The active site of cytochrome P-450 nifedipine oxidase: a model-building study

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A model of the active site of cytochrome P-450 nifedipine oxidase is built on the basis of sequence homology with cytochrome P-450$_{CAM}$. Substrates are docked into the binding pocket, and molecular mechanical energy minimization is performed to analyze the forces between the substrates and the enzyme.

Keywords: cytochrome P-450, sequence homology, nifedipine oxidase, P-450$_{CAM}$, substrate docking, drug metabolism, mutation modeling, Intrasequence Homology Display (IHD)

INTRODUCTION

Cytochrome P-450 is a ubiquitous monooxygenase enzyme.[1,2] It catalyzes the oxidation of compounds both endogenous and exogenous to the body, playing a role in the detoxification of drugs, in the biosynthesis and metabolism of steroid hormones, in the deactivation of certain drugs and in the carcinogenic activation of polycyclic aromatic hydrocarbons. It exists in multiple forms; each may bind different substrates and may differ in regio- and stereoselectivity.

The aim of studies of P-450s is to inhibit selectively undesirable reactions catalyzed by these enzymes. To achieve this goal, precise information about the active site is required. However, P-450s are usually membrane-bound insoluble enzymes, and normal techniques fail to produce purified preparations suitable for experimental structure determinations. The only known exception is P-450$_{CAM}$ from the bacterium *Pseudomonas Putida*, for which a crystal structure has been elucidated.[3] This has prompted several studies on this relatively easily available P-450, assuming that the catalytic cycle is independent from the source of the enzyme. However, the design of selective inhibitors requires detailed knowledge of the active site of the particular enzyme to be blocked. Since direct experimental information on the three-dimensional structure of mammalian P-450s is not likely to be easily available, we have performed a model-building study of the active site of a P-450.

Although P-450s are the subject of intensive research, little information useful in modeling the active site of any mammalian P-450 is available. The choice of P-450 nifedipine oxidase (P-450$_{NF}$) was influenced by the following facts: The primary sequence of this enzyme is known;[4] several substrates of it have been described;[5–7] and it metabolizes important drugs. The successful modeling of its active site is the first step toward the design of inhibitors that affect the metabolism of such substrates and drugs.

P-450$_{NF}$ catalyzes the oxidation of several dihydropyridine derivatives that are widely used in the treatment of hypertension and angina pectoris. It is also active toward steroids such as testosterone, androstenedione, cortisol and estradiol, and it further metabolizes compounds such as quinidine, benzphetamine and aldrin. Its substrate specificity overlaps with other P-450 isozymes.[7,8]

METHODS

Assuming that the backbone coordinates in the vicinity of the active site are more or less the same in both P-450$_{CAM}$, for which crystal structure is known, and in P-450$_{NF}$, we investigated changes in the residues of P-450$_{CAM}$ needed to produce the putative active site of P-450$_{NF}$. Having built the binding site, we investigated its ability to bind the known substrates of the enzyme. Several substrates were docked and molecular mechanical minimizations carried out to avoid unfavorable contacts and to elucidate the nature of the binding forces.

Similarities between P-450$_{CAM}$ and P-450$_{NF}$ were assessed using global and local alignment methods. Global alignments were carried out using Needleman and Wunsch algorithm[9] with the Dayhoff mutation data matrix[10] as scoring matrix and with various matrix bias and break penalties.[11] The RELATE program[12] was run with various window lengths to perform local alignments. The results were evaluated with the help of Intrasequence Homology Display.[13]

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Attention was focused on the residues within 10 Å of the camphor C5 atom, which is hydroxylated by P-450$_{CAM}$. Both side-chain mutations and initial substrate docking were carried out by using the program QUANTA.[14] The AMBER[15] suite of programs was used to perform molecular mechanical minimization. Parameters were implemented by exploiting analogies between substrates and fragments whose parameters are present in the AMBER database. For the $-NO_2$ group, parameters of Ref. 16 were used. Atomic charges were determined by fitting electrostatic potentials to the potentials calculated from AM1[17] semiempirical wavefunctions.[18] No reliable parameters are available for the heme connected to cysteine, so the geometry of this part was kept constant. Since no AM1 parameters are available for transition metals, the charges for the heme-cysteine unit were calculated with the MNDO[19] Hamiltonian replacing the iron by chromium, the closest element for which MNDO parameters exist. The large size of the molecule excluded the possibility of performing MNDO calculation for the whole molecule. The propionate side chains were excised from the heme and the charges were determined separately for the two molecules obtained from the fragments by saturation with H atoms.

Molecular mechanical minimization of the active site–substrate complex indicated huge strain energy due to the outer shell of the enzyme being kept fixed. If the whole enzyme was involved in the minimization, these strains were relieved by small changes in the position of the outer residues. In these calculations, however, the same active site–enzyme structure was maintained with different positions of the outer residues depending on the initial structure of the minimization. It introduces some ambiguity in the calculated energy that is avoided by separating the energy of the active site–substrate complex from the energy of the outer shell residues.

RESULTS

Mutation modeling of P-450$_{NF}$ binding site

Global alignments of the sequences of P-450$_{NF}$ and P-450$_{CAM}$ produced a maximum alignment score of 5.18 in standard deviation units. This number reflects a statistically significant sequence homology between P-450$_{CAM}$ and P-450$_{NF}$ and justifies the procedure followed in predicting the active site of P-450$_{NF}$.

A summary of mutations based on sequence alignments is shown in Table 1, and the resulting substrate-binding pocket is depicted in Figure 1. The two sequences show the highest similarity in the regions of O_2-binding pocket and heme-binding domain.[3,20] The consensus sequences common in P-450 enzymes, namely G--T in the O_2-binding pocket and F--G---C-G in the heme-binding domain, are indeed observed in P-450$_{NF}$.

The mutations required are ambiguous in some cases; residues 322, 395 and 396 (residue sequence numbers, here and later on, refer to those in P-450$_{CAM}$[3]) are separated from the substrate by the heme, so the nature of these residues does not directly affect the properties of the substrate-binding pocket. Residues 181 and 185 may make contact with larger substrates, so these mutations are relevant in determining the features of the binding pocket (see below).

Table 1. Summary of mutations required to produce the P-450$_{NF}$ active site from the P-450$_{CAM}$ active site

Residue sequence number in P-450$_{CAM}$	P-450$_{CAM}$ residue	P-450$_{NF}$ residue	Residue sequence number in P-450$_{NF}$
86–87	PF	VF	101–102
96	Y	V	111
98–102	FIPTS	FMKSA	113–117
112	R	R	130
119	V	F	137
181	T	F,L[a]	193,**200**[a]
185	T	K,L[a]	197,**204**[a]
241	M	Q	298
243–253	GLLLVGGLDTV	IIFIFAGYETT	300–310
295–297	VAD	VVN	359–361
322	Q	—[b]	—[b]
349–351	TFG	PFG	434–436
355–360	HLCLGQ	RNCIGM	440–445
395–396	IV	—[b]	—[b]

[a]Sequence alignments are unable to distinguish between the two residues. Steric requirements of docked substrates suggest the mutations written in bold (see text)

[b]No obvious choice of mutation based on sequence alignments is possible. The residue is not in contact with the substrate (see text)

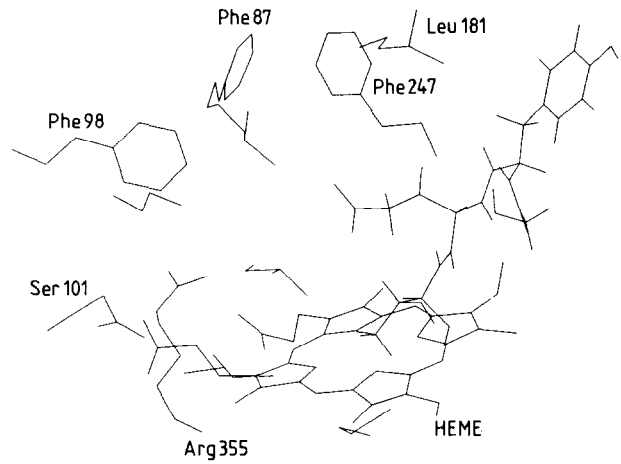

Figure 1. Model of P-450$_{NF}$ active site

In P-450$_{CAM}$ the heme group is kept in place by hydrogen bond donors, namely Arg 112, Arg 299, His 355 and Asp 297.[3] In the mutated structure, His 355 is replaced by Arg and Asp 297 by Asn. The mutated residues can also donate hydrogen bonds, providing a similar environment for the heme as in P-450$_{CAM}$ (Figure 2).

The side chains of Phe 350, Leu 356 and Gln 360 and the polypeptide chain between Phe 350–Gln 360 form a pocket for Cys 357, whose sulphur atom donates an electron pair to the heme iron atom.[3] The mutated residues Phe, Asn and Met have similar lengths and are capable of providing a similar heme-binding pocket.

The mutation of Tyr 96 to Val indicates an important change in substrate specificity. This Tyr residue—forming a hydrogen bond with the carbonyl oxygen atom of camphor—plays an important role in governing the regioselec-

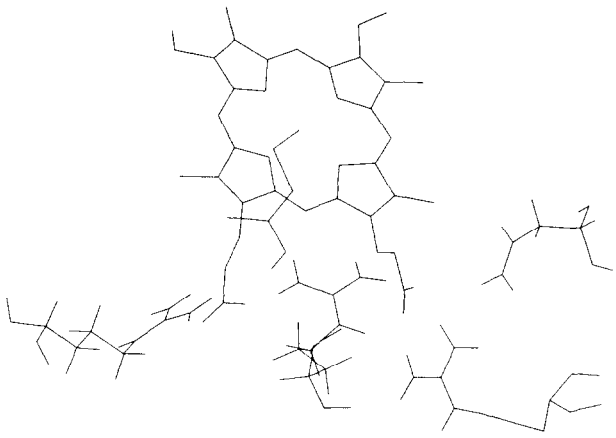

Figure 2. Model of P-450$_{NF}$ heme-binding domain

tivity of camphor oxidation by P-450$_{CAM}$. The replacement of Tyr by Val results in the loss of such hydrogen-bond donation capacity and considerably increases the space available for the substrate.

Summarizing the assessment of mutations, we can conclude that the consensus sequences common in P-450s are conserved, the overall hydrophobicity of the active site is maintained and the space available for the substrate is increased in accordance with the larger size of P-450$_{NF}$ substrates. Further, the mutated residues provide a similar environment for the heme group in P-450$_{NF}$ as in P-450$_{CAM}$ and a similar distortion in the regular alpha-helical hydrogen bonding pattern of the distal helix forming the O_2-binding pocket is essential for all such monooxygenases.

Substrate docking into the putative binding pocket

P-450$_{NF}$ is involved in the metabolism of various molecules showing no apparent structural similarity (Figure 3). A common feature of these molecules, however, is that they contain bulky hydrophobic groups. These facts indicate that polar interactions (e.g., hydrogen bonds) between the enzyme and substrate are not the main interactions involved in substrate binding, because such interactions would impose more rigorous restrictions on the structure of substrates and would result in higher substrate specificity. This assumption is also supported by the observation that some substrates may be attacked at different atoms,[6,7] showing again the absence of forces holding these substrates rigidly in place.

In the course of substrate docking, information from the crystal structure of the P-450$_{CAM}$-camphor complex[3] was utilized. The substrate atom attacked was assumed to have the same position as the C5 atom of camphor in the P-450$_{CAM}$-camphor complex. An additional restriction on the substrate position is that the atom attacked by the activated oxygen has to be accessible to the O_2 sitting in the oxygen-binding pocket formed by residues 248–252. Substrate conformations were sought that fulfill the above conditions and satisfy the steric requirements of the substrate pocket.

The first group of molecules docked into the binding

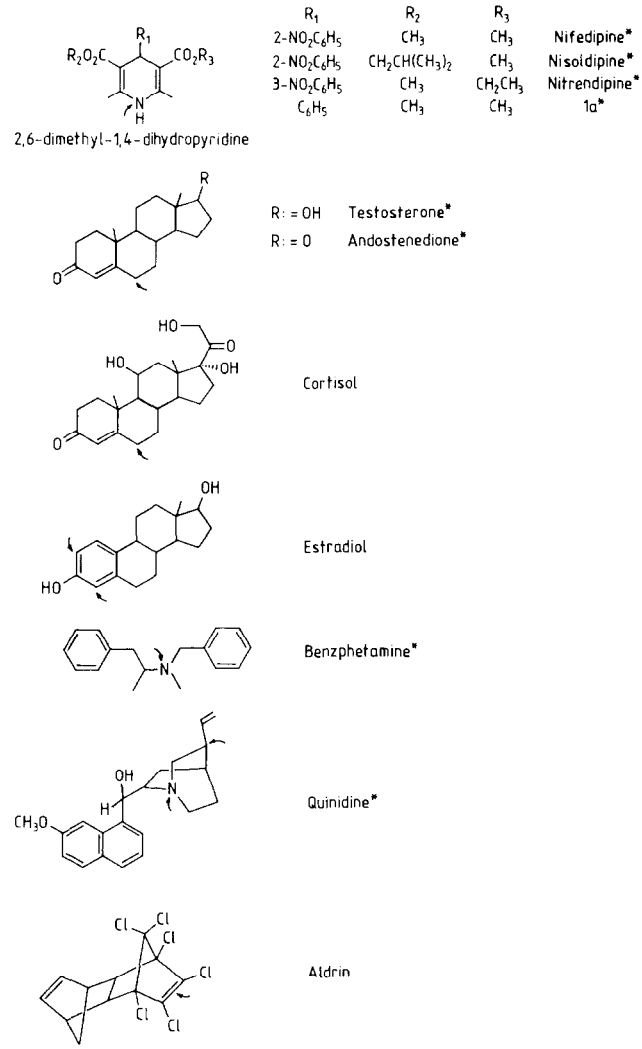

Figure 3. P-450$_{NF}$ substrates. Arrows indicate the primary site of attack by the enzyme[6]

pocket comprises the dihydropyridines (see Figure 3). The metabolism of several dihydropyridines with various side chains has been studied by Bocker and Guengerich.[5] In the case of these molecules, polar interactions may be expected between the ester groups and the enzyme and between the polar substituents of the phenyl group and the enzyme. As discussed previously, the substrate pocket has few polar residues; the two candidates obviously capable of polar interaction are Ser 101 and Arg 355. Tyr 252 is thought to donate a hydrogen bond to the carbonyl oxygen of Gly 248,[3] and even if it contributes to the binding of substrates (the separation of the hydroxyl oxygen of Tyr 252 and the carbonyl oxygen of dihydropyridines is approximately 4 Å), it cannot be responsible for the substrate specificity of P-450$_{NF}$ since this Tyr residue, as part of the oxygen-binding pocket, is highly conserved and is present in all known P-450s. Furthermore, such substrate–Thr 252 interactions would probably disrupt the O_2-binding pocket conformation.

We were able to find a conformation for docked dihydropyridines in which their aromatic groups are in the favorable position of being parallel with Phe 87 and perpendicular

to Phe 98, and their NO_2 group being directed to Ser 101 and Arg 355 (Figure 4). It requires the rotation of the substituted phenyl group from the synperiplanar conformation usually observed in solid state[21] to a position in which its plane is nearly perpendicular to the mirror plane of dihydropyridine ring. The stabilization of this conformation cannot be attributed to the interaction of the $-NO_2$ group with H-bond donors, since 1a also adopts a similar conformation, though it does not contain $-NO_2$ group.

The enzyme substrate complexes obtained by interactive docking were refined by molecular mechanical minimization. The calculations helped to avoid some close contacts resulted by the docking but were unable to give a well-defined position of the $-NO_2$ group with respect to the Ser 101 and Arg 355. The refined structures were dependent on the initial structures predicting several different H-bond patterns with small energy differences. This conclusion is valid for both the $2-NO_2$ and $3-NO_2$ substituted compounds. It is worth noting that no polar group of the enzyme was found in the vicinity of the ester groups of dihydropyridines, showing that these groups are not fundamental in binding. If they are indispensable for the oxidation by $P\text{-}450_{NF}$ then they probably play a role in another stage of the reaction (e.g., they may help to stabilize the intermediate evolving after the first electron abstraction[5]).

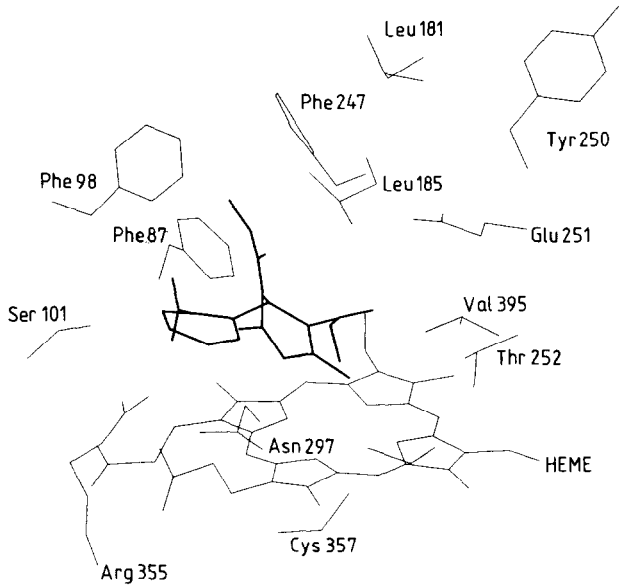

Figure 4. Model fit of nifedipine in $P\text{-}450_{NF}$ active site

Decomposition of the calculated interaction energy yields a ratio of van der Waals to electrostatic interaction energy varying between 2 and 4 (Table 2). The favorable contacts of the dihydropyridine ring with the heme, of the aromatic ring of the substrate with Phe 87 and of the substrate side chains with Phe 98, Phe 247, Val 295 and Val 395 are mainly responsible for the interaction energy.

It should be kept in mind that the calculated binding energy is not equal to the energy change accompanied by the binding of the substrate to the enzyme in aqueous solution. In the case of P-450, where the substrate and the binding pocket are hydrophobic, the main driving force for binding is the increase of entropy due to the disappearance of ordered water–hydrophobic contacts.[22]

The docking of dihydropyridines makes it possible to choose between the residues suggested by sequence alignments for the 181 and 185 positions (Table 1). The fitting of dihydropyridines with long chains in the 3 and 5 positions is possible only if both residues are relatively short, suggesting that both Thr 181 and Thr 185 are mutated to Leu.

The binding of other types of substrates shows similar characteristics. The steroid skeleton of testosterone and androstenedione is in a quasi-parallel position with respect to the heme (Figure 5). The O atom in the 17th position accepts an H-bond from Ser 101. No source of directional forces between the carbonyl oxygen atom of the substrate and the enzyme was found. Although common oxygen atoms in the 3 position hint at the special role of these atoms in the binding, the two possible sites of hydroxylation of estradiol,

Table 2. Substrate–enzyme binding energy calculated by molecular mechanics[a,b]

Compound	Excess energy of substrate[c]	Excess energy of enzyme[c]	Interaction energy			Binding energy
			van der Waals	Electrostatic	Total	
Nifedipine	25.3	7.7	−38.9	−11.3	−50.3	−17.2
S-nisoldipine	34.4	15.0	−35.9	−17.1	−53.0	−3.6
R-nisoldipine	11.5	23.5	−43.0	−8.3	−51.4	−16.2
S-nitrendipine	11.8	14.6	−34.9	−4.5	−39.4	−13.0
R-nitrendipine	4.8	21.2	−33.5	−16.6	−50.1	−25.1
1a	14.6	1.2	−29.6	−3.1	−32.7	−16.9
Testosterone	6.0	12.4	−24.7	−8.0	−32.6	−14.2
Androstenedione	4.5	13.3	−27.7	−5.4	−33.1	−15.3
Quinidine	6.2	11.5	−34.8	−3.5	−38.3	−20.6
S-benzphetamine	1.6	0.0	0.3	−28.0	−27.8	−26.2
R-benzphetamine	6.6	7.0	0.0	−22.8	−22.8	−9.2

[a] See text for the discussion of the meaning of the calculated energies
[b] All energies are in kcal/mol
[c] Calculated as the difference between the energy in the complex and of the separate molecule

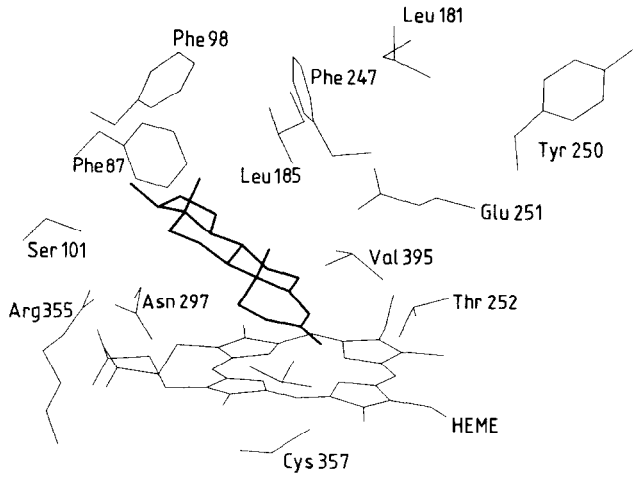

Figure 5. Model fit of testosterone in P-450$_{NF}$ active site

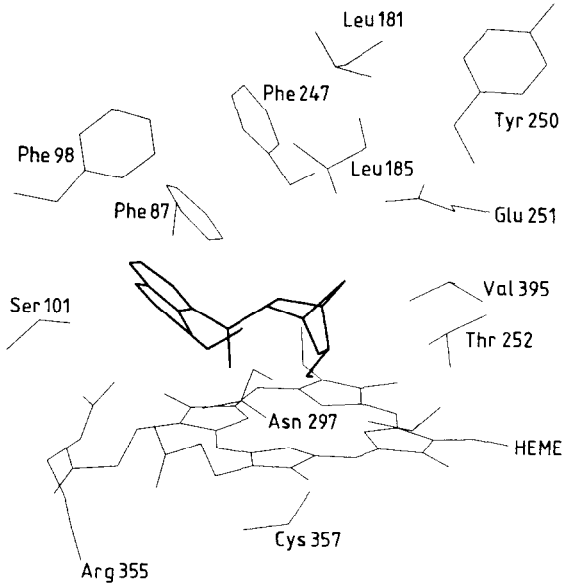

Figure 6. Model fit of quinidine in P-450$_{NF}$ active site

both different from the site of oxidation of other steroids, seem to contradict the model of a rigidly tethered substrate and make the binding with one H-bond acceptable.

P-450$_{NF}$ catalyzes both quinidine 3 hydroxylation and N oxidation. Thus, a position for the docked quinidine was sought in which both possible sites of attack are facing the oxygen-binding pocket. In the resulting complex, the fused rings of the substrates are parallel with Phe 87 with a separation of ~4 Å and their N atoms accept an H-bond from Ser 101 as well as the O atom from Asn 297 (Figure 6).

The metabolism of (+)-benzphetamine by rabbits is 30–35% faster than that of the other enantiomer. Interestingly, the calculated binding energy for the S– and R–benzphetamines bound to P-450$_{NF}$ differs by 17.0 kcal/mol, which may be the origin of a similar difference in the speed of the metabolism of the benzphetamine enantiomers by humans. The S-benzphetamine binds to the model active

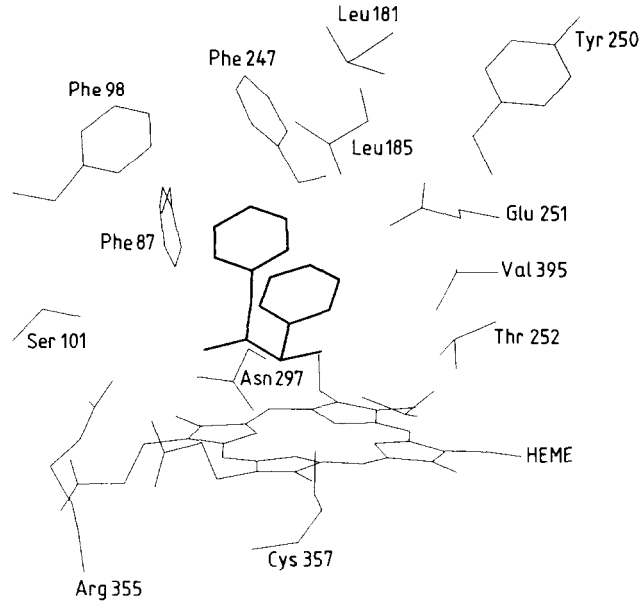

Figure 7. Model fit of S-benzphetamine in P-450$_{NF}$ active site

site (Figure 7) in a slightly distorted conformation, causing no stress in the enzyme and with a considerably higher interaction energy than does the R-benzphetamine (Table 2).

CONCLUSION

A model of the active site of P-450$_{NF}$ has been built based on the mutation of the related P-450$_{CAM}$ tertiary structure. The mutated structure provides a reasonable environment for the heme group and contains a binding pocket acceptable to the features of the substrates. Several substrates of P-450$_{NF}$ were successfully docked into the postulated binding pocket. Molecular mechanical energy minimizations emphasize the importance of van der Waals forces in the enzyme–substrate interaction. Results presented here suggest that a basically correct model of the active site of P-450$_{NF}$ has been built, though further experimental data are required to establish the extent of the validity of the model.

We are happy to provide coordinates of our model for others to refine or subject to experimental test.

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