

# Hydroxylation of fatty acids by microsomal and reconstituted cytochrome P450 2B1

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**Abstract** Understanding the mechanisms by which cytochrome(s) P450 (CYP) discriminate good from poor substrates, and orient them for highly regio- and stereoselective oxidation, has considerable intrinsic and practical importance. Here we present results of a study of fatty acid hydroxylation by CYP2B1 in a reconstituted system and in microsomes from phenobarbital-pretreated rats. The results indicate that 2B1 prefers decanoic acid as the optimum fatty acid substrate (among C<sub>8</sub>–C<sub>16</sub>) and that it hydroxylates all positions five or more methylene groups distant from the carboxylate carbon. That hydroxylation does not occur at carbon atoms closer to the carboxyl group than the C<sub>6</sub> position suggests that these carbons may not reach the ferryl oxygen because the carboxyl group is anchored to a specific site at a fixed distance from the heme iron. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Cytochrome P450 2B1; Fatty acid;  $\omega$ -Hydroxylation; Microsome

## 1. Introduction

Understanding the mechanisms by which different cytochrome P450 (CYP) isozymes discriminate good from poor substrates, and orient them for highly regio- and stereoselective oxidation, has considerable intrinsic and practical importance. We have been interested in elucidating the basis for the pronounced substrate- and regioselectivity of CYP4A enzymes, especially CYP4A1. This isozyme accepts only fatty acids or closely related aliphatic analogs as substrates, exhibits a strong preference for a C<sub>12</sub> chain length and hydroxylates the  $\omega$ -terminal methyl group with high regioselectivity. A fusion protein (f4A1) containing the elements of P450 reductase and CYP4A1 shows identical characteristics and has been probed in our laboratory using more than 40 fatty acid analogs of varying chain length, steric bulk, conformational rigidity and chemical functionality [1–4]. The results have suggested that the enzyme has a specific polar group recognition site (for COO<sup>−</sup>, CH<sub>2</sub>OH, etc.) located the length of an extended C<sub>12</sub> chain from the heme iron, and a moderately congested hydroxylation site, such that  $\omega$ -methyl groups (even those of a *t*-butyl group) can approach the ferryl oxygen

and become hydroxylated, whereas mid-chain methylene groups, regardless of chain length, do so only with great difficulty. A related situation exists for the bacterial fatty acid hydroxylase CYP102, which preferentially hydroxylates longer chains (C<sub>16</sub>–C<sub>18</sub>) at positions  $\omega$ -1,  $\omega$ -2 and  $\omega$ -3 but not  $\omega$ . Here, polar group recognition is provided by R47 while F87 blocks the  $\omega$ -methyl from accessing the ferryl group [5].

Several other mammalian CYPs also hydroxylate fatty acids. For example, CYP2E1, which is well known to oxidize a wide variety of small molecules (e.g. acetaminophen and smaller), hydroxylates fatty acids almost exclusively at the  $\omega$ -1 position and shows a pronounced preference for undecanoic and lauric acids as substrates and similarly sized aliphatic alcohols and ketones as inhibitors [6–10,25]. Interestingly, the T303S mutant of 2E1 shows similar chain length preference but dramatically reduced regioselectivity, forming a series of monohydroxy metabolites from  $\omega$  to  $\omega$ -4 [11,12]. CYP2C2 from rabbit liver hydroxylates fatty acids primarily at the  $\omega$  and  $\omega$ -1 positions and nonanoic acid has the optimum chain length [12]. Little else is known about the substrate specificity of this isozyme, but many other 2C enzymes hydroxylate larger highly non-planar molecules such as steroids, sedative hypnotics, tolbutamide and sulfonamides. CYP2B1, which accounts for  $\geq 75\%$  of the P450 content of microsomes from phenobarbital (PB)-pretreated rats, is well known for accepting a broad array of highly non-planar medium-sized molecules ranging from adamantane to hexobarbital as substrates [13].

In this manuscript we present results of a study of fatty acid hydroxylation by CYP2B1 in purified reconstituted form and in microsomes from PB-pretreated rats. The results indicate that CYP2B1 prefers decanoic acid as the optimum fatty acid substrate (among C<sub>8</sub>–C<sub>16</sub>) and that it hydroxylates all positions five or more methylene groups distant from the carboxylate carbon. That hydroxylation does not occur at carbon atoms closer to the carboxyl group than the C<sub>6</sub> position suggests that these carbons may not reach the ferryl oxygen because the carboxyl group is anchored to a specific site at a fixed distance from the heme iron.

## 2. Materials and methods

### 2.1. Preparation of microsomal fractions

Hepatic microsomes were obtained from livers of PB-treated rats by differential centrifugation as previously described [14]. Pyrophosphate-washed microsomes were suspended in 100 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol. The suspension was divided into small aliquots and stored at  $-70^{\circ}\text{C}$  prior to use with no subsequent loss of enzyme activity.

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Abbreviations: CYP, cytochrome(s) P450; PB, phenobarbital

## 2.2. Enzyme preparations

Electrophoretically homogenous CYP2B1 was isolated as described previously [15]. NADPH-CYP oxidoreductase (OR) was kindly provided by Dr. R.W. Estabrook (University of Texas Southwestern Medical Center at Dallas).

## 2.3. Enzyme assay

CYP concentrations were determined by the method of Omura and Sato using the extinction coefficient  $\epsilon_{450-490} = 91/\text{mM}/\text{cm}$  [16]. NADPH-CYP OR concentrations were measured spectrophotometrically using the extinction coefficient  $\epsilon_{456} = 21.4/\text{mM}/\text{cm}$  [17]. Microsomal protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA).

## 2.4. Fatty acid hydroxylase assay

Identification and quantitation of metabolites were performed using GC/MS as described previously [4]. Incubations were conducted at 37°C in 13×100 mm glass tubes. Briefly, reaction buffer (100 mM potassium phosphate, pH 7.4, containing 10 mM  $\text{MgSO}_4$ ) was pre-heated to 37°C, and aliquots of substrate (100 nmol) and microsomes (1.4–1.7 nmol P450), or 2B1 reconstituted system (1.0 nmol CYP2B1, 1.5 nmol OR and 50  $\mu\text{g}$  DLPC) were added. This mixture was vortexed and incubated for 2 min at 37°C and the reaction started by the addition of NADPH (1  $\mu\text{mol}$ ; 20  $\mu\text{l}$  of 50 mM stock solution in  $\text{H}_2\text{O}$ ). The final incubation volume was 1.0 ml. The mixtures were incubated for 10 min at 37°C (the reaction rate is constant for at least 12 min [4]). After incubation, reactions were quenched with HCl and metabolites were recovered by solid phase extraction, derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide and analyzed by capillary GC/MS. For quantitation, metabolite response factors were measured directly from standards or estimated from closely similar compounds [18].

## 3. Results and discussion

We first examined the hydroxylation of nine saturated straight-chain fatty acids ( $\text{C}_8$ – $\text{C}_{16}$ ) by liver microsomes from PB-induced rats in which the major P450 isozyme present ( $\geq 75\%$  of total) is CYP2B1 [19–22]. The results obtained, presented in Table 1, show several interesting features. All nine substrates were hydroxylated, but with widely differing efficiency. Total turnover was significantly greater for the  $\text{C}_{10}$ ,  $\text{C}_{11}$  and  $\text{C}_{12}$  substrates compared to their shorter or longer homologs. With most substrates the hydroxylation showed moderate to high regioselectivity for the terminal  $\omega$  position. This was unexpected, considering the generally low specificity of CYP2B1 toward a wide range of xenobiotic substrates. This regioselectivity for  $\omega$ -hydroxylation was most pronounced with decanoic and nonanoic acids, where it accounted for 83% of total products. Similar results were observed with nonanoic acid, and to a lesser extent with several other acids. Given that hydroxylation of decanoic acid occurs

on every carbon from  $\text{C}_{10}$  through  $\text{C}_6$ , the product ratio expected on purely statistical grounds would have been 3/11 or 27%  $\omega$ -alcohol, but if one takes into account the intrinsically lower reactivity of methyl vs. methylene hydrogens, this number should be even lower. Thus the  $\omega$ -regioselectivity of CYP2B1 toward medium-chain fatty acids is quite striking indeed.

In addition, a second type of selectivity is revealed in the hydroxylation data of Table 1. Thus, it appears that no hydroxylations occur at carbons within the first six atoms of the fatty acid chain (counting from the carboxylate carbon as  $\text{C}_1$ ). Otherwise, hydroxylation occurs at all positions to some extent. For low-turnover substrates like  $\text{C}_{15}$  and  $\text{C}_{16}$  it was not always possible to detect every possible hydroxylation product. These more strongly hydrophobic acids may have been less available to the enzyme because of enhanced partitioning into microsomal lipid or micelles.

One interpretation of the above results is that CYP2B1 has an active center large enough to accommodate fatty acids up to at least  $\text{C}_{14}$  in total size, probably in a coiled rather than extended conformation, but with the side chains having the conformational freedom to present most or all of the methylene positions to the ferryl oxygen. Thus, for longer fatty acids a larger array of regioisomeric alcohol metabolites is produced. The absence of hydroxylation on the first six carbons of the molecule in all cases may indicate that the carboxylate group is recognized and bound at a site that is located about the length of a pentamethylene chain away from the ferryl oxygen, a situation not unlike that inferred for CYP4A1 [2–4] or observed for CYP102 [5]. Such a view is also consistent with the broad acceptance of many medium-sized non-planar xenobiotics as substrates and/or ligands by CYP2B1.

In reconstituted systems, purified CYP2B1 showed a somewhat similar pattern of selectivity toward fatty acid substrates (Table 2). However, in these experiments, the turnover numbers were lower than those observed with microsomes because we did not attempt to optimize the reconstitution conditions in terms of lipid, detergent, reductase, cytochrome  $b_5$ , etc. Variation of these factors in reconstituted systems is well known to affect turnover rates. One consequence of the lower overall turnover rates is that it became difficult to observe many of the individual minor mid-chain hydroxylation products that were observed in the microsomal system. Nevertheless, with reconstituted CYP2B1, decanoic acid was again hydroxylated preferentially at the  $\omega$  position, and nonanoic acid, the shortest acid studied, was a decidedly poorer substrate overall. With longer fatty acids this preference for  $\omega$ -hydrox-

Table 1  
Fatty acid hydroxylation by PB-induced microsomes

| Fatty acid    | Metabolite produced (pmol product/min/nmol P450) |            |            |            |            |            |            |            |
|---------------|--------------------------------------------------|------------|------------|------------|------------|------------|------------|------------|
|               | $\omega$                                         | $\omega-1$ | $\omega-2$ | $\omega-3$ | $\omega-4$ | $\omega-5$ | $\omega-6$ | Total      |
| Octanoic      | 56 ± 12                                          | 20 ± 3     | 42 ± 19    | nd         | nd         | nd         | nd         | 118 ± 34   |
| Nonanoic      | 268 ± 18                                         | 51 ± 9     | 32 ± 8     | nd         | nd         | nd         | nd         | 351 ± 35   |
| Decanoic      | 1173 ± 394                                       | 88 ± 44    | 45 ± 19    | 70 ± 24    | 32 ± 2     | nd         | nd         | 1409 ± 484 |
| Undecanoic    | 562 ± 206                                        | 303 ± 54   | 75 ± 9     | 91 ± 17    | 61 ± 13    | 21 ± 3     | nd         | 1113 ± 301 |
| Dodecanoic    | 542 ± 284                                        | 592 ± 220  | 163 ± 64   | 107 ± 41   | 61 ± 24    | 27 ± 12    | 21 ± 1     | 1514 ± 645 |
| Tridecanoic   | 194 ± 104                                        | 241 ± 47   | 57 ± 3     | 70 ± 14    | nd         | 18 ± 3     | 20 ± 2     | 600 ± 173  |
| Tetradecanoic | 140 ± 53                                         | 118 ± 5    | 46 ± 10    | 55 ± 13    | 26 ± 8     | 24 ± 4     | 19 ± 1     | 429 ± 88   |
| Pentadecanoic | 81 ± 16                                          | 120 ± 6    | nd         | nd         | nd         | nd         | nd         | 201 ± 22   |
| Hexadecanoic  | 82 ± 46                                          | 41 ± 16    | nd         | nd         | nd         | nd         | nd         | 122 ± 62   |

All values are reported as the mean from four to six experiments ± S.D. nd, not detected. Our level of detection was less than 14 pmol/min/nmol P450

Table 2  
Fatty acid hydroxylation by reconstituted CYP2B1

| Fatty acid    | Metabolite produced (pmol product/min/nmol P450) |              |             |              |
|---------------|--------------------------------------------------|--------------|-------------|--------------|
|               | $\omega$                                         | $\omega$ -1  | $\omega$ -2 | Total        |
| Nonanoic      | nd                                               | 17 $\pm$ 3   | nd          | 17 $\pm$ 3   |
| Decanoic      | 184 $\pm$ 36                                     | 64 $\pm$ 9   | nd          | 248 $\pm$ 43 |
| Undecanoic    | 43 $\pm$ 4                                       | 108 $\pm$ 12 | nd          | 151 $\pm$ 16 |
| Dodecanoic    | 70 $\pm$ 17                                      | 69 $\pm$ 18  | nd          | 139 $\pm$ 35 |
| Tridecanoic   | 49 $\pm$ 18                                      | 209 $\pm$ 3  | nd          | 258 $\pm$ 22 |
| Tetradecanoic | 72 $\pm$ 12                                      | 193 $\pm$ 26 | 20 $\pm$ 3  | 285 $\pm$ 58 |
| Hexadecanoic  | nd                                               | 171 $\pm$ 10 | nd          | 171 $\pm$ 10 |

All values are reported as the mean from three to six experiments  $\pm$  S.D. nd, not detected. Our level of detection was less than 14 pmol/min/nmol P450. Assay conditions as described under Section 2.

ylation was significantly reduced or even shifted to the  $\omega$ -1 position. In contrast, with CYP4A1, the strong preference for  $\omega$ -hydroxylation is maintained for longer fatty acids, even though their turnover decreases significantly [1].

It is well documented that CYP2B1 displays very broad substrate specificity and its active center can accommodate a wide variety of different chemical structures. CaJacob et al. [23] reported P450<sub>b</sub> ('major' form of PB-induced rat CYP) to have  $\omega/\omega$ -1 ratio for hydroxylation of lauric acid of 1:8 (0.13), while in PB-microsomes Okita and Masters reported this ratio as 0.6 [24]. We have found that for lauric acid (C<sub>12</sub>) the  $\omega/\omega$ -1 ratio for both microsomal and reconstituted CYP2B1 is around 1 (Table 1). While microsomal data on the hydroxylation ratio of lauric acid can be considered as roughly similar, the difference in the data concerning the reconstituted system is considerable. CaJacob et al. [23] used in their study PB-induced CYP isolated by the procedure of Waxman and Walsh [25]. Analysis of the N-terminal sequence from this paper revealed that this protocol leads to the isolation of CYP2B2 rather than CYP2B1.

The site of hydroxylation in a fatty acid substrate is apparently determined by the interplay of two factors, one intrinsic to the substrate molecules (thermodynamic), and one intermolecular in nature (steric). The thermodynamic factor (i.e. weaker C–H bonds in methylene vs. methyl groups) strongly favors  $\omega$ -1 hydroxylation; thus,  $\omega$ -hydroxylation occurs when the fatty acid is placed in the active center in such a way that only the  $\omega$ -methyl group can 'reach' ferryl oxygen of P450 heme. By this means, analysis of chain-length dependence with regard to regioselectivity of fatty acid hydroxylation can reveal information about the structure of active center and the distance between entrance to the substrate access channel and the heme ferryl oxygen.

To date four different P450 isozymes (CYP2E1, CYP2C2, CYP4A1 and CYP4A11) have been characterized in terms of specificity toward hydroxylation of fatty acids, and all exhibit distinct regioselectivity and chain-length dependence. While CYP2E1 hydroxylates fatty acids almost exclusively at the  $\omega$ -1 position with lauric acid being the most favorable substrate, CYP2C2 hydroxylates fatty acids at different positions depending on the chain length with nonanoic acid being preferentially hydroxylated at the  $\omega$  position [9,12]. Remarkable selectivity in hydroxylation of the less reactive, terminal methyl group of fatty acids is a characteristic feature of CYP4A isozymes. CYP4A isozymes primarily hydroxylate a very homogenous group of substrates, medium and long-chain fatty acids, prostaglandins and eicosanoids.

It is quite striking that the value of the  $\omega/\omega$ -1 ratio for

microsomal CYP2B1 hydroxylation of decanoic acid is on the same order of magnitude as  $\omega/\omega$ -1 ratio for CYP4A1 hydroxylation of lauric acid. Shortening as well as increasing the chain length causes a sharp decrease in the  $\omega/\omega$ -1 ratio.

Taken together the data presented confirm and extend earlier suggestions [1,26] that the characteristic feature of a P450 active center is the presence of a polar recognition site that can govern positioning of the substrate molecule in the active center, and in case of CYP2B1, the distance between this polar recognition site and the oxo-heme group is approximately the length of an extended 10-carbon chain (ca. 12 Å). In conclusion, we would like to point out the general applicability and ease of use of homologous series of fatty acids and fatty acid analogs as substrates for active center mapping of CYP isozymes.

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