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A preliminary 3D model for cytochrome P450 2D6 constructed by homology model building

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SUMMARY

A homology model building study of cytochrome P450 2D6 has been carried out based on the crystal structure of cytochrome P450 101. The primary sequences of P450 101 and P450 2D6 were aligned by making use of an automated alignment procedure. This alignment was adjusted manually by matching α-helices (C, D, G, I, J, K and L) and β-sheets (β3/β4) of P450 101 that are proposed to be conserved in membrane-bound P450s (Ouzounis and Melvin [Eur. J. Biochem., 198 (1991) 307]) to the corresponding regions in the primary amino acid sequence of P450 2D6. Furthermore, α-helices B, B′ and F were found to be conserved in P450 2D6. No significant homology between the remaining regions of P450 101 and P450 2D6 could be found and these regions were therefore deleted. A 3D model of P450 2D6 was constructed by copying the coordinates of the residues from the crystal structure of P450 101 to the corresponding residues in P450 2D6. The regions without a significant homology with P450 101 were not incorporated into the model. After energy-minimization of the resulting 3D model of P450 2D6, possible active site residues were identified by fitting the substrates debrisoquine and dextrometorphan into the proposed active site. Both substrates could be positioned into a planar pocket near the heme region formed by residues Val^{370}, Pro^{371}, Leu^{372}, Trp^{316}, and part of the oxygen binding site of P450 2D6. Furthermore, the carboxylate group of either Asp^{100} or Asp^{301} was identified as a possible candidate for the proposed interaction with basic nitrogen atom(s) of the substrates. These findings are in accordance with a recently published predictive model for substrates of P450 2D6 [Koymans et al., Chem. Res. Toxicol., 5 (1992) 211].

INTRODUCTION

The metabolism of debrisoquine, sparteine and more than 25 other drugs is impaired in 1–30% of various ethnic populations [1]. Administration of these drugs may lead to severe side effects [2] and is associated with an altered susceptibility of humans to lung and bladder cancer [3, 4]. The

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cytochrome P450 2D6 enzyme system has been shown to be responsible for this human defect in drug oxidation known as the 'debrisoquine/spartein' polymorphism [5, 6].

Screening of drugs and other chemicals for evidence of marked polymorphism of the 'debrisoquine/spartein' type is generally considered to be of great importance. Knowledge of common structural features of known substrates of P450 2D6 is very useful in identifying new and existing drugs that might be susceptible to P450 2D6 polymorphism. Several years ago, two models were proposed [7, 8] which describe some common characteristics of substrates of P450 2D6, based on space-filling substrate models. However, the two models had contradictive elements: in the model of Wolff et al. [7] the distance between basic nitrogen atoms and the oxidation site of P450 2D6 substrates was suggested to be 5Å (the so-called 5Å-substrates), whereas Meyer et al. [8] proposed this distance to be 7Å (7Å-substrates). Recently, we have published a new model for substrates of P450 2D6, which accommodates both 5Å- and 7Å-substrates and which describes the main characteristics of P450 2D6 substrates in terms of steric and electronic criteria [9]. The predictive value of our model was evaluated by predicting the involvement of P450 2D6 in 14 P450-mediated metabolic reactions occurring in four compounds not used to derive the model. The computer-assisted predictions appeared to correlate well with the experimental results.

The model could be validated further by building a 3D structure of P450 2D6 on the basis of the crystal structure of P450 101. Several attempts have been made to build 3D structures of membrane-bound P450s by homology model building based on the crystal structure of P450 101 [10–13]. The overall homology between membrane-bound P450s and P450 101, however, is low (± 15%). Therefore, a realistic 3D model for membrane-bound P450s that is based on the crystal structure of P450 101 cannot be expected [14, 15]. Furthermore, insertion of more than four amino acid residues may cause severe problems as these loop conformations are determined not only by the local sequence but also by the protein and aqueous environment and may be highly flexible [16, 17]. Hence, homology model building studies of membrane-bound P450s based on the crystal structure of P450 101 only have value when restricted to the homologous regions of P450s (the region around the fifth, axial, heme iron ligand and the oxygen binding site) and to those regions where secondary structure elements of P450 101 are clearly conserved [18–20]. In most of the multiple alignments published so far [18, 21–24], the reliability of the alignment in the other regions is questionable. Furthermore, a realistic alignment between membrane-bound P450s and P450 101 should take only the cytosolic part of the membrane-bound P450s into consideration. In addition, the different P450 families are unequally represented in the multiple alignment studies. An approach which would be expected to yield more reliable results uses a consensus sequence for each P450 family in the alignment. In this regard, the recently published multiple alignment by Ouzounis and Melvin [20] seems very promising.

In the present study, the multiple alignment described by Ouzounis and Melvin [20] was used as a guide to build a 3D model of P450 2D6 on the basis of the crystal structure of P450 101. The homology model was constructed using only those regions of P450 101 that are clearly conserved in P450 2D6.

COMPUTATIONAL METHODOLOGY

The coordinates of the crystal structure of P450 101 were taken from the Brookhaven Protein Databank [25]. An initial alignment between the primary amino acid sequences of P450 101 and

P450 2D6 was established by use of an automated sequence alignment procedure (I-align) [26], which forms part of the FASTA suite of sequence analysis programs [27]. This alignment was adjusted manually to match the multiple alignment of Ouzounis and Melvin [20]. Homology building and energy minimization were carried out using Quanta/CHARMm version 3.2 (Polygen Corp., MA, Waltham, June 1991). The simulations were performed on a Silicon Graphics 4D/25GT.

Several stages were distinguished during the course of the homology building study:

Sequence alignment

The automated sequence alignment between P450 101 and P450 2D6 revealed that the region around the fifth, axial, cysteinyl ligand of P450 101 (helix L) and the oxygen binding site (helix I of P450 101), which are highly conserved in cytochromes P450, are also conserved in P450 2D6. Other secondary structure elements of P450 101 that are proposed to be conserved in membrane-bound P450s [20], i.e. helices C, D, G, J and K and β-structures β3/β4 (residues 293–323), were aligned with the corresponding regions in P450 2D6 by manually adjusting the automated alignment when necessary. These regions in the primary amino acid sequence of P450 2D6 were identified by using the consensus sequence of P450 family 2 in the multiple alignment of Ouzounis and Melvin [20].

On the basis of the sequence homology between P450 101 and P450 2D6, three additional secondary structure elements were found to be conserved in P450 2D6, i.e. helices B, B′ and F. In contrast, helices A, E, H, and the β-sheets β1, β2, and β5 of P450 101 were not conserved in P450 2D6 and were deleted from the alignment, as were all insertions and deletions relative to P450 101. As a consequence, the alignment between P450 2D6 and P450 101 only contained helices B, B′, C, D, F, G, I, J, K, L and β-sheets β3/β4 (residues 293–323) of P450 101. This alignment (Fig. 1) was used for the homology model building of P450 2D6 on the basis of the crystal structure of P450 101.

Homology model building

The conserved regions of P450 101 and P450 2D6 were aligned and the coordinates of the 'overlapping' residues were copied from the 'known' P450 101 structure onto the 'unknown' P450 2D6 structure, using the 'Protein Design' module of Quanta/CHARMm version 3.2. The side chains of residues that had to be mutated for P450 2D6 were built as far as possible on basis of the coordinates of the corresponding residues in the crystal structure of P450 101. Subsequent atom positions in the side chain were prescribed by templates for the new residues. Then, a search was made for close contacts (bumps) between non-hydrogen atoms with a cutoff distance of 1.0 Å. The bumps were removed by use of the 'Spin' option of Quanta/CHARMm version 3.2, which rapidly searches for an orientation of the side chain in which the number of bad contacts is minimized. All other atoms were fixed. Rotations were performed around the C^{α}-C^{β} axis of the specified residue with steps of 60° and a cutoff distance of 3.0 Å. Finally, a single-point CHARMm energy calculation was performed to add hydrogen atoms, using the polar hydrogen atom description as implemented in the AMINO.RTF file.

Energy minimization

The 3D structure of P450 2D6 thus obtained was further optimized by gradual energy-minimi-

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P450  2D6      83 G L A A V R E A L V T // 114 P R S Q G V F L //
P450  101      67 R G Q L I R E A Y E D // 89  P R E A G E A Y //

P450  2D6      126 P A W R E Q R R F S V S T L R N L G L G // 149 L E
P450  101      106 P E Q R Q F R A L A N Q V V G M P V V D // 127 L E

P450  2D6      Q W V T E E A A C L C A A F // 198 D D P R F L R L L D
P450  101      N R I Q E L A C S L I E S L // 173 D I P H L K Y L T D

P450  2D6      L A Q // 240 V L R F Q K A F L T Q L D E L L T E H R M
P450  101      Q M T // 192 T F A E A K E A L Y D Y L I P I I E Q R R

P450  2D6      T W // 291 N D E N L R I V V A D L F S A G M V T T S T
P450  101      Q K // 234 T S D E A K R M C G L L L V G G L D T V V N

P450  2D6      T L A W G L L L M I // 323 L H P D V Q R R V Q Q //
P450  101      F L S F S M E F L A // 266 K S P E H R Q E L I E //

P450  2D6      355 Y T T A V I H E V Q R F G // 370 V P L G M T H M T
P450  101      280 R I P A A C E E L L R R F // 293 S L V A D G R I L

P450  2D6      S R D I E V Q G F R I P K G T T L I T N L S // 436 F S
P450  101      T S D Y E F H G V Q L K K G D Q I L L P Q M // 350 F G

P450  2D6      A G R R A C L G E P L A R M E L F L F F T S L L Q H F
P450  101      H G S H L C L G Q H L A R R E I I V T L K E W L T R I
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Fig. 1. Sequence alignment of conserved regions between P450 2D6 and P450 101. These regions include helix B (67–77), B′ (89–96), C (106–125), D (127–142), F (173–185), G (192–214), I (234–265), J (266–276), K (280–292), L (350–378) and sheets β3/β4 (293–323).

zation of all side chains in three stages. The constraints in the three stages were: (1) all non-hydrogen atoms fixed; (2) all backbone atoms and the porphyrin ring fixed; positional harmonic constraints of 10 kcal/mol·Å applied to all other non-hydrogen atoms; (3) all backbone atoms and the porphyrin ring fixed; all other atoms free. At every stage the minimization algorithm consisted of the steepest descent method (max. 100 steps), which rapidly improves a poor conformation, followed by conjugate gradient minimization (max. 100 steps), which has better convergence characteristics than the steepest descent technique. At stage 3 the conjugate gradient algorithm was continued until the root mean square (rms) energy gradient was less than 0.25 kcal/mol·Å. During the calculations a 4*R distance-dependent dielectric constant in combination with a 12 Å non-bonded cutoff was maintained. The non-bonded list was updated every 50 steps. For the other CHARMm parameters default values were taken as implemented in Quanta/CHARMm version 3.2. No water molecules and non-protonated amino acids were incorporated in the calculations. The polar hydrogen description of the amino acids of P450 2D6, as implemented in the AMINO.RTF file, was used (hydrogens bound to carbon are not represented explicitly). The energy-minimized 3D model of P450 2D6 had a CHARMm potential energy of −661 kcal/mol.

The substrates dextrometorphan and debrisoquine were docked manually in the energy-minimized 3D model of P450 2D6. The oxidation sites of both substrates were fixed at a similar position as the oxidation site of camphor in P450 101, i.e. about 4.2 Å from the heme iron atom. Subsequently, the results from our proposed small molecule substrate model of P450 2D6 [9] were used as a guide to manually fit the substrates into the 3D model of P450 2D6.

RESULTS AND DISCUSSION

Much of the insight gained into the mechanism of oxidation and molecular structure of membrane-bound cytochromes P450 has been based on the crystal structure of P450 101 [28], a cytosolic P450 from the bacterium *Pseudomonas putida*. As crystal structures of membrane-bound P450s are not available yet, indirect methods have to be applied to gain insight into the architecture of the active site of an individual membrane-bound P450 isozyme. Several attempts have been made to build a 3D structure of membrane-bound P450s by homology model building on the basis of the crystal structure of P450 101 [10–13].

In two of these studies, concerning P450 17 and P450 1A1 [11, 12], a complete 3D structure of a membrane-bound P450 was determined, including all insertions and deletions. However, because of the low sequence identity between membrane-bound P450s and P450 101 (± 15%), the construction of complete 3D models of membrane-bound P450s from the crystal structure of P450 101 is probably unrealistic. The 3D model of P450 3A4, the nifedipine-oxidizing cytochrome P450 constructed by Ferenczy and Morris [10], only constitutes the active site region of the protein. Furthermore, this model is highly speculative because not all regions of P450 101 that contain active site residues are clearly conserved in membrane-bound P450s. Finally, in the course of these homology model building studies, Graham-Lorence et al. [13] applied an elegant combination of homology building techniques and site-directed mutagenesis results to identify the possible active site residues of P450 19A1, which catalyzes the conversion of androgens to estrogens. These authors constructed the highly conserved heme binding site (helix L) and oxygen binding site (helix I) of P450 19A1 on the basis of the crystal structure of P450 101. Subsequently, the substrate androstenedione was docked into the model by superimposing its oxidation site onto that of camphor. Residues that were found to interact with the substrate were changed by site-directed mutagenesis. In this way, residues Ala^{306}-Ala^{307}-Pro^{308} and Glu^{302} were identified as possible active site residues of P450 19A1.

In the current study, a homology model of P450 2D6 was constructed which was primarily based on conserved structural regions between membrane-bounds P450s and the cytosolic P450 101. All other residues of P450 2D6 were deleted from the 3D model.

Alignment between P450 101 and P450 2D6

Only helices B, B′, C, D, F, G, I, J, K, L, and β-structures β3/β4 of P450 101 aligned in P450 101 and P450 2D6. All other residues of P450 2D6 were deleted from the model. The resulting alignment is shown in Fig. 1.

The active-site region

In a previous molecular modeling study of 16 substrates of P450 2D6, constituting 23 metabolic reactions, several common characteristics of these substrates were described [9]. The main features included: (1) All substrates of P450 2D6 contain at least one basic nitrogen atom, which is proposed to interact with a carboxylate group on the protein. This nitrogen atom interacts with either one of the two oxygen atoms of the carboxylate moiety, thus explaining the binding of substrates with a distance of either 5 Å or 7 Å between the oxidation site and the basic nitrogen atom; (2) The region near the oxidation site is sterically limited and is suggested to be planar; (3)

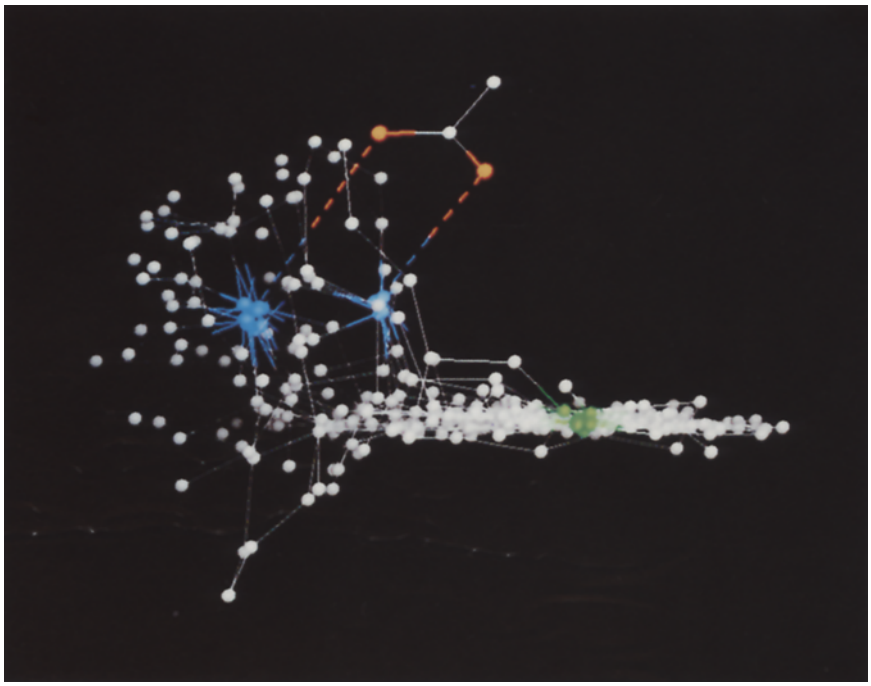

Fig. 2. Active site model of P450 2D6 when all 16 substrates (23 metabolic reactions) investigated in the small molecule modeling study are fitted onto the template molecules debrisoquine and dextrometorphan. The oxidation sites (green) of all substrates are superimposed. The basic nitrogen atoms are either fitted onto the basic nitrogen atom of debrisoquine (blue, back) or onto that of dextrometorphan (blue, front) and interact with one of the two carboxylic oxygen atoms (orange) of a protein carboxylate group. The planar domain near the oxidation site has dimensions of approximately $6.5 \times 7.5 \times 2.5$ Å^3.

It is suggested that there is an electrostatic interaction between this planar part of the substrates and a complementary electrostatic field of P450 2D6. These findings are summarized in Fig. 2.

This substrate model of P450 2D6 was used as a guide to manually dock the substrates dextrometorphan and debrisoquine into the 3D model of P450 2D6 (Fig. 3). A planar pocket (dimensions approximately $9 \times 10 \times 5$ Å^3) was found near the presumed oxidation site of substrates of P450 2D6 in the energy-minimized 3D model. The planar pocket is formed by the oxygen binding site (residues Thr^{309} to Thr^{312}), residues Val^{370}, Pro^{371}, Leu^{372} and Trp^{316} of P450 2D6 (Fig. 3). These latter five residues correspond to Ser^{293}, Leu^{294}, Val^{295} (β-structure β3) and Phe^{259} (helix I) in P450 101 and also form a planar pocket in this cytosolic protein. However, in P450 101 the entrance to the planar pocket is partially blocked by the presence of the side chain of Leu^{294}, which corresponds to Pro^{371} in P450 2D6, lacking a side chain. The planar pocket in the 3D model of P450 2D6 might be the region which is complementary to the previously proposed planar domain in the 3D substrate model (see above, [9]). Furthermore, an electrostatic interaction between the substrate and the protein was proposed to occur in the small molecule model. The side chain of a Gln residue at position 211 in the primary sequence of P450 2D6 might be involved in this interaction (Fig. 3).

Modeling of the active site

The substrates dextrometorphan (7 Å-substrate) and debrisoquine (5 Å-substrate) were docked manually in the 3D model of P450 2D6. Both substrates could be fitted in several ways into the planar pocket with the oxidation sites at a distance of 4.2 Å from the heme iron atom, as judged from by the distance between the camphor C5 atom and the iron atom in the crystal structure of P450 101. No obvious steric hindrance with the remainder of the protein was indicated in these fits. Subsequently, the active-site region of the 3D model of P450 2D6 was screened for the presence of a carboxylate moiety which could interact with the basic nitrogen of the substrates [9]. The carboxylate group of Asp^{301} of P450 2D6 was found to be a possible candidate for this interaction (Fig. 3). This aspartic acid residue forms part of the strongly conserved I-helix and corresponds to alignment position 370 in the multiple alignment of Ouzounis and Melvin [20]. Interestingly, the results of recent computer modeling and site-directed mutagenesis studies predict a Glu residue in P450 19A1 at this alignment position [13]. Furthermore, this alignment position matches the single amino acid substitution (Gly-Asp) via which cytochrome P450 51 is changed into the inactive cytochrome P450 SG1, which is devoid of lanosterol 14-demethylation activity [29].

It should be kept in mind that carboxylate moieties from residues that were deleted from the model could also interact with the basic nitrogen atoms of the substrates of P450 2D6. In this respect, Asp^{100} of P450 2D6 is interesting. This acidic residue forms part of a region between helices B and B′ of P450 2D6. In the camphor-bound P450 101 complex [30], the corresponding region is involved in substrate binding. Furthermore, in the multiple alignment of Ouzounis and Melvin [20], only members of the 2D subfamily and a member of family 4 have an acidic residue at the same position as Asp^{100} in P450 2D6 (alignment position 118). Therefore, the presence of this residue might explain the selectivity of P450 2D6 in metabolizing only substrates with a basic nitrogen atom.

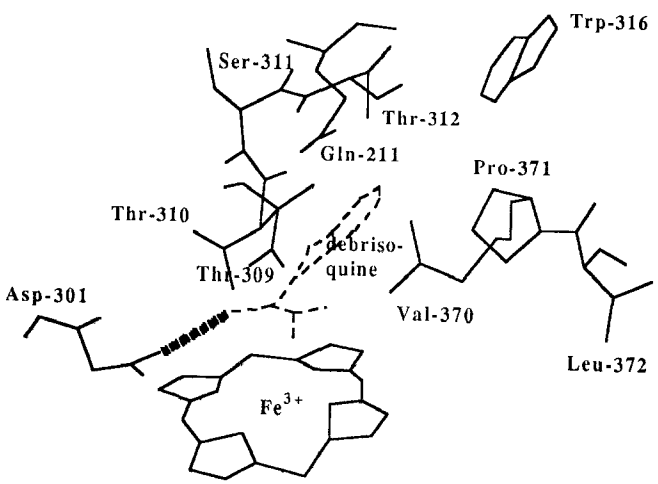

Fig. 3. Topology of the active site of the 3D model of P450 2D6. The substrate debrisoquine is docked into the active site with the aromatic moiety fitted into the planar pocket of the enzyme formed by part of the oxygen binding site (Thr^{309} to Thr^{312}) and residues Val^{370}, Pro^{371}, Leu^{372} and Trp^{316}. A possible interaction between the carboxylate group of Asp^{301} and the basic nitrogen atom of debrisoquine is indicated.

Limitations of the current approach

The assignment of active site residues in the 3D model of P450 2D6 depends upon the validity of the alignment between P450 2D6 and P450 101. Clearly, the assumptions upon which the alignment is based may be only partly valid. Furthermore, the relative positions of α-helices and other core backbone features in the 3D model may be somewhat displaced from their true values. For example, Lesk and Chotia [31] have shown for the globulin family that rigid body shifts occur up to 7 Å and 30° between homologous α-helices. Moreover, some of the regions that are deleted from the model may be expected to influence the final binding of substrates in the proposed active site. No attempts were made to energy-minimize a possible fit of the substrates dextrometorphan and debrisoquine in the active site region of P450 2D6. Nonetheless, the current approach is useful in identifying residues in the P450 2D6 sequence that might be involved in substrate-binding of P450 2D6.

In conclusion, the 3D model of P450 2D6 presented seems complementary to the common structural features of our substrate model of P450 2D6 [9]. Possible active site residues that interact with the substrates dextrometorphan and debrisoquine were identified. Notwithstanding some limitations of the methods used, the homology model of P450 2D6 presented here provides a useful approach for further experiments to validate the assumptions upon which the model is based. In this context, site-directed mutagenesis of Asp^{301} or Asp^{100} of P450 2D6 to either an aliphatic, aromatic, or basic residue should abolish its activity, whereas mutation of these aspartic acids to glutamic acid should result in retained activity towards several substrates. The planar pocket to which substrates are supposed to bind could be investigated by site-directed mutagenesis or by the synthesis of new substrates.

Note added in proof

After submission of this manuscript, the literature was reviewed by Lewis and Moereels [32]. The alignment they presented largely resembled the multiple alignment of Ouzounis and Melvin [20]. Importantly, the position of the proposed active site residues was similar with the exception of the Gln residue at position 211 in the primary sequence of P450 2D6. The side chain of this residue was proposed to be involved in an electrostatic interaction between P450 2D6 substrates and the protein.

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