Schagger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.
Segel, I., & Johnson, M. (1963) *Anal. Biochem. 5*, 330–337.
Shively, J. E., Ed. (1986) *Methods of Protein Microcharacterization*, pp 1–456, Humana Press, Clinton, NJ.
Silverman, R. B., & Nandi, D. L. (1988) *Biochem. Biophys. Res. Commun. 155*, 1248–1254.
Snyder, L. M., Fortier, N. L., Leb, L., McKenney, J., Trainor, J., Sheerin, H., & Mohandas, M. (1988) *Biochim. Biophys. Acta 937*, 229–240.
Staal, G. E. J., Koster, J. F., Banziger, C. J. M., & Van Milligen-Boersma (1972) *Biochim. Biophys. Acta. 276*, 113–123.
Tischler, M. E., & Allen, D. K. (1985) *Enzyme 34*, 220–223.
Toth, K. M., Clifford, D. P., Berger, E. M., White, C. W., & Repine, J. E. (1984) *J. Clin. Invest. 74*, 292–295.
Valentine, W., Toohey, J., Paglia, D., Nakatani, M., & Brockway, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1394–1398.
Van Kampen, E. J., & Zijlstra, W. G. (1961) *Clin. Chim. Acta 6*, 538–544.
Wagner, G. M., Lubin, B. M. & Chiu, D. T.-Y. (1988) in *Cellular Antioxidant Defense Mechanisms*, (Chow, C. K., Ed.) Vol. I, Chapter 13, pp 185–195, CRC Press, Boca Raton.
Well, W. W., Xu, D. P., Yang, Y., & Rocque, P. A. (1990) *J. Biol. Chem. 265*, 15361–15364.
Ziegler, D. M. (1985) *Annu. Rev. Biochem. 54*, 305–329.

A Hypervariable Region of P450IIC5 Confers Progesterone 21-Hydroxylase Activity to P450IIC1†

Thomas Kronbach,‡,§ Byron Kemper,‖ and Eric F. Johnson*,‡

Division of Biochemistry, Department of Molecular and Experimental Medicine, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037, and Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Received December 18, 1990; Revised Manuscript Received April 9, 1991

ABSTRACT: Cytochrome P450IIC5 is a hepatic progesterone 21-hydroxylase while the 95% identical P450IIC4 has a >10-fold higher K_m for progesterone 21-hydroxylation and the 74% identical P450IIC1 does not hydroxylate progesterone at detectable rates. Previous work demonstrated that the apparent K_m of P450IIC4 for progesterone 21-hydroxylation can be markedly improved by replacing a valine at position 113 with an alanine which is present at this position in P450IIC5. In the present studies, a single point mutation in cytochrome P450IIC1 that changed valine at position 113 to alanine conferred progesterone 21-hydroxylase activity to this enzyme. Although the catalytic activity was less than that of P450IIC5, these results indicate the residue 113 plays a critical role in the determination of the substrate/product selectivity in subfamily IIC P450s. By alignment with the sequence of P450cam, the segment of the polypeptide, residues 95–123, containing residue 113 corresponds to a substrate-contacting loop in the bacterial enzyme. The region containing residue 113, which is highly variable among family II P450s, may also be a substrate-contacting loop in the mammalian cytochromes P450. The exchange of this hypervariable region of cytochrome P450IIC1, residues 95–123, with that of P450IIC5 enhanced the 21-hydroxylase activity of the cells transfected with this chimera to levels similar to those of cells transfected with the plasmid encoding P450IIC5. Kinetic analysis of microsomes isolated from the transfected cells showed that the apparent K_m for progesterone 21-hydroxylation of the chimera was indistinguishable from that of P450IIC5. This suggests that despite their low amino acid similarity with P450cam, the eukaryotic P450 enzymes share some of the functional organization of the bacterial enzyme and that variation in this region is one of the mechanisms by which the eukaryotic cytochromes P450 have derived their multisubstrate specificity.

The evolution of the enzymes of the cytochrome P450 (P450)[1] superfamily has led to a multitude of catalytic functions (Nebert & Gonzalez, 1987). Highly similar isozymes frequently metabolize different substrates, and a single isozyme is often active in the metabolism of numerous structurally diverse substrates. This divergence in function seems to be independent of the divergence in primary structure in that some closely related forms are catalytically distinct and more distantly related forms catalyze the same reaction with similar efficiency. For example, cytochrome P450IIC5 is

† Supported by USPHS Grants GM31001 (E.F.J.) and GM35897 (B.K.). Facilities for computer-assisted analysis and the synthesis of oligonucleotides are supported in part by GCRC Grant M01 RR00833 and by the Sam and Rose Stein Charitable Trust, respectively.

* Address correspondence to this author.
‡ Research Institute of Scripps Clinic.
§ Present address: Department of Drug Metabolism, Goedecke AG, Mooswaldallee 1-9, 7800 Freiburg, Federal Republic of Germany.
‖ University of Illinois of Urbana–Champaign.

[1] The generic term "P450" is used to indicate a cytochrome P-450. Individual forms of P450 are designated according to the uniform system of nomenclature described by Nebert et al. (1991) with the following exceptions: The common name P450cam is used for P450CI. P450IIA4 and P450IIA5 are designated as $P450_{15\alpha}$ and $P450_{coh}$ to distinguish the steroid 15α-hydroxylase and the coumarin 7-hydroxylase, respectively (Lindberg & Negishi, 1989). Mutations are designated by indicating the one-letter abbreviation for the residue that was replaced, its position in the sequence, and the one-letter designation of the new residue in the indicated order.

0006-2960/91/0430-6097$02.50/0

unique among the hepatic P450s in that it catalyzes the conversion of progesterone to deoxycorticosterone (Dieter et al., 1982a) at rates similar to the adrenal 21-hydroxylase (Bumpus & Dus, 1982) although it shows <30% sequence identity with this enzyme (Tukey et al., 1985; Higashi et al., 1986). On the other hand, P450IIC4 is 95% identical with P450IIC5 (Johnson et al., 1987) but shows a more than 10-fold higher K_m for progesterone 21-hydroxylation (Kronbach et al., 1989), and P450IIC1 (Leighton et al., 1984) which is 74% identical with P450IIC5 shows no detectable 21-hydroxylase activity (Kronbach et al., 1990). We have previously exploited the similarity in amino acid sequence and functional difference of P450IIC5 and P450IIC4 to identify by the expression of chimeric enzymes and mutants a substrate binding domain (Kronbach et al., 1989) in which Ala-113 is a major determinant of the difference in the apparent K_m of P450IIC4 and P450IIC5 for the substrate progesterone (Kronbach & Johnson, 1991). As the N-terminal 128 amino acids of P450IIC5 can confer 21-hydroxylase activity to the 74% identical P450IIC1 when this chimera is expressed (Kronbach et al., 1990), residue 113 may again play a crucial role in determining this characteristic function. We, therefore, decided to test the role of this residue on the catalytic activity of P450IIC1 toward progesterone by constructing the corresponding mutant.

Materials and Methods

Chimeric genes were constructed in pBluescript or pCMV plasmids. Single-stranded DNA was prepared from pBluescript by use of helper phage R408 (Stratagene) or M13KO7 (Pharmacia). Site-directed mutagenesis was performed with the phosphorothionate method (Taylor et al., 1985) using reagents purchased from Amersham.

A *Bgl*II site was introduced into P450IIC5 and P450IIC1 with the respective oligonucleotides 5′-TCCAAGATCtACCAGGGC-3 and 5′-CCCAGATCtACCAAGGC-3′. Val-113 of P450IIC1 was mutated to Ala with the oligonucleotide 5′-TGCTGAAAgCGATTCCATA-3′, and G117A and R119T mutations were introduced either into P450IIC1 or into P450IIC1-V113A with the mixed oligonucleotide 5′-TCTCCTTCCAT(g/c)TCTTT(g/c)CATTGCTGAAA-3′. Lower case letters designate the mutant nucleotide. Mutants were identified and verified by dideoxy sequencing using T7 polymerase (Pharmacia). COS1 cells were grown in T75 or T225 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco), MEM–nonessential amino acids (Gibco), 10 mM HEPES, and penicillin/streptomycin (each 50 units/mL). The cells were transfected by the DEAE-dextran method including chloroquine and a DMSO shock (Cullen, 1987). In some experiments, 1 μg of the plasmid pSV2AL-Δ5′ (de Wet et al., 1987) was included in the transfected DNA, and the activity of the luciferase expressed from this plasmid was determined (de Wet et al., 1987) in order to monitor differences in transfection efficiency.

Microsomal fractions were prepared from cells 48–72 h after transfection by differential centrifugation. The cells were harvested by scraping them into 10 mL of cold (4 °C) phosphate-buffered saline and 0.1 M sodium phosphate, pH 7.4, containing 0.15 M NaCl after mild trypsinization. The suspension was spun for 5 min at approximately 500*g* in a clinical centrifuge (IEC), the supernatant was aspirated, and the cells were resuspended in 500 μL of 250 mM sucrose and 1 mM EDTA. This suspension was homogenized by using a 1-mL glass, straight-wall tissue grinder (no. 440412, Radnoti, Monravia, CA) at approximately 200 rpm in an ice–water bath (15 strokes). Debris was precipitated at 500*g* for 5 min at 4 °C. The supernatant was then collected and centrifuged for 60 min at 150000*g* at 4 °C in 1.5-mL polyallomer tubes in a table-top ultracentrifuge (Beckman TL100). The pellet was homogenized at a protein concentration close to 10 mg/mL with a "pellet pestle" (Kontes) in a 1.5-mL polypropylene tube at 200 rpm in an ice–water bath. The homogenization buffer contained 100 mM potassium phosphate, pH 7.4, 1 mM EDTA, and 20% glycerol. A typical yield is 50–70 μg of protein as determined by the BCA microassay (Pierce) for one confluent T75 flask. The method described previously (Kronbach et al., 1989) had employed an additional 10000*g* spin. We find that the method described here yields slightly lower specific content with a 2-fold increased yield of enzyme from COS1 cells over that used earlier (Kronbach et al., 1989).

Progesterone 21-hydroxylase activity was determined in vivo in the cell culture flask at 48 h after transfection by supplementation of the culture medium for 2 or 4 h with 10 μM [^{14}C]progesterone (NEN, 60 Ci/mol) as described earlier (Kronbach et al., 1989).

The substrate dependence of progesterone 21-hydroxylation was determined in vitro for microsomes as described earlier (Kronbach et al., 1989). Briefly, 25 μg of microsomal protein was incubated in a final volume of 100 μL of 50 mM potassium phosphate, pH 7.4, with varying amounts of [^{14}C]-progesterone and an NADPH regenerating system (Kronbach et al., 1987) for 30 min. Immediately before the reaction was stopped by extraction with chloroform, 8 nmol of progesterone and 4 nmol of 21-hydroxyprogesterone were added. The products were separated by TLC and visualized under UV illumination by the quenching of the fluorescence of the TLC indicator. The regions containing substrate and product were cut from the flexible TLC plate (Bakerflex IB2-F, Baker) and quantified by scintillation counting. The values for the apparent K_m and V_{max} were estimated by nonlinear, least-squares fitting of the Michaelis–Menten equation to the experimental results using the program Sigma Plot 4.0 (Jandel Scientific).

Microsomal estradiol 2-hydroxylase activity was measured with 50 μg of microsomal protein in 100-μL assay volume at a concentration of 10 μM 17β-[4-^{14}C]estradiol (Amersham, 55 Ci/mol) under the conditions described above for progesterone except that the assay buffer contained 1 mM ascorbic acid to prevent nonenzymatic oxidation of 2-hydroxyestradiol. The sample was incubated for 60 min at 37 °C, and the reaction mixture was extracted with 1 mL of chloroform and processed by thin-layer chromatography and autoradiography as described (Schwab & Johnson, 1985).

Immunoblotting was performed by electrophoretically transferring 20 μg of microsomal protein separated on a 10% SDS gel (Laemmli, 1970) onto nitrocellulose (Towbin et al., 1979). Free binding sites were blocked with a blocking solution which consisted of 2% nonfat dry milk, 0.05% Tween 20, and 0.01% sodium azide in phosphate buffered saline, and the proteins were detected with an antiserum raised in a pig developed against P450IIC3. The primary antibody was detected with ^{125}I-labeled protein A (ICN, >30 Ci/g) and autoradiography.

Results

To test whether a single mutation at position 113 in P450IIC1 would confer progesterone 21-hydroxylase activity to this enzyme, Val-113 of P450IIC1 was mutated to Ala, the corresponding residue in P450IIC5. P450IIC1 and the mutant enzyme were expressed in COS1 cells, and 2 days after transfection, the cell culture medium was supplemented for 4 h with 10 μM [^{14}C]progesterone. Although P450IIC1 was devoid of measurable 21-hydroxylase activity, we found that

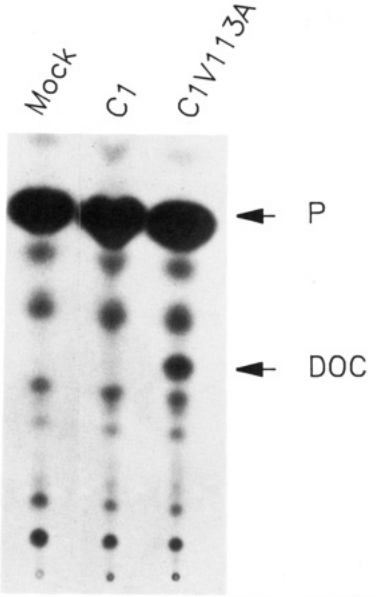

FIGURE 1: Catalytic activity of P450IIC1 and P450IIC1-V113A expressed in COS1 cells. Progesterone 21-hydroxylase activity of COS1 cells transfected without DNA (Mock) or with plasmids encoding P450IIC1 (C1) or P450IIC1-V113A (C1-V113A). Two days after transfection, the cell culture medium was supplemented with 10 μM [^{14}C]progesterone. After incubation for 4 h, the products were extracted and analyzed by TLC. An autoradiogram of the TLC is shown. P denotes the mobility of progesterone; DOC indicates the mobility of 21-hydroxyprogesterone (deoxycorticosterone). The lanes shown were taken from the same autoradiographic image.

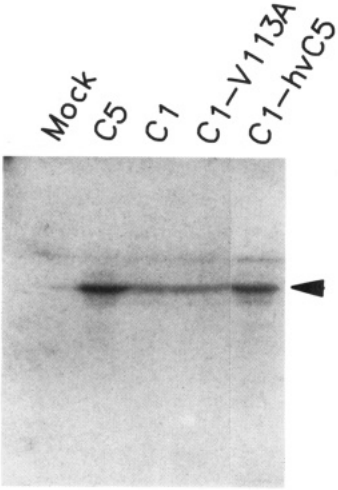

FIGURE 2: Immunoblot of the microsomes prepared from COS1 cells transfected without DNA (Mock) or with P450IIC5 (C5), P450IIC1 (C1), P450IIC1-V113A (C1-V113A), and P450IIC1-hvC5 (C1-hvC5). Twenty micrograms of microsomal protein was separated on a denaturing 10% SDS gel and transferred to nitrocellulose. The expressed proteins were detected with a pig anti-P450IIC3 antibody and ^{125}I-labeled protein A. The lanes shown were taken from the same autoradiographic image of the blot. The arrowhead indicates the mobility of the P450 proteins.

the mutant had low but clearly detectable 21-hydroxylase activity (Figure 1). Similar levels of P450IIC1 and P450IIC1-V113A were expressed as judged by immunoblotting (Figure 2). This indicates that the progesterone 21-hydroxylase activity is not due to a difference in the level of the enzyme expressed but is an intrinsic property of P450IIC1-V113A. The level of activity of this mutant was roughly 5–10% of the activity of P450IIC5 expressed under similar conditions. The TLC system separates 21-hydroxyprogesterone from progesterone and the 2α-, 17α-, 6β-, and 16α-hydroxy and 17α,21-dihydroxy metabolites. It is,

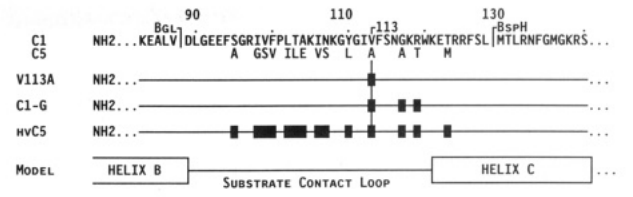

FIGURE 3: Partial sequence comparison of P450IIC1 and P450IIC5 in the hypervariable region and schema for the generation of the mutants and chimeras. The sequence of P450IIC1 is shown, whereas only differences found in the sequence of P450IIC5 are shown below it. The numbering above the sequence indicates the residue number. C1-V113A was obtained by site-directed mutagenesis of the P450IIC1 cDNA. Additional constructs described in this paper are also displayed. C1-G was obtained by the introduction of G117A and R119T mutations in V113A. The restriction sites used for the construction of chimera C1-hvC5 are indicated. The *Bgl*II site was introduced as a silent mutation in both cDNAs by site-directed mutagenesis. Topological features of P450cam (model) which have been predicted to occur among the mammalian P450s (Edwards et al., 1989) are shown below the schema of the constructs.

therefore, very likely that the metabolite of P450IIC1-V113A shown in Figure 2 is 21-hydroxyprogesterone. Thus, the single Val to Ala change at position 113 conferred the substrate/product specificity, characteristic for P450IIC5, to P450IIC1.

Since the activity of the mutant was low when compared to P450IIC5 and the apparent K_m could not be estimated, we asked which additional part(s) of the primary structure of P450IIC5 would yield a progesterone 21-hydroxylase with an activity similar to P450IIC5. Although the Val-113 mutation had been shown previously to alter the apparent K_m of P450IIC4 for progesterone to a value that approached that of P450IIC5, this change in the context of two adjacent mutations produced a chimera, G, with an apparent K_m that was indistinguishable from that of P450IIC5 (Kronbach et al., 1989). Mutations were, therefore, introduced into P450IIC1 in order to make a chimera between P450IIC5 and P450IIC1 that corresponds to chimera G between P450IIC5 and P450IIC4 (Figure 3). The mutations G117A and R119T were introduced into P450IIC1 near the Val-113 mutation either alone or in combination with the V113A change (C1-G, Figure 3). These changes did not, however, alter the activity of P450IIC1 or P450IIC1-V113A (data not shown). It was, therefore, likely that a larger segment of P450IIC5 was necessary to generate a more efficient chimeric enzyme.

The segment around Ala-113 of P450IIC5, residues 91–123, by the alignment of primary sequence and secondary structure predictions (Nelson & Strobel, 1989; Gotoh & Fujii-Kuriyama, 1989) corresponds to a substrate-contacting loop in the structure of P450cam determined by X-ray diffraction (Figure 3). This loop contains Tyr-96, a residue of P450cam which contributes to substrate binding by donating a hydrogen bond to the substrate camphor. The substrate-contacting loop lies on the surface of P450cam between helices B and C, and its flexibility may permit the formation of a substrate access channel (Poulos et al., 1987). The corresponding segment of P450IIC5 is a segment where the primary structures of subfamily IIC P450 enzymes differ most and which is at least partly localized on the surface of P450IIC4 and P450IIC5 as judged by epitope mapping (Kronbach & Johnson, 1991). We have suggested (Kronbach & Johnson, 1991) that this surface localization and loop structure would allow extensive genetic variation without disrupting the overall topology of the mammalian cytochromes P450. This also suggested to us that this region of the eukaryotic P450s forms a substrate-contacting loop, and we suspected that this region which contains residue 113 encodes substrate specificity not only between closely

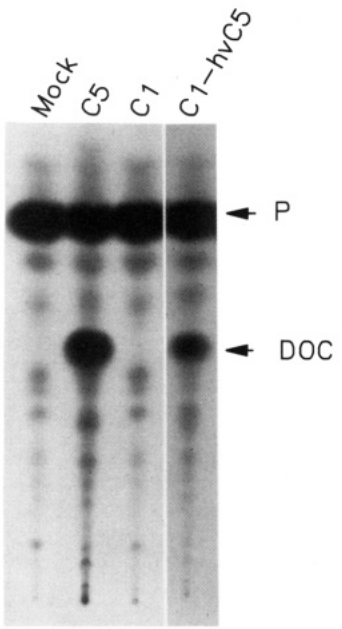

FIGURE 4: Catalytic activity of P450IIC1 and P450IIC1-hvC5 expressed in COS1 cells. COS1 cells were transfected without (Mock) or with DNA coding for P450IIC5 (C5), P450IIC1 (C1), and P450IIC1-hvC5 (C1-hvC5). Two days after transfection, the culture medium was supplemented with 10 μM [^{14}C]progesterone and incubated for 2 h. The products were analyzed after extraction with chloroform by radiometric TLC (Kronbach et al., 1989; Dieter et al., 1982b). The lanes shown were taken from the same autoradiographic image of the thin-layer chromatography plate. P denotes the mobility of progesterone; DOC indicates the mobility of 21-hydroxyprogesterone (deoxycorticosterone).

related P450s but also in the family II cytochromes P450 in general.

To test this, a chimera was constructed between P450IIC5 and P450IIC1, P450IIC1-hvC5, that replaced the hypervariable region of P450IIC1, amino acids 95–123, with that of P450IIC5 (Figure 3). When this chimera was expressed in COS1 cells, it exhibited the high regiospecificity of P450IIC5 for progesterone 21-hydroxylation and a level of activity only slightly lower than that of P450IIC5 (Figure 4). In other experiments, the yield of 21-hydroxyprogesterone from 2 μM progesterone in triplicate 1-h incubations was 1.0 ± 0.1 nmol/10^6 cells for both P450IIC5 and P450IIC1-hvC5. However, the relative transfection efficiency for P450IIC5 was roughly 2-fold lower as judged by the activity of luciferase expressed from pSV2AL-Δ5′ (de Wet et al., 1987) cotransfected with the plasmid encoding each P450. Thus, the corrected activity of P450IIC5 is 2-fold greater than that of P450IIC1-hvC5.

To define the catalytic differences for progesterone between P450IIC1-hvC5 and the wild type, P450IIC5, microsomal fractions were prepared from the COS1 cells which had been transfected with the expression plasmid encoding P450IIC1-hvC5, and the substrate-dependent formation of 21-hydroxyprogesterone was assayed. The apparent K_m determined for P450IIC1-hvC5 is 1.5 μM (Figure 5), which is similar to that of P450IIC5, 1.7 μM (Kronbach et al., 1989; Dieter et al., 1982a). This indicates that the replacement of residues 95–123 of P450IIC1 with those of P450IIC5 is sufficient to confer to this chimeric protein a progesterone 21-hydroxylase activity with the apparent K_m of P450IIC5.

A direct comparison of V_{max} values is complicated by the difficult estimation of the amount of catalytically active enzyme in the microsomal preparations from the transfected cells and by the fact that we do not know if our polyclonal antiserum

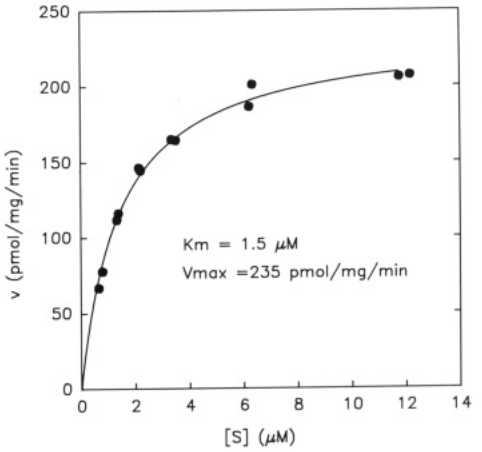

FIGURE 5: Substrate-dependent formation of 21-hydroxyprogesterone from progesterone by microsomes prepared from COS1 cells transfected with P450IIC1-hvC5. Two days after transfection, COS1 cells were harvested, and microsomes were prepared as described under Materials and Methods. K_m and V_{max} were estimated by nonlinear, least-squares fitting of the Michaelis–Menten equation to the experimentally determined points shown on the graph. The curve presents this fit.

reacts equally well with both P450IIC1 and P450IIC5. However, the V_{max} of 235 pmol mg^{-1} min^{-1} shown in Figure 5 compares favorably with values between 280 and 450 pmol mg^{-1} min^{-1} obtained by using microsomes from COS1 cells which had been transfected with the plasmid encoding P450IIC5 using the same conditions as for P450IIC1-hvC5, and a similar intensity of antibody binding is apparent in immunoblotting (Figure 2). Moreover, the activity of the cells assayed in vivo is similar for P450IIC5 and P450IIC1-hvC5 transfectants (Figure 4). This suggests that the 115 amino acid differences between the wild-type P450IIC5 and P450IIC1-hvC5 do not have a major influence on the progesterone 21-hydroxylase activity of this chimeric enzyme and that the new enzymatic characteristics for this substrate are largely determined by amino acids in the hypervariable region, residues 95–123. In contrast, when the chimera was tested for estradiol 2-hydroxylase activity, none was detected, as is the case for P450IIC1. This reaction is readily apparent for P450IIC5 expressed in microsomes from COS1 cells (not shown). Thus, the chimera exhibits a distinct substrate profile when compared to P450IIC5.

Discussion

We demonstrate here that a single amino acid change is sufficient to confer progesterone 21-hydroxylase activity to P450IIC1. Although the resulting activity was low when compared to that of P450IIC5, this result supports the findings of earlier studies which had implicated the residues at this exact alignment position as determinants of substrate selectivity among family II P450s. In rabbit P450IIC4 and P450IIC5, residue 113 is responsible for most of the >10-fold difference in the apparent K_m for progesterone 21-hydroxylation (Kronbach & Johnson, 1991). In addition, a Val-Ala difference at position 117 between the mouse cytochromes $P450_{coh}$ and $P450_{15\alpha}$ (Lindberg & Negishi, 1989) and a Ile-Phe difference at position 114 in rat P450IIB1 (Aoyama et al., 1989) have both been shown to play a critical role in determining the catalytic activity of the respective enzymes. These differences align with residue 113 of P450IIC5 (Nelson & Strobel, 1989; Gotoh & Fujii-Kuriyama, 1989; Kronbach & Johnson, 1991) as shown in Figure 6. The change in $P450_{coh}$ of Val-117 to Ala decreased the coumarin hydroxylase activity 3-fold, and the converse change in $P450_{15\alpha}$ conferred coumarin

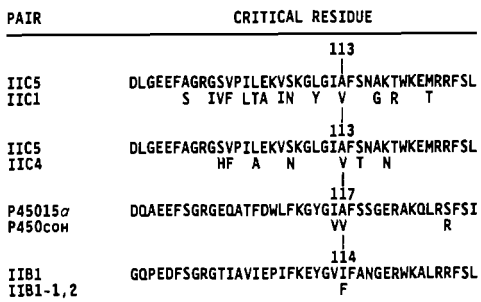

FIGURE 6: Partial sequence comparison of cytochromes P450 in which changes in amino acid residues equivalent to residue 113 of P450IIC5 affect the catalytic activity of the enzyme. The alignment of the critical residue is shown by the vertical line together with the corresponding position in the sequence. The segment shown corresponds to that exchanged between P450IIC5 and P450IIC1 in C1-hvC5. P450IIC4 and IIC5 are from Kronbach and Johnson (1991), $P450_{coh}$ and $P450_{15\alpha}$ are from Lindberg and Negishi (1989), and P450IIB1 and P450IIB1-1,2 are taken from Aoyama et al. (1989).

7-hydroxylase activity at a level 25% of that in $P450_{coh}$ (Lindberg & Negishi, 1989). This difference which occurs naturally between allelic forms of $P450_{coh}$ (Negishi et al., 1990) is likely to underlie, in part, phenotypic differences of coumarin hydroxylation in the mouse (Miles et al., 1990; Wood & Taylor, 1979; Wood, 1979). An Ile-114 to Phe change, in combination with the Leu-58 to Phe mutation in P450IIB1, changed the ratio between 16α and 16β metabolites of testosterone by about 50-fold (Aoyama et al., 1989). This suggests that this residue may in general be a critical residue for substrate recognition and that P450IIC5 shares the three-dimensional organization of P450IIC1, P450IIC4, P450IIB1, $P450_{coh}$, and $P450_{15\alpha}$.

Although this single mutation conferred progesterone 21-hydroxylase activity to P450IIC1, alterations of adjacent residues to correspond to those of P450IIC5 were necessary in order to confer the full activity and apparent K_m of P450IIC5 to P450IIC1. This segment which contains critical residue 113 is a highly variable region among P450s in subfamily IIC. Such high variability in primary structure could indicate that this part of the protein is mostly localized on the surface of the enzyme where genetic variation can occur without interfering with the folding of the protein. Experimental evidence for this hypothesis is provided by two findings. First, antibodies derived against peptides corresponding to amino acids 108–115 and 121–131 of P450IIB1 recognize the microsomal protein as judged by immunoelectron microscopy and enzyme-linked immunosorbent assays (De Lemos-Chiarandini et al., 1987). Second, residues Ser-115 and Lys-118 of P450IIC5 are part of the epitope of a monoclonal antibody directed against this protein (Kronbach & Johnson, 1991). The alignment with P450cam and comparison with the X-ray structure indicated that this region of the mammalian cytochromes P450 corresponds to a substrate-contacting loop of the bacterial protein (Poulos et al., 1987). This loop contains three contact residues, Phe-87, Phe-98, and Tyr-96. The last residue, Tyr-96, orients the substrate for regiospecific hydroxylation through hydrogen bonding (Atkins & Sligar, 1988). This flexible loop is also thought to control the access of the substrate to its binding site in P450cam (Poulos et al., 1986). Amino acids 95–123 may encode a similar domain in the eukaryotic cytochromes P450 which could accommodate large sequence variation and simultaneously contribute to differences of substrate specificity exhibited by P450 enzymes.

When residues 95–123 of P450IIC1 were replaced by those from P450IIC5, the resultant chimeric enzyme showed enzyme kinetic parameters for progesterone 21-hydroxylation similar to those of the wild-type P450IIC5. There are 14 differences between P450IIC1 and P450IIC5 in this region from amino acids 95 to 123 (Figure 3). The importance of each of the changed amino acids has not yet been tested; however, the changes of G117A and R119T in addition to V113A in P450IIC1 did not increase the activity of the 21-hydroxylase. Thus, we do not know how many of the 13 differences are required in addition to the V113A to obtain the kinetic properties of P450IIC1-hvC5. One or more of these differences could confer 21-hydroxylase activity to P450IIC1 in the absence of the V113A change. P450IIC4 exhibits a low affinity 21-hydroxylase activity with a valine at residue 113 (Kronbach et al., 1989), and it has been observed that changes in any one of three amino acids conferred low but detectable coumarin 7-hydroxylase activity to $P450_{15\alpha}$ (Lindberg & Negishi, 1989). It is possible, therefore, that other critical amino acids could be defined by changes at positions other than 113 that might confer 21-hydroxylase activity to P450IIC1.

It is clear, however, that the segment 95–123 is not the only determinant of substrate and product specificity in these enzymes. The lack of estradiol 2-hydroxylase activity of P450IIC1-hvC5 indicates that different parts of the primary structure of P450IIC5 determine the catalytic activity toward estradiol. Other regions in the primary structures of P450s have been implicated in substrate binding (Uno et al., 1990; Uno & Imai, 1989; Imai et al., 1989; Kronbach et al., 1990). In addition to a change at residue 117, single amino acid changes at residues 209 or 365 could confer coumarin hydroxylase activity to $P450_{15\alpha}$ (Lindberg & Negishi, 1989). Moreover, we have shown that the 128 amino-terminal residues of P450IIC5 can confer progesterone 21-hydroxylase activity to P450IIC1 but a similar chimera in which the same 128 amino acids had been fused to P450IIC2 did not catalyze progesterone 21-hydroxylation (Kronbach et al., 1990). We have localized this difference to the 28 carboxy-terminal amino acids in P450IIC1 and P450IIC2 (Kronbach, Kemper, and Johnson, unpublished results).

The successful construction of an efficient enzyme by the exchange of a highly variable domain of P450IIC1 with that of P450IIC5, amino acids 93–125, suggests that this region of the cytochrome P450 can accommodate great sequence variability without disrupting the proper folding of the enzyme. In support of this, Uno and Imai (1989) demonstrated that inclusion of this segment, codons 89–125, of P450IIC2 in chimeras with P450IIC14 was required to produce an active laurate (ω-1) hydroxylase although additional segments of P450IIC2 were also required. This suggests that this highly variable region of the family II cytochromes P450 is one of the structural elements by which the P450 enzymes have derived their multisubstrate specificity through genetic variation.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of J. Collins and D. Williams, and we thank K. Griffin for critical reading of the manuscript.

REFERENCES

Aoyama, T., Korzekwa, K., Nagata, K., Adesnik, M., Reiss, A., Lapenson, D. P., Gillette, J., Gelboin, H. V., Waxman, D. J., & Gonzalez, F. J. (1989) *J. Biol. Chem. 264*, 21327.

Atkins, W. M., & Sligar, S. G. (1988) *J. Biol. Chem. 263*, 18842.

Bumpus, J. A., & Dus, K. M. (1982) *J. Biol. Chem. 257*, 12696.

Cullen, B. R. (1987) *Methods Enzymol. 152*, 684.

De Lemos-Chiarandini, C., Frey, A. B., Sabatini, D. D., & Kreibich, G. (1987) *J. Cell Biol. 104*, 209.
de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., & Subramani, S. (1987) *Mol. Cell. Biol. 7*, 725.
Dieter, H. H., Muller-Eberhard, U., & Johnson, E. F. (1982a) *Biochem. Biophys. Res. Commun. 105*, 515.
Dieter, H. H., Muller-Eberhard, U., & Johnson, E. F. (1982b) *Science 217*, 741.
Edwards, R. J., Murray, B. P., Boobis, A. R., & Davies, D. S. (1989) *Biochemistry 28*, 3762.
Gotoh, O., & Fujii-Kuriyama, Y. (1989) in *Frontiers in biotransformation. Basis and mechanisms of regulation of cytochrome P-450* (Ruckpaul, K., & Rein, H., Eds.) pp 195–243, Taylor & Francis, New York.
Higashi, Y., Yoshioka, H., Yamane, M., Gotoh, O., & Fujii-Kuriyama, Y. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2841.
Imai, Y., Uno, T., Nakamura, M., & Yokota, H. (1989) *Drug Metab. Rev. 20*, 467.
Johnson, E. F., Barnes, H. J., Griffin, K. J., Okino, S., & Tukey, R. H. (1987) *J. Biol. Chem. 262*, 5918.
Kronbach, T., & Johnson, E. F. (1991) *J. Biol. Chem. 266*, 6215.
Kronbach, T., Mathys, D., Gut, J., Catin, T., & Meyer, U. A. (1987) *Anal. Biochem. 162*, 24.
Kronbach, T., Larabee, T. M., & Johnson, E. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8262.
Kronbach, T., Kemper, B., & Johnson, E. F. (1990) in *Current Research in Protein Chemistry: Techniques, Structure, and Function* (Villafranca, J. J., Ed.) pp 481–488, Academic Press Inc., San Diego.
Laemmli, U. K. (1970) *Nature 227*, 680.
Leighton, J. K., DeBrunner-Vossbrinck, B. A., & Kemper, B. (1984) *Biochemistry 23*, 204.
Lindberg, R. L. P., & Negishi, M. (1989) *Nature 339*, 632.
Miles, J. S., Moss, J. E., Meehan, R. R., & Wolf, C. R. (1990) *Genomics 7*, 445.
Nebert, D. W., & Gonzalez, F. J. (1987) *Annu. Rev. Biochem. 56*, 945.
Nebert, D. W., Nelson, D. R., Coon, M. J., Estabrook, R. W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R., & Waxman, D. J. (1991) *DNA 10*, 1.
Negishi, M., Lindberg, R., Lang, M., Wong, G., Itakura, T., & Yoshioka, H. (1990) in *Drug Metabolizing Enzymes: Genetics, regulation and toxicology. Proceedings of the VIIIth International Symposium on Microsomes and Drug Oxidations* (Ingelman-Sundberg, M., Gustafsson, J.-A., & Orrenius, S., Eds.) p 15, Karolinska Institutet, Stockholm, Sweden.
Nelson, D. R., & Strobel, H. W. (1989) *Biochemistry 28*, 656.
Poulos, T. L., Finzel, B. C., & Howard, A. J. (1986) *Biochemistry 25*, 5314.
Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195*, 687.
Schwab, G. E., & Johnson, E. F. (1985) *Arch. Biochem. Biophys. 237*, 17.
Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8765.
Towbin, H., Theophil, S., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350.
Tukey, R. H., Okino, S. T., Barnes, H. J., Griffin, K. J., & Johnson, E. F. (1985) *J. Biol. Chem. 260*, 13347.
Uno, T., & Imai, Y. (1989) *J. Biochem. 106*, 569.
Uno, T., Yokota, H., & Imai, Y. (1990) *Biochem. Biophys. Res. Commun. 167*, 498.
Wood, A. W. (1979) *J. Biol. Chem. 254*, 5641.
Wood, A. W., & Taylor, B. A. (1979) *J. Biol. Chem. 254*, 5647.