliphatic cytochrome P-450 dependent monooxygenase activity. However, the lack of incorporation of molecular oxygen into 6=OP was surprising. As this reaction did not occur in an argon atmosphere or in the absence of cytochrome P-450g or NADPH, and was not inhibited by catalase, mannitol, or superoxide dismutase, we can only conclude that this enzymatic reaction depends upon cytochrome P-450g and O_2.

Hydroxylation at a carbon–hydrogen bond on the same carbon as a hydroxyl group results in the formation of a *gem*-diol, which upon interaction with an acidic or basic molecule loses H_2O to form a keto product. In the absence of steric and electronic factors, the probability of losing either of the *gem*-diol hydroxy groups should be equal, and 50% of the ^{18}O, originating from molecular oxygen, would be retained in the product. In the presence of significant steric and/or electronic effects, all of the oxygen derived from molecular oxygen may be retained or lost. Determination of whether (1) enzyme conformational factors directing the stereochemistry of a *gem*-diol collapse, (2) substrate directs loss of H_2O due to 1,3-diaxial ring interactions,[3] and/or (3) 6=OP arises from a non-*gem*-diol mechanism involving loss of a second hydrogen atom which may be facilitated by the conjugation effects of the adjacent α,β-unsaturated system awaits further clarification.

Cytochrome P-450g Dependent Microsomal Metabolism. After observation of the concerted oxidation of progesterone by cytochrome P-450g in a reconstituted enzyme system, we investigated the potential contribution of this enzyme to the microsomal metabolism of progesterone. Cytochrome P-450g is a noninducible, sex-specific enzyme developmentally regulated in male rats where hepatic microsomal levels of cytochrome P-450g rise from less than 1% in 3-week-old rats to >10% of total cytochrome P-450 in 6-week-old adult animals. There are at least two subpopulations of adult male Long Evans rats, one which expresses low levels (<1%) of cytochrome P-450g and the other high levels (≥10%) (Bandiera et al., 1986). Genetic studies utilizing ACI/Hsd (high P-450g) and WF/Hsd (low P-450g) inbred rats have indicated that high levels of cytochrome P-450g are inherited as an additive trait and the responsible gene is autosomal (Rampersaud et al., 1987).

Microsomal cytochrome P-450g dependent progesterone metabolism was investigated in preparations isolated from individual adult male Long Evans rats with high or low levels of cytochrome P-450g (Table III). Major metabolites in both high- and low-P-450g animals included 16α-OHP and 6β-OHP. The turnover numbers for metabolism at these positions were not influenced significantly by the microsomal content of P-450g. 6=OP was a minor metabolite in both sets of microsomes, the formation of which was not reproducible in duplicate incubations; thus, quantitation of this metabolite was not attempted. 6β,16α-diOHP and 6=O,16α-OHP were not detected as metabolites in microsomes from animals with high or low levels of cytochrome P-450g. Moreover, a monospecific antibody raised against P-450g did not significantly inhibit microsomal progesterone metabolism in either set of microsomes (Table III). These data were somewhat surprising as the activities of other cytochrome P-450 isozymes which efficiently hydroxylate progesterone in the reconstituted system were inhibited in microsomes by their corresponding antibodies (Swinney et al., 1987). Thus, further studies were performed in an attempt to determine why cytochrome P-450g does not metabolize progesterone in intact microsomes.

Table III: Effect of Anti-P-450g IgG on Progesterone Metabolism in Microsomes Containing High and Low Levels of Cytochrome P-450g[a]

% of total P-450 as P-450g	anti-P-450g	progesterone metabolites [pmol min^{-1} (nmol of P-450)$^{-1}$ or (% control act.)]			
		6β,16α	6=O,16α	16α	6β
10.9 ± 1.0	–	ND[b]	ND	1185 ± 275	1090 ± 337
	+			(95)	(100)
0.7 ± 0.1	–	ND	ND	1318 ± 355	1018 ± 261
	+			(87)	(103)

[a] Turnover numbers are expressed as the mean ± standard deviation from incubation of microsomes obtained from individual 10-week-old male rats (n = 3) with previously determined P-450g levels (Bandiera et al., 1986). Incubations contained 0.2 μM P-450, 250 μM progesterone, and 2 mg/mL anti-P-450g IgG and were incubated for 5 min. [b] ND = not detected.

Addition of purified cytochrome P-450g to microsomes with high cytochrome P-450g levels using a 20-min preincubation in an attempt to incorporate the enzyme into the microsomes did not result in an increase in progesterone oxidation (data not shown). Solubilization of microsomes in sodium cholate as well as the addition of antibody to solubilized microsomes (to possibly expose an inhibitory antibody binding site buried in the membrane) did not produce any evidence for cytochrome P-450g dependent microsomal progesterone metabolism. Addition of NADH also did not result in any cytochrome P-450g associated activity (data not shown). All the above experiments were run at a progesterone concentration of 250 μM. It was possible that one or more cytochrome P-450 isozymes of higher $K_{m,app}$ as well as higher efficiency may be metabolizing the substrate to 6β- and 16α-OHP. Therefore, incubations were also run at a substrate concentration of 10 μM, a concentration still above the $K_{m,app}$ for cytochrome P-450g dependent metabolism of progesterone but equal to or below the $K_{m,app}$ of several other isozymes involved in progesterone hydroxylation (Swinney et al., 1987). Once again, no difference was observed in the metabolism of progesterone by microsomes with high or low levels of cytochrome P-450g.

A difference between progesterone metabolism in microsomes with high or low levels of cytochrome P-450g was observed upon addition of purified NADPH–cytochrome P-450 reductase. The tertiary product of cytochrome P-450g catalyzed oxidation of progesterone, 6=O,16α-OHP, was observed to be formed exclusively by microsomes with high levels of cytochrome P-450g and, as shown in Figure 8, increased linearly over 8-fold while the other metabolies increased to a

[3] The steroid B ring is in a chair conformation with the 19-methyl group and 6β-hydroxy group axial. The cis 1,3-diaxial arrangement of these two groups causes a strong repulsion between the two with the result being an adoption of equatorial geometry of the 6β-hydroxy group through loss of the 6α-hydroxy group.

Table IV: Effect of Added NADPH–Cytochrome P-450 Reductase and Anti-P-450g IgG on Progesterone Metabolism in Microsomes Containing High and Low Levels of Cytochrome P-450g[a]

microsomes	NADPH–cytochrome P-450 reductase added	anti-P-450g	progesterone metabolites [pmol min^{-1} (nmol of P-450)$^{-1}$ or (% control act.)]			
			6β,16α	6=O,16α	16α	6β
high P-450g	–	–	169 ± 37	1 ± 2	1746 ± 296	2365 ± 160
	+	–	787 ± 197	31 ± 10	3055 ± 281	3814 ± 145
	+	+	(90)	(0)	(92)	(104)
low P-450g	–	–	216 ± 68	ND[b]	1614 ± 97	3191 ± 518
	+	–	728 ± 129	ND	2511 ± 174	4660 ± 162
	+	+	(104)	ND	(82)	(102)

[a] Turnover numbers are expressed as the mean ± standard deviation from incubation of microsomes obtained from individual 10-week-old male rats (n = 3) with previously determined P-450g levels (Bandiera et al., 1986). Microsomes (0.5 nmol of P-450) were preincubated with or without NADPH–cytochrome P-450 reductase (1.3 nmol) for 20 min at 37 °C. Anti-P-450g (5 mg/mL) was then added, and the samples were preincubated at 25 °C for 10 min. Progesterone metabolism (250 μM) was performed for 20 min at 37 °C. Only progesterone metabolites that are formed by purified cytochrome P-450g in the reconstituted system are shown. [b] ND = not detected.

Table V: Steroid Oxidation by Cytochrome P-450g[a]

substrate	total metabolism [nmol min^{-1} (nmol of cytochrome P-450g)$^{-1}$]	substrate	total metabolism [nmol min^{-1} (nmol of cytochrome P-450g)$^{-1}$]
progesterone	42.6 (6)	15α-hydroxyprogesterone	11.5 (3)
2α-hydroxyprogesterone	30.8 (4)	16α-hydroxyprogesterone	32.9 (2)
6α-hydroxyprogesterone	11.6 (1)	16-ketoprogesterone	3.5 (1)
6β-hydroxyprogesterone	85.7 (3)	17α-hydroxyprogesterone	28.9 (3)
6-ketoprogesterone	11.3 (1)	21-hydroxyprogesterone	46.8 (3)
11β-hydroxyprogesterone	25.2 (4)		

[a] The minimum number of products formed from each substrate is shown in parentheses. Turnover numbers were calculated by assuming equal extinctions for each metabolite at 254 nm. Five-minute incubations contained 0.1 μM cytochrome P-450g, 0.4 μM NADPH–cytochrome P-450 reductase, 16 μM phospholipid, 1 μM NADPH, and 50 μM of each substrate.

much lesser extent in the presence of added NADPH–cytochrome P-450 reductase. Table IV shows the effect of addition of NADPH–cytochrome P-450 reductase (1.3 nmol, 4500 units) and anti-P-450g on the rate of progesterone metabolism by microsomes with high or low levels of cytochrome P-450g. Addition of reductase increased the rate of formation of all products in microsomes with high cytochrome P-450g levels, and 6=O,16α-OHP was increased the most dramatically, from 1 ± 2 to 31 ± 10 pmol min^{-1} (nmol of P-450g)$^{-1}$. The rates of formation of all products were also increased in microsomes with low cytochrome P-450g levels with the exception of 6=O,16α-OHP. This product was not observed in these microsomes irrespective of the concentration of added NADPH–cytochrome P-450 reductase. Addition of anti-P-450g completely inhibited formation of this product in microsomes with high levels of cytochrome P-450g but did not significantly inhibit the formation of any of the other metabolites.[4]

To further pursue the cause of the inactivity of cytochrome P-450g in microsomes without added reductase, we determined the $K_{d,app}$ of purified cytochromes P-450h and P-450g for NADPH–cytochrome P-450 reductase, reasoning that cytochrome P-450g may be inactive in microsomes due to a much higher association constant relative to the other isozymes. However, the $K_{d,app}$ values for cytochromes P-450g and P-450h were similar, approximately 0.2 μM (data not shown), indicating that the lack of activity of cytochrome P-450g in microsomes is probably not due to a low affinity of the hemoprotein for the reductase. The experiments also suggest that the concerted mechanism is not a result of saturating

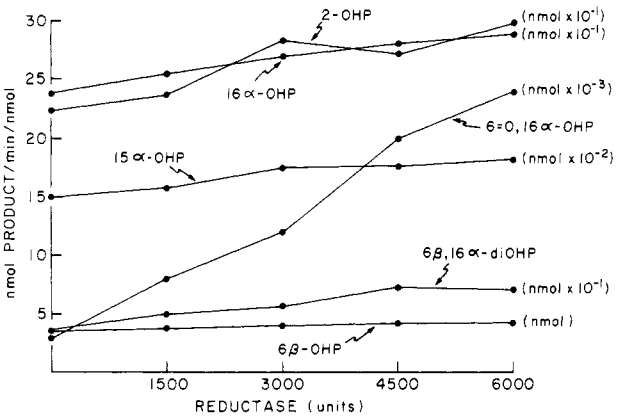

FIGURE 8: Effect of added reductase on progesterone metabolism in microsomes from adult male rats. 0.5 μM microsomal cytochrome P-450 obtained from adult male rats was preincubated for 20 min at 37 °C with NADPH–cytochrome P-450 reductase and then incubated in the presence of NADPH and 250 μM progesterone for 20 min at 37 °C.

NADPH–cytochrome P-450 reductase as the ratio of primary to secondary to tertiary metabolites did not change when the ratio of NADPH–cytochrome P-450 reductase to cytochrome P-450g was less than 1. Although the reason for the lack of catalytic activity of microsomal cytochrome P-450g for progesterone metabolism under normal conditions is unknown at this time, the results presented (Table IV) eliminate the possibility that the substrate selectivity of cytochrome P-450g has been altered during purification. Whether these perplexing results are specific for progesterone metabolism remains to be evaluated. Cytochrome P-450g has relatively poor catalytic activity for several xenobiotic substrates (Ryan et al., 1984), so that the above experiments cannot be performed with another substrate at the present time.

Substrate Selectivity and Active-Site Considerations. The substrate selectivity for cytochrome P-450g dependent oxidation was investigated with a series of derivatives of proge-

[4] Despite the fact that 6β,16α-diOHP is a metabolite of progesterone formed by purified cytochrome P-450g, it is clear from the results in Table IV that other cytochrome P-450 isozymes are responsible for this reaction in microsomes. It is interesting to note that 6β,16α-dihydroxyandrostenedione is a product of the *sequential* oxidation of androstenedione in rat liver microsomes (Sheets & Estabrook, 1985).

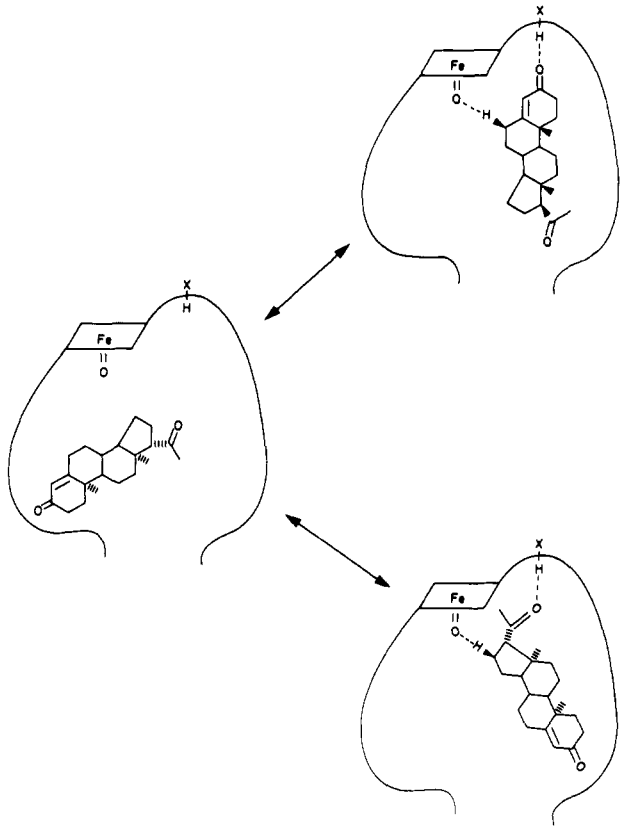

FIGURE 9: Proposed active-site model of cytochrome P-450g.

sterone. The compounds, which included an oxygenated derivative on each of the steroid rings as well as the C-17 side chain, were all efficiently metabolized by the enzyme (Table V). The products formed were not identified in most instances because of a lack of available standards, and as a result, only the minimum number of products could be estimated by the number of peaks absorbing at 254 nm in the two different HPLC systems utilized (systems I and II). It is interesting to note that while this enzyme oxidizes C-21 steroids with high turnover numbers, the many non-steroid substrates previously investigated are relatively poor substrates for this enzyme (Ryan et al., 1984).

Cytochrome P-450g also appears to be unique in that it further metabolizes the primary oxidation products. Wood et al. (1983) reported that the secondary metabolism of several monohydroxytestosterone metabolites by the five cytochrome P-450 isozymes investigated (cytochromes P-450a, P-450b, P-450c, P-450d, and P-450e) was, in general, not accomplished by the isozymes which were responsible for the primary oxidation. For example, cytochrome P-450b catalyzed 16α-hydroxylation of testosterone, but upon incubation of 16α-hydroxytestosterone in a reconstituted system containing cytochrome P-450b, no further metabolism of this product was observed. Cytochrome P-450g, however, appears to be highly flexible in its ability to oxidize potential products of progesterone oxidation.

We have previously postulated that there may be one or more groups in the active site of cytochrome P-450g that can hydrogen bond to the carbonyl functionalities of the molecule to direct 6β- and D-ring oxidations (Swinney et al., 1987). Evidence for this hypothesis came from the fact that the positions of hydroxylation were of similar stereochemical distance from the carbonyl groups and that testosterone, when subject to cytochrome P-450g dependent oxidation, was metabolized predominantly to the 6β-regioisomer and only poorly to D-ring regioisomers. Progesterone, with its two distal carbonyl groups, was metabolized approximately equally at the 6β- and 16α-positions, data which were consistent with the lack of a distal carbonyl associated with the D ring of testosterone. Accordingly, the high capacity of this enzyme for oxygenated steroids may in fact be due to the enzyme's ability to hydrogen bond to these oxysteroids.

Another interesting observation was that 6α-OHP was metabolized to a product that did not comigrate with 6=OP, whereas 6β-OHP was readily oxidized to 6=OP. This suggests that although many potential steroid substrates are oxidized by cytochrome P-450g, the binding of each is highly selective, leading to regioselective product formation. Thus, progesterone can bind in such a manner to achieve hydroxylation at the 6β-position, and 6β-OHP can presumably bind in an orientation conducive to oxidation at the 6α-position (formation of 6=OP), but 6α-OHP cannot bind in an orientation conducive to 6β-oxidation. This suggests that when a substrate binds in an orientation to allow 6-oxidation, presumably through hydrogen bonding at the 3-carbonyl, a steric interaction exists which can prevent binding in a catalytically competent mode if a hydroxy group is bound to the 6-position.

The converted enzymatic mechanism becomes more fascinating when the stereochemical factors which would allow for the multiple hydroxylations are considered. For instance, the concerted hydroxylation to form 6β,16α-diOHP may occur with a first hydroxylation at the 16α-position followed by a hydroxylation at the other end of the molecule, on the opposite face (6β). For this to occur without the molecule leaving the enzyme active site, the molecule must *flip and rotate* in the active site. Accordingly, the active site must have a large enough space to allow this freedom of rotation. Thus, the enzyme must bind with high specificity to allow for highly regioselective metabolism and at the same time allow enough free space for rotation of a large molecule. With these considerations, we propose that the substrate will first bind in the active site in a relatively unrestricted manner and then equilibrate into a catalytically competent conformation with the assistance of hydrogen bonding to carbonyl groups. After hydroxylation, the intermediate product may flip and rotate in the unrestricted active site (while still in the vicinity of the active site it prevents entrance of another substrate molecule) before diffusing into another catalytic competent orientation (Figure 9). This proposal does not preclude that there may be two distinct sites—a binding site and a catalytic site. Research is presently under way in an attempt to further define the factors that influence the unique stereochemical characteristics of this enzyme active site.

It is interesting to note that the process of biosynthesis of pregnenolone from cholesterol by cytochrome P-450$_{scc}$ and the biosynthesis of estrone from androstenedione by aromatase [cf. Hall (1985)] have much in common with the oxidation of progesterone by cytochrome P-450g. The substrates are sterols whose importance in eukaryotic organism's homeostasis is well documented, the enzymes all belong to the cyrochrome P-450 superfamily, each process involves three oxidations by NADPH and molecular oxygen, and the oxidations occur in a concerted kinetic manner.

ACKNOWLEDGMENTS

We thank Dr. Jane Sayer for critical reading of the manuscript and Cathy Michaud for excellent assistance in the preparation of the manuscript.

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Mechanism-Based Inactivation of Horseradish Peroxidase by Sodium Azide. Formation of *meso*-Azidoprotoporphyrin IX†

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Received February 1, 1988; Revised Manuscript Received March 11, 1988

ABSTRACT: Catalytic turnover of sodium azide by horseradish peroxidase, which produces the azidyl radical, results in inactivation of the enzyme with K_I = 1.47 mM and k_{inact} = 0.69 min^{-1}. Inactivation of 80% of the enzyme requires approximately 60 equiv each of NaN_3 and H_2O_2. The enzyme is completely inactivated by higher concentrations of these two agents. *meso*-Azidoheme as well as some residual heme are obtained when the prosthetic group of the partially inactivated enzyme is isolated and characterized. Reconstitution of horseradish peroxidase with *meso*-azidoheme yields an enzyme without detectable catalytic activity even though reconstitution with heme itself gives fully active enzyme. The finding that catalytically generated nitrogen radicals add to the meso carbon of heme shows that biological meso additions are not restricted to carbon radicals. The analogous addition of oxygen radicals may trigger the normal and/or pathological degradation of heme.

The azide anion functions in different situations as a substrate, reversible inhibitor, or irreversible inhibitor of catalytic hemoproteins like catalase and horseradish peroxidase. In the case of catalase, coordination of the azide anion to the prosthetic heme[1] iron atom inhibits the catalytic dismutation of H_2O_2 (Deisseroth & Dounce, 1970). If H_2O_2 is present, however, the azide anion is also oxidized by catalase to nitrous oxide, nitric oxide, and nitrogen gas (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Keilin & Nicholls, 1958; Rein et al., 1968). Spin trapping studies suggest that these azide metabolites are formed by reaction of the catalytically generated azidyl radical with molecular oxygen (Kalyanaraman et al., 1985). In contrast, azide does not bind at neutral pH to the prosthetic heme iron atom of horseradish peroxidase (Morishima et al., 1977). Nevertheless, horseradish peroxidase is at least as vulnerable as catalase to inhibition by this agent because it is inactivated rather than simply inhibited by azide. Brill and Weinryb reported in 1967 that horseradish peroxidase is inactivated by a H_2O_2-independent reaction (but see below) of the azide anion with a methionine residue of the protein. Spin trapping studies have shown that horseradish peroxidase, like catalase, oxidizes the azide anion to the azidyl radical (Kalyanaraman et al., 1985). The one-electron oxidation of azide appears to be a general peroxidatic reaction because it is also catalyzed by chloroperoxidase, myeloperoxidase, and lactoperoxidase (Kalyanaraman et al., 1985).

We recently demonstrated that horseradish peroxidase is inactivated during the catalytic turnover of phenyl- and al-

† This research was supported by Grant GM 32488 from the National Institutes of Health. The mass spectra were obtained in the Biomedical, Bioorganic Mass Spectrometry Facility of the University of California, San Francisco, with support from Grants RR 01614 and P-30 DK 26743 from the National Institutes of Health.

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[1] Abbreviations: heme, iron protoporphyrin IX regardless of the iron oxidation and ligation states; HPLC, high-pressure liquid chromatography; HRP, horseradish peroxidase.

0006-2960/88/0427-5470$01.50/0