Two Catalytically Distinct Subforms of Cytochrome P-450 3b As Obtained from Inbred Rabbits†

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ABSTRACT: Cytochrome P-450 3b has been shown previously to exist in one of two catalytically and structurally distinct forms. Preparations of P-450 3b obtained from outbred New Zealand White rabbits exhibit both high-efficiency (V_{max}/K_m) progesterone 6β- and 16α-hydroxylases and a low-efficiency 16α-hydroxylase. In contrast, P-450 3b prepared from a genetically defined strain of rabbits, IIIVO/J, does not display the high-efficiency 6β- and 16α-hydroxylase activities. This appears to reflect the inheritance of one of two catalytically distinct subforms of P-450 3b. In the present study, we provide information with respect to the characterization of these progesterone hydroxylases as they occur in two highly inbred strains of rabbits, B/J and III/J. The identification of these strains as sources of these subforms was achieved by utilizing a monoclonal antibody to P-450 3b that produced a distinct pattern of inhibition for each phenotype. The functional and structural attributes of these subforms of P-450 3b indicate that whereas only the low-efficiency 16α-hydroxylase is expressed in B/J rabbits, both of the catalytically distinct subforms of P-450 3b are expressed in the III/J rabbits. Thus, it is unlikely that these enzymic variants reflect allomorphic forms of P-450 3b.

The existence of multiple forms of the cytochrome P-450 monooxygenases has been established by an extensive array of experimental techniques. These include SDS[1]–polyacrylamide gel electrophoresis, amino acid sequence analysis, and catalytic assessments (Lu & West, 1980). Through the application of even more exacting methodologies, such as is afforded by cDNA probes (Leighton et al., 1984), monoclonal antibodies (Reubi et al., 1984a,b), and kinetic analysis conjoined with specific modulators of enzyme activity (Dieter & Johnson, 1982), subtle differences in both structure and function among the various electrophoretic classes of cytochrome P-450 are beginning to be revealed. Thus, the breadth of cytochrome P-450 multiplicity continues to grow.

Previous investigations from this laboratory have demonstrated that two or more electrophoretically equivalent, catalytically distinct forms comprise rabbit liver cytochrome P-450 3b. Microsomes and purified P-450 3b obtained from outbred New Zealand White (NZW) rabbit livers exhibit a high-efficiency, low-K_m progesterone 6β-hydroxylase and a 16α-hydroxylase activity that manifests the kinetic properties of at least two enzymes, functionally competent subunits, or independently regulated active sites, each of which displays a characteristic apparent K_m differing by greater than 1 order of magnitude. Microsomes and P-450 3b prepared from a genetically defined strain of rabbits, IIIVO/J, express only a low-affinity progesterone 16α-hydroxylase (Dieter & Johnson, 1982). The high-affinity 6β- and 16α-hydroxylases can be selectively inhibited by 16α-methylprogesterone, whereas the low-affinity 16α-hydroxylase appears to be slightly stimulated by this compound (Dieter & Johnson, 1982). In contrast, the naturally occurring metabolite of progesterone, 5β-pregnane-3β,20α-diol, selectively increases the overall efficiency, V_{max}/K_m, of the low-affinity 16α-hydroxylase activity of microsomes and of P-450 3b obtained from the livers of IIIVO/J rabbits (Johnson et al., 1983). Hereafter, the variant forms of P-450 3b will be denoted as either the 6βH or 6βL phenotype, reflecting their relative capacity to 6β-hydroxylate progesterone.

The only structural difference noted between the variant P-450 3bs prepared from NZW and IIIVO/J rabbits is the complete absence of a single peptide in the IIIVO/J P-450 3b tryptic peptide chromatograms consistently observed in the corresponding chromatograms obtained from NZW P-450 3b, peptide 14 (Dieter & Johnson, 1982). This difference is preserved whether the P-450 3b is purified by conventional ion-exchange chromatography or by the use of immunoaffinity chromatography utilizing a monoclonal antibody covalently bound to Sepharose (Reubi et al., 1984a).

In the present investigation, we endeavored to identify inbred strains of rabbits that express either of the 6β phenotypes. Having identified two strains that type true for either the 6βL or 6βH P-450 3b subform, we isolated the P-450 3b therefrom by conventional ion-exchange chromatography and subjected each to extensive kinetic characterization with respect to progesterone metabolism in order to determine the segregation of kinetic properties of P-450 3b as they occur for the homozygous inbred strains. The relationship of observed activities and structural differences extant between the two subforms is discussed.

Experimental Procedures

Materials. [4-^{14}C]Progesterone (56 mCi/mmol) and organic counting scintillant were obtained from Amersham. TPCK-treated trypsin was purchased from Worthington Diagnostics. Cyanogen bromide activated Sepharose and Na_4-NADPH were from Sigma. The μBondapak C_{18} column was obtained from Millipore. HPLC-grade water and acetonitrile were from Burdick & Jackson. New Zealand White rabbits were purchased from local breeders. Rabbits of strains IIIVO/J, III/J, OS/J, B/J, X/J, and WH/J were obtained

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[1] Abbreviations: NZW, New Zealand White; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate.

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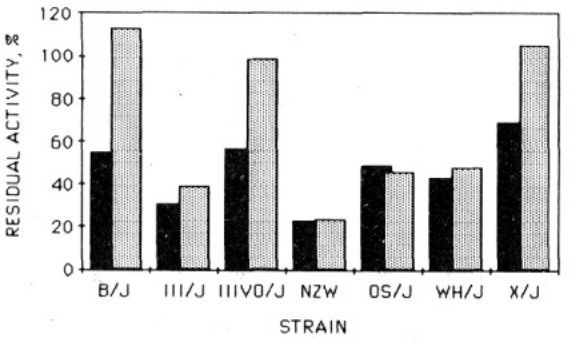

FIGURE 1: Effect of a monoclonal antibody to P-450 3b on the microsomal metabolism of progesterone. Microsomes from each indicated strain of rabbits were assayed for progesterone 16α- (black) and 6β- (stippled) hydroxylations in the absence and presence of 100 μg of a monoclonal antibody to P-450 3b, 8-27. Residual activity refers to the percent amount 16α- and 6β-hydroxylase activity observed in the presence of the antibody relative to that observed in the absence of added antibody. The activities observed in the absence of added antibody were as follows: B/J, 0.43 and 0.57; III/J, 0.74 and 2.00; IIIVO/J, 0.52 and 1.12; NZW, 0.80 and 1.62; OS/J, 0.48 and 1.99; WH/J, 0.61 and 1.58; X/J, 0.27 and 0.28. Each of the aforementioned activities are expressed as nanomoles of product formed per minute per milligram of microsomes for 16α- and 6β-hydroxyprogesterones, respectively. Progesterone was present at an initial concentration of 10 μM. Specific details regarding the assay of progesterone are given under Methods.

from Jackson Laboratories. The inbreeding coefficients for the latter strains are >0.98, 1.0, >0.90, 1.0, >0.91, and >0.85, respectively (Altman & Katz, 1979). Animals, materials, and chemicals needed for the production and characterization of a monoclonal antibody to P-450 3b have been described (Reubi et al., 1984a).

Methods. The isolation and preparation of microsomes was as described previously (Dieter & Johnson, 1982). The subforms of P-450 3b were purified by ion-exchange chromatography as reported (Dieter & Johnson, 1982). NADPH-cytochrome P-450 reductase was purified as described (Yasukochi & Masters, 1976) with minor modifications (Johnson et al., 1979). The production of a monoclonal antibody to P-450 3b, 8-27, and the techniques utilized in the phenotyping of P-450 3b as it occurs in microsomes were as described (Reubi et al., 1984a). Methods for the assay of progesterone metabolism by microsomes and reconstituted P-450 3b have also been described (Dieter & Johnson, 1982). Tryptic peptide mapping of P-450 3b by reverse-phase HPLC was as previously reported (Ozols et al., 1981).

RESULTS

Interstrain Differences in the Catalytic Properties of P-450 3b. The metabolism of progesterone was assessed in vitro for liver microsomes prepared from seven individual strains of rabbits, NZW, IIIVO/J, III/J, WH/J, OS/J, X/J, and B/J, by utilizing an initial substrate concentration of 10 μM, in both the presence and the absence of the 8-27 monoclonal antibody. This antibody has previously been demonstrated to be inhibitory toward P-450 3b supported metabolism (Reubi et al., 1984a). In the absence of added antibody, each microsomal sample was metabolically competent in the conversion of progesterone to its 6β- and 16α-hydroxyl derivatives. When 100 μg of the 8-27 antibody was included in the reaction mixtures, the 6β-hydroxylase activities were inhibited by 50–75% for the microsomes obtained from most outbred NZW rabbits and the inbred strains III/J, OS/J, and WH/J (Figure 1). The corresponding activity in microsomes obtained from strains B/J, X/J, and IIIVO/J was essentially unaffected by the addition of antibody. The 16α-hydroxylation of progesterone was inhibited in all strains of rabbits by at least 30% to almost 75%, thus confirming the presence of P-450 3b in each strain. A third hydroxylated metabolite, deoxycorticosterone, was produced at a rate of <0.4 nmol min^{-1} mg^{-1} by microsomes from each strain. The formation of the latter is principally attributed to P-450 1 (Reubi et al., 1984a), and its rate of formation was uneffected by the antibody to P-450 3b.

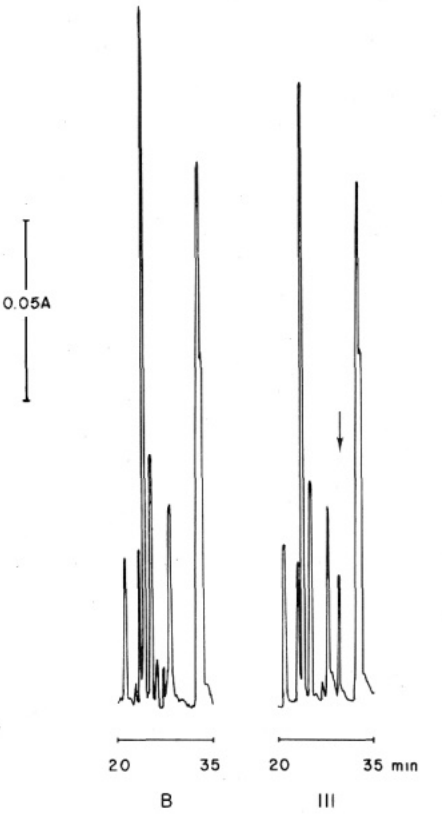

FIGURE 2: Tryptic peptide mapping. Liver microsomes from each indicated strain, containing approximately 200 nmol of total P-450, were solubilized and reacted with the 8-27 antibody covalently bound to Sepharose as described under Methods. The sequestered P-450 3b was then eluted, reacted with trypsin, and mapped by reverse-phase HPLC as described under Methods. Only the portions of the chromatogram in which peptide 14 is normally encountered are shown. The arrow indicates the time for the normal elution of peptide 14.

Peptide Mapping Studies. Previous work has shown that the P-450 3b purified from outbred NZW rabbits may be differentiated from the P-450 3b obtained from strain IIIVO/J by comparison of their tryptic peptide maps (Dieter & Johnson, 1982). Furthermore, we have shown that this difference is maintained for these preparations of P-450 3b when isolated directly from solubilized microsomes by means of immunoaffinity chromtography utilizing the 8-27 antibody covalently linked to Sepharose (Reubi et al., 1984b). Therefore, each microsomal preparation was subjected to immunopurification by these means to sequester the P-450 3b antigen. The P-450 3b samples were then reacted with trypsin and the resulting peptide fragments chromatographed by reverse-phase HPLC. P-450 3b obtained from rabbits that contained an inhibitable 6β-hydroxylase activity, NZW, III/J, WH/J, and OS/J (see previous section), possessed peptide 14 (Dieter & Johnson, 1982), whereas those microsomes containing a noninhibitable 6β-hydroxylase activity yield preparations of P-450 3b deficient in peptide 14, namely, those microsomes obtained from strains IIIVO/J, B/J, and X/J. Figure 2 illustrates representative tryptic peptide profiles for each phenotype.

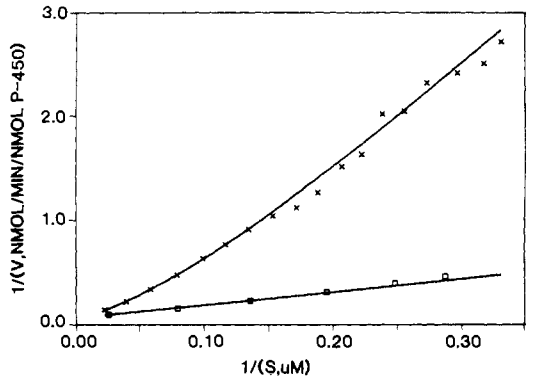

FIGURE 3: Lineweaver–Burk analysis of the 16α-hydroxylation of progesterone as catalyzed by B/J P-450 3b. The reconstitution and assay protocols are described under Methods. Control activity is represented by (×) whereas (□) refers to the presence of 10 μM 5β-pregnane-3β,20α-diol. The curves were calculated as described under Results.

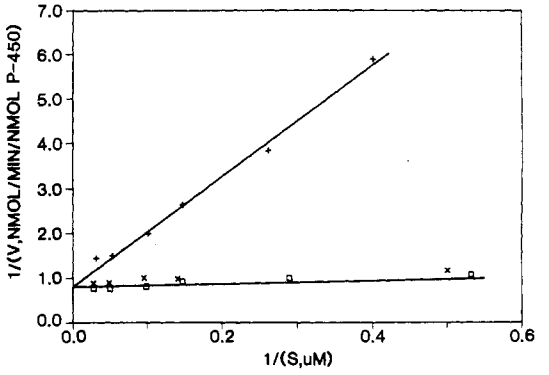

FIGURE 4: Lineweaver–Burk analysis of the 6β-hydroxylation of progesterone as catalyzed by III/J P-450 3b. P-450 3b was reconstituted and assayed as described under Methods. The control activity is represented by (□) while (+) corresponds to the presence of 10 μM 16α-methylprogesterone or (×) 10 μM 5β-pregnane-3β,20α-diol.

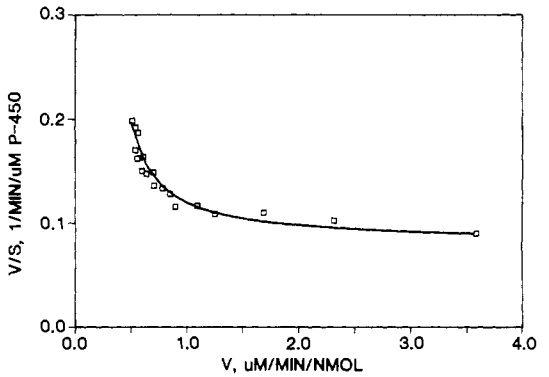

FIGURE 5: Eadie–Hofstee plot of the 16α-hydroxylation of progesterone as catalyzed by III/J P-450 3b. P-450 3b was reconstituted and assayed as described under Methods. The continuous curve was generated by assuming that the curvilinear data is the sum of that arising from two enzymes, one of which has the kinetic properties of the B/J P-450 3b 16α-hydroxylase and a second exhibiting a K_m of 0.3 μM and a V_{max} of 0.5 mol min^{-1} (mol of P-450)$^{-1}$. Each enzyme was assumed to comprise 45% and 50% of the preparation, respectively, as deduced by iterative fitting.

Thus, on the basis of the antibody inhibition study and the corroborating evidence provided by the peptide mapping experiments, we conclude that strains III/J, OS/J, and WH/J exhibit the 6βH phenotype, whereas strains IIIVO/J, B/J, and X/J represent the 6βL phenotype. Strains III/J and B/J were selected for further investigation because these strains are each highly inbred (coefficient of inbreeding 1.0) (Altman & Katz, 1979). Thus, it is probable that each is homozygous for the genetic loci coding for the particular subform of P-450 3b. In order to evaluate the kinetic properties governing the catalysis of progesterone by these P-450 subforms, we purified both the III/J and B/J P-450 3b by conventional ion-exchange chromatography so as to allow for reconstitution studies. The purified preparations of P-450 were apparently homogeneous as evidenced by a single band on SDS–polyacrylamide gel electrophoresis. The specific content of cytochrome P-450 averaged 18 nmol/mg of protein for both the III/J and B/J preparations.

Reconstitution Studies. The P-450 3b prepared from B/J rabbits was reconstituted with NADPH-cytochrome P-450 reductase and dilauroyl-*L*-α-lecithin and was assayed for progesterone catabolism by utilizing varying concentrations of the substrate. As expected, the B/J P-450 3b is competent in only the 16α-hydroxylation pathway. Lineweaver–Burk analysis of this activity revealed a curvilinear dependence of the inverse of the rate of the reaction on the inverse of the substrate concentration, Figure 3. Inclusion of 10 μM 5β-pregnane-3β,20α-diol in the incubation mixture resulted in a decrease in the slope and greatly diminished the curvature seen in the absence of added 5β-pregnane-3β,20α-diol. The activated enzyme exhibits an apparent V_{max} of 20 nmol min^{-1} (nmol of P-450)$^{-1}$ and a K_m of 20 μM. The curvature seen in the absence of the allosteric effector probably reflects activation of the enzyme by the substrate. This activation, which becomes more pronounced as the concentration of progesterone is increased, obscures the kinetic characteristics of the unactivated enzyme although an estimate for K_m/V_{max} of >10 can be made from the slope of the line at low substrate concentrations. The line shown in Figure 3 was calculated by assuming that the substrate activated the enzyme to a form with the same kinetic characteristics as those displayed by the enzyme in the presence of 5β-pregnane-3β,20α-diol. The unactivated enzyme was assumed to exhibit a first-order dependence on substrate concentration, V_{max}/K_m = 0.085, over the entire range of values with an effector binding site exhibiting a K_d of 50 μM for progesterone. Similar fits can be obtained where the unactivated enzyme exhibits a variety of K_m or V_{max} values constrained to yield a ratio of K_m/V_{max} of 10, provided appropriate changes are made in the binding constant for the substrate at the effector site.

When the III/J P-450 3b was reconstituted in the presence of progesterone, spanning a range similar to that used for the B/J P-450 3b, subsequent Lineweaver–Burk analysis revealed that the 6β-hydroxylase is a high efficiency, V_{max}/K_m ca. 1.0 min nmol of P-450 3b, high-affinity activity, apparent K_m < 1.0 μM, Figure 4. The 16α-hydroxylase activity was more complex. Double-reciprocal analysis of this activity showed a clear biphasicity. This is perhaps better illustrated by examining the data in a V vs. $V/[S]$ plot, Figure 5. This particular means of plotting data is especially well suited in such instances where deviations from linearity are encountered in classic double-reciprocal plots. As shown in Figure 5, the kinetic parameters governing each phase for the III/J P-450 3b supported 16α-hydroxylation of progesterone are quite disparate. Note that the ratio of V_{max}/K_m for the low-efficiency phase is similar (about 0.1) to that of the B/J P-450 3b, whereas that of the second component is greater (>0.4).

The line shown in Figure 5 was calculated by assuming that the observed activity is the sum of that arising from two enzyme, one of which has the kinetic properties exhibited by B/J P-450 3b and a second that exhibits a K_m of 0.3 μM and V_{max} = 0.5 nmol min^{-1} (nmol of P-450)$^{-1}$. Each enzyme was as-

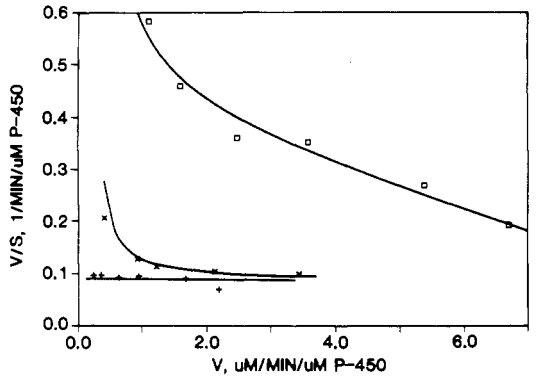

FIGURE 6: Eadie–Hofstee plot of the 16α-hydroxylation of progesterone as catalyzed by III/J P-450 3b. P-450 3b was reconstituted and assayed as described under Methods. (×) indicates the control activity whereas (+) denotes the presence of 10 μM 16α-methylprogesterone; (□) corresponds to the presence of 10 μM 5β-pregnane-3β,20α-diol. The data for the control activity were fit as in Figure 5. The curve for the 5β-pregnane-3β,20α-diol-activated enzyme was generated by constraining the kinetic properties of the high-affinity component and modifying those of the low-affinity phase in conformance with those of the B/J P-450 3b observed in the presence of 5β-pregnane-3β,20α-diol.

sumed to comprise 45% and 55% of the preparation, respectively. These values were chosen by successive approximation.

Reconstitution experiments conducted in the presence of 10 μM 16α-methylprogesterone slightly stimulated the 16α-hydroxylase activity of the B/J P-450 3b, resulting in a more linear double-reciprocal plot. The apparent first-order rate constant, V_{max}/K_m, for this activity was estimated to be approximately 0.17 min nmol of P-450. When the III/J P-450 3b was assayed in the presence of similar amounts of 16α-methylprogesterone, competitive inhibition of the 6β-hydroxylase activity resulted, reducing the apparent K_m for the substrate by 1 order of magnitude, Figure 4. The 16α-methylprogesterone also inhibited the high-affinity 16α-hydroxylase activity of the III/J P-450 3b, whereas the low-affinity activity was essentially unchanged. Thus, the curvilinear V vs. $V/[S]$ plot obtained in the absence of added 16α-methylprogesterone becomes linear in the presence of 16α-methylprogesterone, revealing only the low-affinity 16α-hydroxylase activity, Figure 6. As shown, the low-affinity 16α-hydroxylase appears to exhibit a first-order dependence on substrate concentration with a V_{max}/K_m of 0.1 min nmol of P-450. This activity cannot be inhibited further by increasing the concentration of the inhibitor. The apparent first-order rate constant expressed per mole of III/J P-450 3b determined under these conditions is roughly half that of the B/J P-450 3b, reflecting the dilution of the preparation by the enzyme catalyzing 6β-hydroxylation.

The inclusion of 10 μM 5β-pregnane-3β,20α-diol to the III/J P-450 3b had no detectable effect on the 6β-hydroxylase activity, Figure 4. However, the 16α-hydroxylase activity was profoundly effected, Figure 6. The line shown was calculated by constraining the properties of the high-affinity phase and altering those of the low-affinity phase in conformance with those of the activated form of the B/J P-450 3b seen in the presence of the allosteric-effector. Thus, 5β-pregnane-3β,20α-diol acted as a selective positive effector of the low-efficiency 16α-hydroxylase activity of the III/J P-450 3b, Figure 5.

The III/J P-450 3b was also assayed in the presence of both 16α-methylprogesterone and 5β-pregnane-3β,20α-diol simultaneously. The 6β-hydroxylase activity was, as expected, inhibited in a competitive manner (not shown). The 16α-hydroxylase activities also behaved as would be predicted from the previous experiments. The high-affinity activity was completely inhibited while the remaining low-affinity activity was stimulated as seen for the enzyme from B/J rabbits. The resulting Lineweaver–Burk plot was essentially linear with an apparent K_m of 30 μM and V_{max} = 8 nmol min^{-1} (nmol of P-450)$^{-1}$. The latter being roughly 50% of that seen for B/J P-450 3b. Taken together, these results suggest that the two enzymes comprising P-450 3b can be modulated independently by competitive inhibition or selective activation.

Discussion

Previous investigations dealing with the catabolism of progesterone as catalyzed by cytochrome P-450 3b obtained from outbred, NZW rabbits, resulted in the identification of two catalytically distinct subforms that apparently comprise this electrophoretic class of cytochrome P-450 (Dieter & Johnson, 1982). Kinetic assessments of this P-450 3b revealed that it consists of a high-efficiency 6β-hydroxylase in addition to a low-affinity 16α-hydroxylase. Thus, double-reciprocal plots of the latter activity appear biphasic, reflecting the catalytic contributions of each subform. Only one subform of P-450 3b was obtained from a partially inbred strain of rabbits, IIIVO/J (coefficient of inbreeding >0.98) (Altman & Katz, 1979). This subform has a greatly decreased affinity for progesterone and contains only a low-efficiency 16α-hydroxylase activity (Dieter & Johnson, 1982). As a means for discussing each subform of P-450 3b, they will be denoted as 6βH representing the high-affinity 6β- and 16α-hydroxylases and 6βL representing the low-affinity 16α-hydroxylase. It should be pointed out that the kinetic constants describing the 16α-hydroxylase activity ascribed to this subform are commensurate with those observed for the low-affinity 16α-hydroxylase associated with the 6βH subform.

Since the 6βH subform is obtained from an outbred population of animals, little can be stated with respect to the genetic segregation of activities associated with this form of P-450 as juxtaposed to those associated with the 6β^- subform, the latter of which would probably be homozygous at the alleles encoding the P-450 3b in as much as the inbreeding coefficient of the IIIVO/J rabbits from which it is obtained is >0.98 (Altman & Katz, 1979). Through the agency of a monoclonal antibody that recognizes an epitope restricted to each subform of P-450 3b and is inhibitory toward P-450 3b supported metabolism, we were able to select a source of liver microsomes from a panel of genetically defined strains of rabbits that is phenotypically 6βH. Inasmuch as the strain selected, III/J, is highly inbred (coefficient of inbreeding 1.0), it is probable that it is homozygous for the genetic loci encoding P-450 3b. Corroboration of its identity as a 6β^+ subform was provided by tryptic peptide mapping studies in which the characteristic 6β^+ peptide 14 was encountered. It should be noted that this in itself also corroborates the existence of this particular peptide as characteristic for the 6βH P-450 3b since it was concordant with the enzyme phenotype for several strains of rabbit.

Thus, the purification of this cytochrome P-450 in a manner that preserves its catalytic activity permitted us to delineate the segregation among genetically defined strains of rabbits of activities toward progesterone by this subform interposed with those activities previously described for the 6β^- subform (Dieter & Johnson, 1982).

Kinetic analysis revealed that the III/J P-450 3b possesses properties remarkably similar to those described for the P-450 3b isolated from the outbred NZW rabbits. To wit, the 6β-hydroxylase activity was low K_m, high V_{max}, and the 16α-hydroxylase activity was biphasic when analyzed by double-reciprocal analysis. Certain assumptions regarding the rela-

tionship between the two enzymes can be made from the inhibition, activation, and or simultaneous inhibition–activation studies.

The action of 16α-methylprogesterone was to inhibit both the high-efficiency 6β- and 16α-hydroxylase activities. The low-efficiency 16α-hydroxylase was largely uneffected. Although inhibition of the 6β-hydroxylase was clearly competitive, the exact nature of inhibition for the high-efficiency 16α-hydroxylase is difficult to identify. This is a result of the interference of such analysis by the low-efficiency 16α-hydroxylase. Further evidence of this nature is provided by the experiments with 5β-pregnane-3β,20α-diol. This compound, a naturally occurring catabolic product of progesterone, has been shown to be the most potent of allosteric effectors studied of the 16α-hydroxylase activity of P-450 3b, obtained from strain IIIVO/J (Johnson et al., 1983). With the III/J P-450 3b, only the low-efficiency 16α-hydroxylase appeared to be effected. Again, the precise nature of the activation caused by this compound was difficult to assess because of the simultaneous expression of two distinct 16α-hydroxylases. However, the combined inhibition–activation study resulted in inhibition of the high-affinity moiety of this activity while stimulating that of the residual low-affinity activity. These results are consistent with the properties of a second enzyme being added to those of the 16α-hydroxylase expressed in strain B/J. An alternative explanation would require a structural difference in P-450 3b leading to complex negative and positive effector interactions to produce the observed effects by 16α-methylprogesterone and 5β-pregnane-3β,20α-diol on the activity of III/J P-450 3b.

The segregation of 6βH and 6βL phenotypes among inbred rabbits independently of the expression of the P-450 3b catalyzing the 16α-hydroxylation of progesterone that is stimulated by the pregnanediol leads us to propose two distinct genetic loci encoding P-450 3b. It is likely that a heritable difference between these strains affects one of these loci yielding phenotypic differences in 6β-hydroxylase activity.

Registry No. Cytochrome P-450, 9035-51-2; progesterone 6β-hydroxylase, 9082-59-1; progesterone 16α-hydroxylase, 9082-60-4; progesterone, 57-83-0.

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Electron Paramagnetic Resonance Spectrum of the Iron Protein of Nitrogenase: Existence of a $g = 4$ Spectral Component and Its Effect on Spin Quantization†

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ABSTRACT: The electron paramagnetic resonance (EPR) spectra of 11 separately prepared and highly purified samples of *Azotobacter vinelandii* iron protein, Av_2, were studied as a function of protein concentration, temperature, and microwave power. Reductive and oxidative EPR titrations of Av_2 demonstrated that the signal height at $g = 1.94$ and integrated spin intensity (spin per mole of Av_2) responded precisely and predictably to the degree of oxidation or reduction of Av_2. When the EPR signals from all 11 samples were integrated in the $g = 2$ region of the spectrum, spin per mole values near 0.25 were obtained. Low-temperature (below 12 K) and low-power levels (below 0.8 mW) caused significant signal distortion, a phenomenon peculiar to reduced Av_2 and certain of the $[Fe_4S_4(SR)_4]^{3-}$ synthetic model compounds. Wide-field scans of Av_2 disclosed the presence of a resonance near $g = 4$ in all Av_2 samples, and signal integrations that include this low-field resonance significantly increased the spin per mole values (uncorrected for g value) of Av_2. Similar effects were observed with Av_2 in the presence of MgATP and for certain $[Fe_4S_4(SR)_4]^{3-}$ clusters.

The Fe protein component of the nitrogenase system is a two-subunit protein (α_2) containing a single iron–sulfur cluster which is thought to be of the Fe_4S_4-type and to be bound by cysteine residues from each of the two subunits (Hausinger & Howard, 1983). The protein is redox active and has been reported to undergo a well-defined electron-transfer reaction with an $E_{1/2}$ value of ca. −300 mV vs. NHE (Zumft et al., 1974; Lowe et al., 1980; G. D. Watt, unpublished results). Binding of MgATP to the dithionite-reduced protein lowers the redox potential by ca. 100 mV while concomitantly changing the EPR signal due to the iron–sulfur cluster from a rhombic to an axial form (Lowe et al., 1980; Mortenson &

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