ORIGINAL ARTICLE

# Structural analysis of binding of a diamantoid substrate to cytochrome P450 2B4: Possible role of Arg 133 in modulation of function and activity of this enzyme

**Miroslav Šulc, Jiří Hudeček, Marie Stiborová, Petr Hodek**

Department of Biochemistry, Faculty of Science, Charles University of Prague, Praha 2, Czech Republic

*Correspondence to:* Jiří Hudeček, PhD.
Department of Biochemistry, Faculty of Science, Charles University of Prague,
Hlavova 2030, CZ-128 40, Praha 2, Czech Republic
TEL.: +420 221 951 285, FAX: +420 221 951 283
E-MAIL: hudecek@natur.cuni.cz

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## Abstract

**OBJECTIVES**: Understanding the enzyme mechanism of P450 enzymes needs a detailed knowledge of substrate-enzyme interactions. Here, we examined the interaction of cytochrome P450 2B4 with a diamantanoid substrate.

**METHODS**: The interaction was followed using a photoactivable label, 3-azidiamantane. After photochemically driven reaction, the labeled enzyme was cleaved by trypsine and the labeled peptides separated by HPLC and indentified with the help of radioactivity (for tritiated label) and mass spectrometry. The results were analysed on the basis of the known X-ray structures for mammalian cytochromes P450.

**RESULTS**: Identification of labeled peptides has shown that the probe (binding as a substrate to the enzyme) was attached to fragments: 30–48 (the most likely positions of the label are Leu44, Gln45 and Asp47), 127–140 (with Arg133 labeled, as indicated by mass spectrometry), 359–373 and 434–443 (the exact position of the label unknown). The structural comparison indicates considerable differences in Arg133 interaction with heme propionates, connected with binding of the substrate. Labeling of this residue may thus reflect its involvement in modulation of cytochrome P450 activity.

**CONCLUSION**: The results show existence of additional binding sites for substrate on cytochrome P450 2B4, located close to the surface of the enzyme.

**Abbreviations**

| | |
|---|---|
| BF | - bifonazole |
| CID | - collision-induced dissociation |
| CPI | - 4-(4-chlorophenyl)imidazole |
| HPLC | - high performance liquid chromatography |
| MALDI | - matrix assisted laser desorption ionization |
| MS | - mass spectrometry |
| P450 | - cytochrome P450 |
| TFA | - trifluoracetic acid |
| TOF | - time of flight |
| TPCK | - 1-chloro-3-tosylamido-4-phenyl-2-butanone |

## INTRODUCTION

Superfamily of cytochromes P450 heme enzymes (P450, EC 1.14.14.1) comprises ubiquitous terminal oxidases/oxygenases, acting in multicomponent electron transfer chains (Ortiz de Montellano, 2005). Among various forms of these enzymes, much research interest is attracted by the eukaryotic liver microsomal enzymes, participating in the metabolism of many drugs, xenobiotics and other nonpolar compounds. In particular, knowledge of the details of interaction between the enzyme and substrate may help to understand the specificity of individual P450 forms and the regulation of the rather complicated mechanism of action of these enzymes, which involves (i) binding of the substrate, (ii) electron transfer from the NADPH-P450 reductase and reduction of the heme iron, (iii) binding of dioxygen and its activation by partial reduction, resulting in the hydroxylation of the substrate.

Obviously, the knowledge of three-dimensional structure of the enzyme is extremely important for this purpose. For long time, the P450 research had to rely on the extrapolation of knowledge from the prokaryotic enzymes (in particular the cytochrome P450cam), which are easier to crystallize than the integral membrane microsomal P450s. However, in the last decade several crystal structures of microsomal enzymes became available, among them also some forms without and with substrate or inhibitor bound (for a review, see e.g. Otyepka *et al.* 2007).

Regrettably, this newly available structural information does not alleviate all problems: the crystallized molecules are as a rule genetically engineered chimeras lacking at least the membrane-binding part. Therefore many aspects of membrane P450 systems, the dynamic aspects of the enzyme-substrate interaction and the effects of the flexibility of the enzyme are still not solved completely (Guengerich 2004). Therefore, the "indirect" experimental methods may still represent a substantial complement to the "direct" crystallographic information. Examples are spectroscopic methods (absorption, fluorescence, Raman spectroscopy) and chemical modifications (cross-linking, photoaffinity labeling).

Recently, we reported the results of photoaffinity labeling of the rabbit liver microsomal cytochrome P450 2B4 with a substrate analog 3-azidiamantane (Hodek *et al.*, 2007). The phenobarbital-inducible P450 2B4 is a frequent model and was used in numerous metabolic and structural studies. In the present paper, we analyze the results obtained by photoaffinity labeling further, using the available information on crystal structures of complexes of this enzyme and other mammalian microsomal P450s with inhibitors/substrates.

## MATERIAL AND METHODS

Cytochrome P450 2B4 was isolated from liver microsomes of rabbits pretreated with phenobarbital, and purified by the method of Haugen and Coon (1976). Specific content was 12.2 nmol of P450, as determined spectrophotometrically by the method of Omura and Sato (1964), per mg of total protein, determined by the bicinchonine method (Smith *et al.*, 1985) with bovine serum albumin as a standard. The photolabile probe (3-azidiamantane) and its tritium-labeled compound were prepared as described previously (Hodek and Smrček, 1999).

### *Photoaffinity labeling*

The details have been described previously (Hodek *et al.*, 2007). Briefly, the reaction mixture contained P450 (1 nmol/l) in potassium phosphate buffer (0.1 M, 20% glycerol, pH 7.4) and the equimolar concentration of the label (3-azidiamantane or its tritiated analog). After incubation, the mixture was photoactivated. In competition experiments, an equimolar amount of diamantane was added to the reaction vessel. After the reaction, the excess of unreacted label and the detergent were removed with chromatography on hydroxyapatite, and the sample dialysed.

### *Trypsin digestion*

The sample was digested with TPCK-treated trypsin as described previously (Hodek *et al.*, 2007). The reaction was stopped by addition of TFA (final concentration 1%), and the sample was evaporated and stored at –20 °C.

### *HPLC separation of labeled peptides*

The sample after trypsinolysis was extracted with 10% acetonitrile (with 1% TFA), and the extract separated on a C-18 reverse-phase column, using a linear gradient of acetonitrile (0–75% in 62.5 min) with 0.1% of TFA. The fractions (radioactivity, 214 nm absorption) were pooled and re-chromatographed under similar conditions (with presence of 0.1% ammonium acetate, pH 6.0). Finally, the radioactive fractions were re-chromatographed again and sequenced, using the Edman N-terminal method on a Protein Sequencer LF 6000D (Beckman Instruments, USA). Similar procedure was undertaken with the non-radiactive labeled material, the fractions were collected and subjected to mass spectrometric analysis (MALDI-TOF).

MALDI-TOF analyses were performed on a Bruker-Biflex (Bruker-Daltonics, USA) equipped with a 337 nm nitrogen laser and a delayed extraction ion source. As a MALDI matrix, a 10 mg/ml solution of α-cyano-4-hydroxycinnamic acid in aqueous 30% acetonitrile and 0.1% TFA (v/v) was used. Positive-ion mass spectra of peptide maps were collected in the reflection mode. The sample was loaded on the target, allowed to dry at ambient temperature and covered with the matrix solution. Spectra were externally calibrated by employing the monoisotopic $(M+H)^+$ ion of a peptide standard (human angiotensin I). Every recovered spectrum was compared with theoretical peptide maps and fractions with ghost peaks were additionally analyzed by LC–MS/MS.

The diluted and MALDI-TOF analyzed HPLC fractions were loaded on a LC–MS/MS system (LC$^{Q}$DECA, ThermoQuest, San José, USA) and tandem mass spectra were acquired. HPLC fraction (previously analyzed on the MALDI-TOF) were applied (5 μl) on the capillary column (0.1 × 150 mm) packed with 10 cm of C18 RP resin MAGIC AQ (MichromBioresources, USA). Peptides were separated using gradient elution: 65 min from 5% acetonitrile/0.5% acetic acid to 40% acetonitrile/ 0.4% acetic acid, 15 min from 40% acetonitrile/0.5% acetic acid to 70% acetonitrile/0.4% acetic acid. The column was directly connected to an LC$^{Q}$DECA ion trap mass spectrometer equipped with a nanoelectrospray ion source. Spray voltage was held at 1.2 kV, tube lens voltage was 30 V. The heated capillary was kept at 175 °C with a voltage 10 V. Collision energy was kept at 42 U and the activation time was 30 ms. Positive-ion full scans were acquired over an m/z range 350–1 600. Collisions were done for the top three most intense ions in each full MS scan. Dynamic exclusion was enabled with repeat count of 2.

*Analysis of crystallographic data:*

The crystallographic coordinates for cytochromes P450 were taken from the respective Protein Data Bank files (http://www.rcsb.org/pdb). Only the data with resolution of 2.7 Å or better were included. The following files were analyzed: CYP2A6 (files 1Z10, 1Z11, 2FDU, 2FDV, 2FDW, 2FDY, 2PG6), CYP2B4 (1SUO, 1PO5, 2BDM), 2C5 (1DT6, 1N6B, 1NRG), CYP2C9 (1OG2, 1OG5, 1R9O) and CYP3A4 (1TQN, 1W0E, 1W0F, 1W0G ). The structures were inspected and the interatomic distances measured manually using the program RasTop ver. 2.1.

## RESULTS AND DISCUSSION

Identification of labeled parts of P450 2B4: The peptides, obtained by trypsinolysis of the cytochrome P450 2B4 photolabeled by tritiated 3-azidiamantane, were separated and purified by HPLC. The results of sequencing of these peptides and identification of their position within the P450 sequence are summarized in Table 1.

In order to get more information about the exact positions of label attachment, the labeling (with non-radioactive 3-azidiamantane) was performed under conditions of competiton of substrate (diamantane) and the peptides isolated by HPLC were subjected to mass spectrometric analysis. For two peptides, corresponding to the peptides C and D in the Table 1, the MS analysis found specific fragments marked by the *m/z* difference (as compared with the fragments found in absence of the label or in presence of competing substrate) corresponding to the diamantane carbene.

The covalent structure of suspected labeled peptides of the peptide C was determined using tandem MS (MS/MS). Daughter ion spectra of peptide C fragmented by collision-induced dissociation (CID) are shown in Figure 1. Data confirmed the deduced amino acid sequence of the labeled peptide and indicated that Arg133 is covalently modified with the diamantane moiety. In addition, the CID spectrum of oxidized peptide C showed the characteristic loss of methanesulfenic acid from the side chain of oxidized Met132 (Figure 1)

Localization of labeled residues within the peptides: First, we should consider the general properties of the photoaffinity label and its mode of binding to

**Table 1.** Tryptic peptides of P450 2B4 labeled with diamantane

| Fragment peptide | Sequenced peptide[a] | Sequence[b] |
|---|---|---|
| Peptide A | A1 (359–373) | LGDLIPFGVPHTVTK |
| Peptide B | B1 (30–38) | LPPGPSPLP |
| | B2 (30–48) | LPPGPSPLPVLGNLLQM*R |
| | B3 (30–48) | LPPGPSPLPVLGNLL*MDR |
| | B4 (30–48) | LPPGPSPLPVLGNLLQM*R |
| | B5 (30–46) | LPPGPSPLPVLGNL*QM |
| | B6 (30–45) | *PPGPSPLPVLGNLL*M |
| | B7 (30–48) | LPPGPSPLPVLGNLLQM*R |
| Peptide C | MS (127–140) | FSLATM**R**DFGMGKR |
| Peptide D | MS (434–443) | RICLGEGIAR |

[a] In parentheses, the position in P450 2B4 sequence is indicated. MS – sequenced by mass spectrometry.
[b] Asterisk indicate residues unidentified by Edman sequencing (possibly labeled with diamantane).
In bold, the labeled Arg133 identified by mass spectrometry.

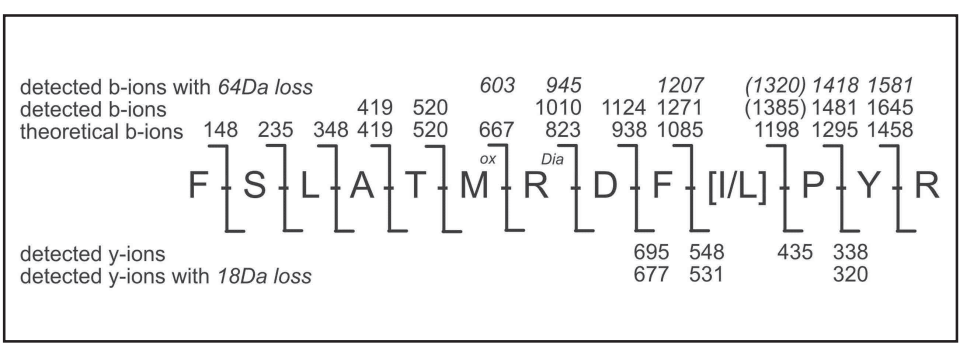


**Figure 1.** Scheme of the fragmentation of peptide C, based on CID daughter spectrum of the species with m/z 1819.3. The calculated and observed m/z values of b7 and following ions in b-series differ by 186, indicating the covalent attachment of the probe to Arg133. The loss of methanesulfenic acid (64) from Met132 (italics) is also shown. Parentheses indicate low peak intensity.

the P450 2B4. Previously, we have shown (Hodek and Smrček, 1999) that the photoactivated compound reacts readily with hydrocarbons (hexane). On the other hand, the electrophilic character of the carbene intermediate formed by photolysis directs it preferrentially to the electron-rich compounds (alcohols, water, dioxygen). Due to the short lifetime of the carbene intermediate, this effectively excludes a possibility that the activated label could "migrate" from the bulk solvent towards the P450 molecule (or along the protein surface). Therefore, the sites labeled in the enzyme should be the same as where the label was bound before the photoactivation (or, more exactly, the atoms spatially accessible by the carbene end of the label).

Our previous studies have shown that the diamantoid label binds with a high specificity to the substrate-binding site, and the introduction of diazirine group does not change the binding properties significantly (Hodek and Smrček, 1999). This was proven both by the values of spectroscopic dissociation constant (indicating the extent of low to high spin conversion upon binding) and the inhibitory effect of the label on P450 enzymatic activity.

The sequencing of peptides isolated after re-chromatography has readily identified their position within the primary structure. For the radioactive fractions eluted between 34.0 and 34.5 minutes, the sequence was deduced from the mass spectrometry experiment.

Structural interpretation of the results needs to identify as closely as possible the residues, labeled in the experiment. This task is rather straightforward for the fragment peptide C, where the arginine residue (Arg133) was clearly indicated as labeled with diamantane by mass spectrometry. The situation is less positive for the fragment peptide D, where the labeled position could not be directly indicated. The peptide has been shown as labeled both by MALDI and by the incorporation of radioactivity, and the labeling was demonstrated to be specific (competitive saturation with diamantane). Thus, one has to accept as a fact that the labeled position is in the labeled fragment (434–443).

For the peptide B, the identification of individual labeled residues might be helped if we realize that (i) for the residues labeled in the side chain, their analytical identification after cleavage is likely to be often impossible, and (ii) next to the label incorporated in the peptide backbone, both the clevage with trypsin and in the Edman method may not be possible. Thus, we may expect the residues Leu44 (B5, B6), Gln45 (B3), Asp47 (B2, B4, B7) and possibly also Leu30 (B6) being labeled. (In parenthesses, the respective isolated peptides are indicated, cf. Table 1). In the last case (B6), the labeling of Leu30 is not very probable, as the peptide with two strongly hydrophobic diamantane moieties attached would likely not be eluted with the elution time comparable (or lower) to the single-labeled peptide of the same sequence. Thus, the fact that the leucine residue in N-terminal position 30 was not identified in the sequence has probably some other causes. The existence of three different isolated peptides with the same position Asp47 labeled (B2, B4, B7) might be explained with the possibility of various atoms in the same residue being labeled, and the isomeric peptides differing in their elution times. Another alternative comes from the fact that there are two additional trypsin-cleavable positions located next to the C-terminus of the sequence found for fragment peptide B, namely Lys49 and Arg53. Therefore, some of the three peptides might

**Table 2.** Distances between arginine and propionate in P450 2B4

| Enzyme | Chain | Arg 98 | | Arg 125 | | Arg 133 | | Arg 434 | |
|---|---|---|---|---|---|---|---|---|---|
| Substrate-free | A | 8.1 | 8.8 | 13.4 | 11.3 | 11.3 | 9.5 | 5.5 | 5.0 |
| | | 6.1 | 7.1 | 11.3 | 9.2 | 10.0 | 8.5 | 4.9 | **4.0** |
| | D | **3.7** | **2.8** | 9.2 | 7.0 | 4.7 | **3.8** | 6.3 | 6.9 |
| | | **2.7** | **3.2** | 8.3 | 6.1 | 6.2 | 5.4 | 5.7 | 5.5 |
| CPI-bound | A | **2.9** | 2.7 | 7.2 | 8.6 | 19.2 | 18.9 | 6.3 | 7.6 |
| | | 4.7 | 4.9 | 8.0 | 8.9 | 19.3 | 18.6 | 8.1 | 9.2 |
| | D | 5.1 | **2.9** | 4.6 | 6.9 | 16.8 | 16.9 | 5.7 | **2.9** |
| | | 6.9 | 4.7 | **2.8** | 5.0 | 14.7 | 14.9 | 5.0 | **4.0** |
| Bifonazole-bound | A | 9.4 | 9.6 | 12.3 | 11.9 | 20.8 | 22.2 | 6.4 | 5.6 |
| | | 11.4 | 11.5 | 14.1 | 13.6 | 18.6 | 20.0 | 7.9 | 7.7 |
| | D | 8.0 | 8.5 | 8.7 | 8.0 | 16.6 | 14.5 | 4.3 | **3.0** |
| | | 9.6 | 10.2 | 8.3 | 7.0 | 15.0 | 12.8 | 4.7 | **4.0** |

Values are distances (in Å) between carboxyl oxygen of the heme propionate (A or D chain) and the guanidine nitrogen of the indicated arginine side chain. The interchangeable atoms (O1 and O2, NH1 and NH2) have been arranged to emphasize the similarity of interactions. The distances below 4 Å ("salt-bridges") are shown in bold.

be in fact longer, although the sequencing was not possible beyond the position 48 (e.g., because of labeling in the Lys49 residue). For the peptide B1, no residue was indicated as being labeled (although the incorporated radioactivity shows the presence of the label). A possible explanation is that the modified position is at the protein backbone next to the Val39, preventing the Edman sequencing beyond the position 38.

Also in the peptide A1, no label position is apparent, although the fragment peptide contains the label. Possibly, the label was lost (by hydrolysis) during the sequencing. Likely candidates for such a labile label bonding would be the threonine residues 370 and 372. An alternative explanation is similar to the B1 peptide: there is another trypsin cleveage site C-terminally to the 373 position. Thus, also here a labeling of the electron-rich Asp374 might stop sequencing, and the fragment peptide A can be in fact longer.

To locate the modified residues in the CYP molecule, and to gain some insight into their possible functional importance, we compared our experimental data with the crystal structures for the substrate-free P450 2B4 (PDB code 1PO5), and for the enzyme with bound inhibitors: CPI and BF, codes 1SUO, 2BDM, respectively.

Comparing our results with the inhibitor-bound structures, we should stress that whereas both CPI and BF react as **ligands** of the heme iron in CYP2B4 (producing the type II difference spectra upon binding), diamantane and diamantane-diazirine are bound as **substrates** (slightly apart from the iron). The size of diamantane molecule (and of its derivative) approximately fits between that of both inhibitors, but the diamantane structure is much more rigid.

The seemingly surprising fact that no residue was labeled inside of the known substrate-binding site (i.e., opposite to the cysteinate) might be explained by the presence of water molecules inside of this cavity. Such (electron-rich) water molecule(s) are likely to react preferentially with the photoactivated label and effectively quench it. The "open" conformation of the P450 2B4 would present a good opportunity for water molecules to "interfere" with the substrate (label) binding, and presence of such molecules and their role in enzyme activity has been postulated (Oprea *et al.*, 1997)

Nonetheless, the other labeled sites had high affinity towards the substrate, although they are mostly located close to the enzyme surface, and their possible functional significance is less clear.

The peptide A (359–373) is practically coincident with the "substrate-recognition site 5", postulated by Gotoh (1992). It likely marks the entrance to one of the recently proposed (Cojocaru *et al.*, 2007) substrate-access channels (2b/2e). The labeling of the proline-rich peptide B (30 to 48) is slightly more difficult to interpret. The most likely positions of labeling of this peptide are between Leu44 and Asp47. This "pre-helix region", just before the A-helix, was formerly reported to interact with the membrane and, at the same time, to facilitate the entrance of hydrophobic substrates into P450 substrate cavity (Dai *et al.*, 1998). Thus, both peptide A and peptide B labeling might be basically understood in the framework of substrate access/egress pathways.

The more intriguing questions are connected with the remaining two peptides: D (434–443) and C. Both are located on the same side as the cysteinate ligand of the heme iron, i.e., **opposite** to the conventional active site. Here, the access would be possible only after massive rearrangements in the vicinity of the heme.

This region is known as the place of interaction of P450s with reductase and/or with cytochrome $b_5$. For cytochrome P450 2B4 itself, there is a solid experimental evidence of involvement of residues on the proximal surface (including Arg133) in this interaction (Bridges *et al.*, 1998).

The Arg133 (and other arginine residues located in these two labeled peptides) may thus play role(s) in binding of P450 redox partners or in electron transfer towards the heme. Direct binding of the substrate to this region might interfere with this binding and might serve as a sort of control mechanism in the initial steps of the P450 enzyme action.

There is an additional role for Arg133 in P450 2B4. This residue is partly involved in the interplay of electrostatic pairing of the heme propionates, characteristic for cytochromes P450 (Poulos *et al.*, 1987, Hasemann *et al.*, 1994, Lounnas and Wade 1997). The role of heme propionates in function of P450 and other heme enzymes has been discussed and studied recently (Guallar and Olsen 2006, Harada *et al.*, 2008).

To understand more deeply the involvement of Arg133 side chain in substrate binding in P450 2B4, we analysed the behavior of this residue in detail, comparing its position (along with other heme-propionate interacting arginine residues) in the crystal structure of the substrate-free and inhibitor-bound enzyme (Table 2).

Interestingly, the situation in P450 2B4 is very different from other mammalian P450, for which the crystal structures are available. Whereas in most cases binding of substrate/inhibitor is accompanied only by small changes in the salt-bridges pattern (within one Å, data not shown), in P450 2B4 the Arg133 is moved away from the heme towards the surface (where it can be labeled) and its place might be occupied by Arg125 (as in the CPI-bound "closed form"). Also other arginine chains are subject to large movements.

Such rearrangements of the strongly charged arginine side chains in the vicinity of heme and of the interaction surface with the reductase might play an important role in regulation of the activity of this P450 form. Similar impact on the P450 reaction might have the competiton between the reductase or cytochrome $b_5$ and the substrate molecule, or, alternatively, some sort of "preparative" interaction of substrate (or other hydrophobic molecules – e.g., a detergent) prior to sub-

strate binding in the active site. Such interactions might be important also in the regulatory/differentiating role found for cytochrome $b_5$ recently (Stiborová 2006). In this respect, the bifonazole-bound P450 2B4 (PDB file 2BDM) is rather instructive. Here, three molecules of substrate are bound (in addition to four detergent molecules). If similar interactions indeed occur, and how the individual P450s might differ in this respect, has to be solved by a future research.

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