A Refined Substrate Model for Human Cytochrome P450 2D6

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Received July 30, 1996[⊗]

Cytochromes P450 (P450s) constitute a large superfamily of heme-containing enzymes, capable of oxidizing and reducing a variety of substrates. Cytochrome P450 2D6 is a polymorphic member of the P450 superfamily and is absent in 5−9% of the Caucasian population as a result of a recessive inheritance of gene mutations. Recently, the importance of aspartic acid 301 (Asp^{301}) for the catalytic activity of P450 2D6, as indicated by a preliminary homology model, was confirmed by site-directed mutagenesis experiments. In this study, the heme moiety and the I-helix containing Asp^{301} were incorporated into the previously derived substrate model for P450 2D6, in the spatial orientations as derived from a recently improved protein model for P450 2D6, thereby incorporating steric restrictions and orientational preferences into the substrate model. The direction of well-defined hydrogen bonds formed between Asp^{301} and basic nitrogen atoms of P450 2D6 substrates was incorporated into the substrate model as well. Also, the position(s) of the basic nitrogen atom(s) of the substrates was/were allowed more flexibility. This was established through the attachment of an aspartic acid residue (representing Asp^{301}) to the (protonated) basic nitrogen atom(s) of the substrates and superimposing the C_α- and C_β-atoms of this aspartic acid residue in the fitting procedure instead of the basic nitrogen atoms. A variety of 8 substrates of P450 2D6 (comprising 17 known P450 2D6 dependent metabolic pathways) has been incorporated successfully into this refined and more restrictive substrate model.

Introduction

Cytochromes P450 (P450s)[1] constitute a large superfamily of heme-containing isoenzymes, capable of oxidizing and reducing a variety of substrates, of both endogenous and exogenous origin (*1*−*5*). Characteristics which allow a vast number of compounds to be metabolized by a limited number of isoenzymes include a broad substrate specificity and a broad regio- and stereoselectivity (*6*). Cytochromes P450 generally detoxify potentially dangerous compounds, but protoxins may also be activated into their ultimate toxins by P450s (*7*−*9*).

Cytochrome P450 2D6 (P450 2D6 (*10*)) is a polymorphic member of the P450 superfamily and is absent in 5−9% of the Caucasian population as a result of a recessive inheritance of gene mutations. Several inactivating alleles have been reported (*11*). These alleles result in a deficiency in drug oxidation known as the debrisoquine/sparteine polymorphism, which affects the metabolism of numerous drugs. Poor metabolizers, which possess two nonfunctional P450 2D6 alleles, show a diminished metabolism of these drugs compared to extensive metabolizers which have at least one functional allele. Various antiarrhythmics, β-adrenoceptor antagonists, antidepressants, opiates, and neuroleptics are known to be substrates for human P450 2D6 (*1*−*3*). These compounds represent a variety of chemical structures, common characteristics being the presence of at least one basic nitrogen atom, a distance of 5 Å or 7 Å between the basic nitrogen atom and the site of oxidation, a flat hydrophobic area near the site of oxidation, and a negative molecular electrostatic potential (MEP) above the planar part of the molecule (*12*, *13*).

Theoretical models predicting the possible involvement of P450 2D6 in the metabolism of drugs or drug candidates are important in drug discovery and drug development. Several small molecule models have meanwhile been published using the chemical structure of a variety of substrates or inhibitors (*12*−*16*). The first models were based on substrates containing a basic nitrogen atom at a distance of either 5 Å (*14*) or 7 Å (*15*) from the site of oxidation and aromatic rings which were positioned coplanar in the models (*14*, *15*). An extended model, using debrisoquine as template, was derived by Islam *et al.*, who indicated that a range in between 5 Å and 7 Å is allowed for the distance between the basic nitrogen atom and the site of oxidation (*16*). A more complete small molecule model for P450 2D6 substrates was derived by Koymans *et al.*, who indicated the presence of negative molecular potentials in the vicinity of the site

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⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1997.
[1] Abbreviations: ADF, Amsterdam density functional; Asp^{301}, aspartic acid 301; CSD, Cambridge Structural Database; DMA, distributed multipole analysis; DZ, double zeta; $\Delta C_\alpha/\Delta C_\beta$, distance between C_α/C_β atoms of the aspartic acid residue attached to the substrate and corresponding atoms in Asp^{301} after flexible fitting procedure; $\Delta r_{(ox-ox)}$, distance between site of oxidation above heme moiety (ring B in Figure 1, ring between pyrrole rings A and B) and site of oxidation in the substrate after rigid fitting procedure; ΔE or $\Delta E_{(opt-fit)}$, density functional energy difference between minimal energy conformation and fitted conformation; GAMESS-UK, Generalised Atomic and Molecular Electronic Structure System, U.K. version; GBR 12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine; LDA, local density approximation; MEP, molecular electrostatic potential; P450, cytochrome P450; P450 2D6, cytochrome P450 2D6; RHF, restricted Hartree Fock; SV, split valence; SZ, single zeta.

S0893-228x(96)00129-4 CCC: $14.00

of oxidation in the substrates and suggested a hypothetical carboxylate group within the protein to be responsible for the well-defined distance of either 5 Å or 7 Å between the basic nitrogen atom and the site of oxidation (*12*). The latter model used debrisoquine and dextromethorphan as templates for the 5 Å and 7 Å substrates, respectively. The sites of oxidation of the two templates and 14 other substrates were superimposed, and the area next to the site of oxidation was fitted coplanar. The basic nitrogen atoms of the substrates were matched either with the basic nitrogen atom of debrisoquine or with the basic nitrogen atom of dextromethorphan. In the final model the two basic nitrogen atoms were approximately 2.5 Å apart. In total, 16 substrates, comprising 23 P450 2D6 dependent reactions, were used to derive this substrate model (*12*). The predictive value of the substrate model was established with almost perfect predictions on 4 compounds comprising 16 possible metabolic products formed by P450 2D6 (*12*). The predictive value was further verified as the predicted metabolites of GBR 12909 (1-[2-[bis(4-fluorophenyl)-methoxy]ethyl]-4-(3-phenylpropyl)piperazine) were confirmed experimentally in metabolism studies using heterologously expressed P450 2D6 (*17*). Recently, a new series of substrates and inhibitors was added to this model.[2] Parallel to the substrate models for P450 2D6, an inhibitor model has been derived as well. The template for this inhibitor model was derived by fitting 6 relatively flexible reversible inhibitors of P450 2D6 onto each other (*13*). The basic nitrogen atoms were matched while the aromatic planes were kept coplanar.

In order to determine the nature and the properties of possible interaction sites in the protein for substrates and inhibitors, three-dimensional representations of the entire active site of P450 2D6 have been constructed using homology modeling techniques (*18*−*21*). The most recent homology models for P450 2D6 are based on the crystal structures of three bacterial P450s, P450 101, P450 102, and P450 108 (*20*, *21*). One specific amino acid, Asp^{301}, was proposed to be a crucial amino acid in the active site of P450 2D6, probably by forming an ionic hydrogen bond with basic nitrogen atoms of substrates (or inhibitors) (*16*, *18*, *20*, *21*). This proposal has been confirmed by recent site-directed mutagenesis experiments (*22*).

For the heme-bound oxygen atom which is incorporated into substrates during metabolism by P450s, two possible positions have been proposed: one perpendicular to the plane of the heme moiety and directly above the central iron atom (*23*) and a second one with the oxygen atom bridged in between the iron atom and one of the four heme nitrogen atoms (*24*, *25*). Both proposals for the position of the heme-bound oxygen atom can in principle be accommodated in the above-mentioned protein model for P450 2D6 (*21*). The protein model suggests the presence of 2 different sites, possibly explaining the occurrence of both 5 Å and 7 Å substrates, i.e., one above ring A (Figure 1, ring between pyrrole rings A and D) and another one above ring B (Figure 1, ring between pyrrole rings A and B) of the heme moiety of P450 2D6 (*21*), respectively. The presence of two distinct sites of oxidation within the P450 2D6 protein, however, would require an adjustment of the substrate model for P450 2D6 (*12*, *17*).[2]

[2] de Groot, M. J., Bijloo, G. J., van Acker, F. A. A., Fonseca Guerra, C., Snijders, J. G., and Vermeulen, N. P. E. (1996) Extension of a predictive substrate model for human cytochrome P450 2D6. *Xenobiotica* (submitted) and unpublished results.

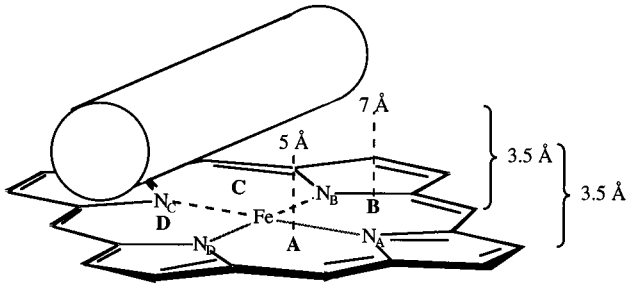

Figure 1. Dummy-atom positions above ring A (ring between pyrrole rings A and D) and ring B (ring between pyrrole rings A and B) of the heme moiety initially used to fit the oxidation sites of 5 Å and 7 Å substrates (labeled "5 Å" and "7 Å", respectively). Within the P450 2D6 protein model (*21*) only the positions above rings A and B of the heme moiety are accessible to the substrates due to steric hindrance by the I-helix (schematically indicated by a cylinder), which is positioned over rings C and D.

The aim of the present study was to investigate the possibility of different sites of oxidation for the 5 Å and 7 Å substrates of P450 2D6 by reinvestigating the previously derived substrate model (*12*, *17*)[2] and to use the three-dimensional information obtained for the active site from the recently described protein model of P450 2D6 (*21*) to refine the substrate model. The heme moiety and the I-helix were incorporated into the substrate model, thus adding steric restrictions and orientational preferences. The directions of well-defined hydrogen bonds between Asp^{301} (part of the I-helix) and basic nitrogen atoms of P450 2D6 substrates were also added to the resulting refined substrate model.

Computational Methods

Compounds and Conformational Analysis. The compounds used in the present small molecule modeling study are shown in Figure 2. The initial conformations of these compounds were either generated using the molecular modeling package ChemX (*26*), retrieved from the Cambridge Structural Database (CSD (*27*)), or derived from the conformations of the substrates used in the substrate model derived by Koymans *et al.* (*12*) or de Groot *et al.* (*17*).[2] Macromodel (*28*) was used for conformational analysis studies on all substrates. If necessary, the ring closure option was used, and a large number of conformations was generated by changing all rotatable bonds with increments of 30°. These conformations were energy minimized with the Amber forcefield (*29*, *30*), using Batchmin (*31*).

Active Site Region of P450 2D6. The coordinates of the heme moiety and the I-helix of P450 2D6 (containing Asp^{301}) were taken from the recently published protein model for P450 2D6 (*21*). At 3.5 Å above the plane of the heme, centered above rings A and B of the heme moiety (Figure 1), dummy atoms were defined, which were used as hypothetical sites of oxidation (as suggested by the protein model (*21*)) for the 5 Å and 7 Å substrates, respectively. The distance between these hypothetical sites of oxidation and the heme moiety is in close agreement with the observed distance between the oxidation site in camphor (above ring A) and the heme moiety in the crystal structure of P450 101 (*32*).

Fitting Procedures. The molecular modeling program ChemX (*26*) was used for rigid and flexible fitting procedures. In the rigid fitting procedure, compounds are fitted only allowing global rotations and translations. The flexible fitting minimizer uses the ChemX nonbonded energy forcefield (*26*) and minimizes the internal nonbonded energy of the fitted molecule with respect to user-defined exocyclic dihedral angles and enforced penalty functions (restraints).

Since in the previously derived protein model for P450 2D6 (*21*) Asp^{301} is hydrogen bonded to the basic nitrogen atom(s) of

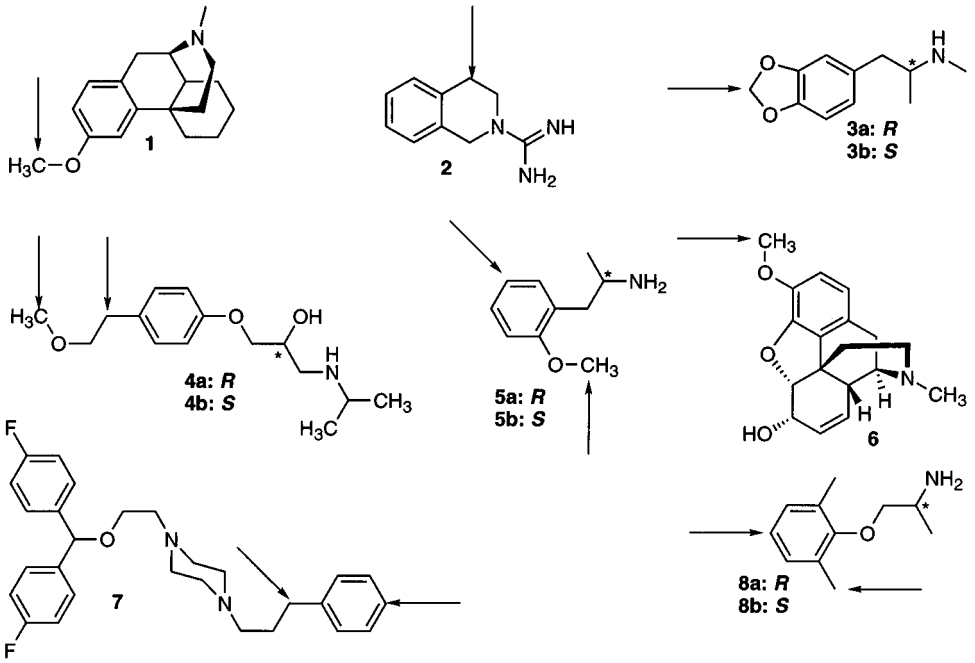

Figure 2. Substrates used in this study: dextromethorphan (**1**), debrisoquine (**2**), MDMA (**3**), metoprolol (**4**), methoxyphenamine (**5**), codeine (**6**), GBR 12909 (**7**), and mexiletine (**8**). Major sites of oxidation by P450 2D6 are indicated with arrows. The benzylic hydroxylation of **8** has not been investigated in this study.

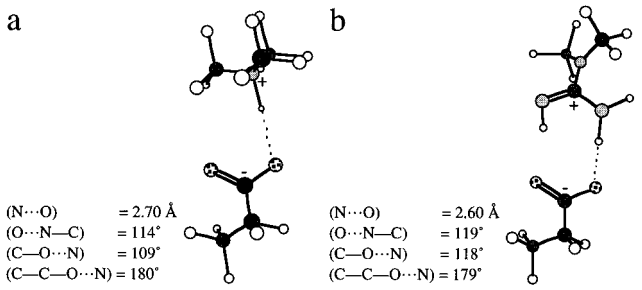

Figure 3. Representation of the applied geometric constraints for ionic hydrogen bonding interactions between part of an aspartic acid residue and (a, left) a protonated tertiary amino group or (b, right) a protonated guanidino group. Adapted from ter Laak *et al.* (*33*). Carbon atoms are colored black, hydrogen atoms white, nitrogen atoms light gray, and oxygen atoms dotted.

the substrates, an aspartic acid residue was attached to the (protonated) basic nitrogen atoms present in the P450 2D6 substrates in the small molecule model, using a method recently developed by ter Laak *et al.* (*33*), employing conventional ChemX routines (*26*). Substrates containing a single basic nitrogen atom were coupled to the carboxylate group, as indicated in Figure 3a. In this case, the orientation of the attached aspartic acid residue relative to the substrate was based on geometric constraints derived from a statistical analysis (GSTAT88) on 23 structures obtained from the CSD (*33*), describing the interaction between a tertiary amino group and a carboxylate moiety. Compounds containing two basic nitrogen atoms (e.g., guanidines) were coupled to the aspartic acid residue, as indicated in Figure 3b. In this case, the geometry of the carboxylate−guanidinium complex was obtained from an *ab initio* geometry optimization (*33*). In all cases, a virtual bond was established between the hydrogen donating nitrogen atom of the substrate and the hydrogen accepting oxygen atom of the aspartate moiety in order to allow for rotation around this bond in the flexible fitting procedures. Overall, this allows for larger flexibility in the possible positions of the basic nitrogen atom(s).

The C_α- and C_β-atoms of the aspartic acid residues coupled to the templates (dextromethorphan (**1**) and debrisoquine (**2**), Figure 2) were matched with the corresponding atoms in Asp^{301} present in the I-helix by employing a rigid fitting procedure. In a subsequent step, the known sites of oxidation of **1** (a 7 Å substrate) and **2** (a 5 Å substrate) were matched with the dummy atoms defined above rings B (labeled "7 Å") and A (labeled "5 Å"), respectively (Figure 1), using a flexible fitting procedure. The relative orientation of **1** and **2** was then manipulated in an attempt to superimpose the flat hydrophobic regions present in both substrates (in an orientation similar to the orientation of the flat hydrophobic region as indicated by docking studies in a P450 2D6 protein model (*21*)), while simultaneously keeping the position of the sites of oxidation of **1** and **2** fixed and allowing a change in the position of the superimposed C_α- and C_β-atoms of less than 0.5 Å. These criteria could not be met while keeping the site of oxidation of **2** fixed on the dummy atom above ring A ("5 Å" in Figure 1). Satisfactory results were, however, obtained with the site of oxidation of **2** fitted onto the dummy atom above ring B ("7 Å" in Figure 1). Consequently, the 5 Å dummy atom above ring A was no longer used in the fitting procedures. To facilitate the coplanar fitting of the flat hydrophobic regions present in the various substrates, a 10 Å × 10 Å grid was defined through the flat hydrophobic region of **1**. This grid was merely used to easily superimpose the planar regions of all investigated substrates. The size of the grid was arbitrarily chosen, and there is no guarantee or necessity that the entire grid area is accessible to all substrates. After fitting a new substrate onto the grid, close contacts between the fitted substrate and the heme moiety and/or the I-helix were checked and, if necessary, removed.

A refined substrate model was obtained by fitting compounds **3**−**8** onto template **1** and the above-defined grid employing similar rigid and flexible fitting procedures (*26*). First, the site of oxidation in the substrate was matched (rigidly) with the "7 Å" dummy oxidation site (Figure 1), after which the flat region of the substrate was rigidly superimposed onto the grid, while keeping the site of oxidation fixed on the "7 Å" dummy atom. Subsequently, the C_α- and C_β-atoms of the aspartic acid residue attached to the substrate were superimposed onto the corresponding atoms in Asp^{301} in a flexible fitting procedure. In general, conformations with distances exceeding 0.5 Å between the superimposed atoms were rejected. An exception was made for the C_β-atom of the attached aspartic acid residue of 5 Å substrates as explained below. For this atom a distance of 1.0 Å to the C_β-atom of Asp^{301} was allowed. After checking for close contacts between the substrate and the protein parts of the model (I-helix and heme moiety) and reorientating the substrate slightly when necessary, the fitted conformation was accepted

when the total energy was below a predefined limit (see next paragraph).

Energy Calculations. The parallel version of the density functional program package ADF (Amsterdam density functional) (*34*, *35*), implemented on IBM/RS6000 workstations, a parallel cluster of 6 IBM/RS6000 workstations, a CRAY-YMP supercomputer, a CRAY C98 supercomputer, and an 8-node IBM/9076-SP1, was used for the calculations described. The density functional used was the local density approximation (LDA) by Vosko, Wilk, and Nusair (*36*). The geometries of the (unprotonated) substrates obtained after a conformational analysis and geometry optimization with Macromodel (*28*) were optimized with ADF using the single zeta (SZ) basis set. This optimization was followed by a single point energy calculation using the double zeta (DZ) basis set including nonlocal gradient corrections according to Becke (*37*) and Perdew (*38*). For the fitted conformations of all substrates, a similar single point energy calculation was performed. Generally, a fit was accepted when the density functional energy difference (ΔE or $\Delta E_{(\mathrm{opt-fit})}$) between minimal energy conformation and fitted conformation (unprotonated, without the attached aspartic acid residue) did not exceed 10 kcal/mol. In specific cases, however, larger energy differences were accepted.

Molecular Electrostatic Potentials (MEPs). MEPs based on Coulomb point charge interactions between the compound under investigation and a positive point charge were calculated with ChemX (*26*) on the van der Waals surface for a series of compounds using partial atomic charges derived from *ab initio* DMA (distributed multipole analysis) calculations (*39*)) at the RHF (restricted Hartree Fock) level in an SV (split valence) 6-31G (*40*, *41*) basis set using the *ab initio* quantum chemical program package GAMESS-UK (Generalised Atomic and Molecular Electronic Structure System U.K. version (*42*, *43*)).

Results and Discussion

The aim of the present study was to investigate the possibility of different sites of oxidation for the 5 Å and 7 Å substrates of P450 2D6 by reinvestigating the previously derived substrate model (*12*, *17*)[2] and to use the three-dimensional information obtained for the active site from the recently described protein model of P450 2D6 (*21*) for refining the substrate model. Additional information was used consisting of: (a) the relative spatial orientation of the heme moiety and the I-helix in the protein, thus adding steric restrictions and orientational preferences to the substrate model, (b) the direction of well-defined hydrogen bonds between Asp^{301} (located in the I-helix) and the basic nitrogen atoms of P450 2D6 substrates, and (c) the possibility of two different sites of oxidation as suggested by the protein model (*21*).

Template Molecules for a Refined Substrate Model for P450 2D6. Dextromethorphan (**1**) was fitted into a model consisting of the I-helix containing Asp^{301}, the heme moiety, and two possible oxidation sites represented by dummy atoms (Figure 1). The template (**1**) was fitted in several steps (see also Computational Methods). Initially, the C_α- and C_β-atoms of the aspartic acid residue attached to the basic nitrogen atom of **1** were superimposed on the corresponding atoms of Asp^{301}. Consecutively, the site of oxidation of **1** was matched in a flexible fitting procedure with the so-called "7 Å" oxidation site (see Figure 1), only allowing rotation around the $C_\alpha-C_\beta$ bond and the hydrogen bond. Then the orientation of **1** was adjusted without changing the position of the oxidation site relative to the heme moiety until the flat hydrophobic region of **1** was positioned in a similar orientation as the planar region derived from docking experiments in a recently published protein model for P450 2D6 (*21*). A flexible fit followed in order to retain a satisfactory match of the C_α- and C_β-atoms again. The angle between the plane of the heme moiety and the plane of the hydrophobic region of **1** appeared to be 29.5°. The plane of the hydrophobic region of **1** was subsequently used to define a grid (10 Å × 10 Å) in order to facilitate the coplanar fitting of the planar regions of the remaining substrates.

As described under Computational Methods, initial attempts to fit the second template molecule (debrisoquine (**2**)) into the model containing **1** using the "5 Å" oxidation site (Figure 1) were unsuccessful. It was impossible to superimpose the C_α- and C_β-atoms of the attached aspartic acid residue onto the corresponding atoms of Asp^{301}, while simultaneously keeping the site of oxidation fixed onto the "5 Å" oxidation site and keeping the planar region of **2** coplanar to the grid. An acceptable fit for **2** could, however, be obtained when the "7 Å" oxidation site was used, although a distance below 1.0 Å could not be established between the C_β-atoms of **2** and Asp^{301}. The angle $C_{\beta(Asp^{301})}-C_\alpha-C_{\beta(\text{attached Asp residue})}$ was 38°. Since all other criteria were met, i.e., an acceptable match for the site of oxidation and the C_α-atom, reasonable hydrogen bonds between the two nitrogen atoms of the guanidino moiety of **2** and the carboxylate group of Asp^{301}, and the planar region of **2** matching in a coplanar fashion onto the above-mentioned grid, the rather large discrepancy for the C_β-atom was tolerated. Preliminary fits indicated that none of the other substrates (**3**−**8**, see below) could satisfactorily be fitted into the model when their oxidation sites were matched with the "5 Å" dummy atom. Therefore, the 5 Å dummy atom was omitted and all so-called 5 Å and 7 Å substrates were fitted using a single site of oxidation ("7 Å" in Figure 1) in the model. Figure 4a shows the refined substrate model for P450 2D6 with the heme moiety, the grid used to match planar regions, and the fitted template molecules **1** (7 Å substrate) and **2** (5 Å substrate).

Other Substrates. The P450 2D6 substrates **3**−**8** were then fitted into the refined substrate model. For these compounds, the sites of oxidation were matched with the site of oxidation of **1** ("7 Å" in Figure 1). Their flat hydrophobic regions were matched coplanar with the plane defined by the grid. Subsequently, the C_α- and C_β-atoms of the attached aspartic acid residues were superimposed on the corresponding atoms of Asp^{301} employing a flexible fitting procedure. The resulting fits were adjusted to minimize close contacts between substrates on the one hand and the I-helix and the heme moiety on the other hand. The results of the rigid fit ($\Delta r_{(\mathrm{ox-ox})}$) and the flexible fit ($\Delta C_\alpha$ and ΔC_β) are shown in Table 1. Generally, the results of the flexible fits were within the defined criteria (see Computational Methods). In some cases, such as for **6**, we allowed for a minimal deviation from our initial criteria (+0.1 Å). Codeine (**6**) could be accommodated only very poorly into the refined substrate model (and also in the original substrate model)[2] and will be discussed further below. Table 1 also gives the energy difference (ΔE) between the fitted and minimal energy conformation of each substrate.

It was stated earlier (see Computational Methods) that an increase of 10 kcal/mol is maximally allowed for ΔE. This limit was arbitrarily chosen and has been used for the original substrate model which was based on semiempirical energy calculations (*12*). In the early stages of P450 substrate modeling, it turned out that conformations of substrates which gave acceptable energy differences (e.g., ΔE < 10 kcal/mol) when calculated with

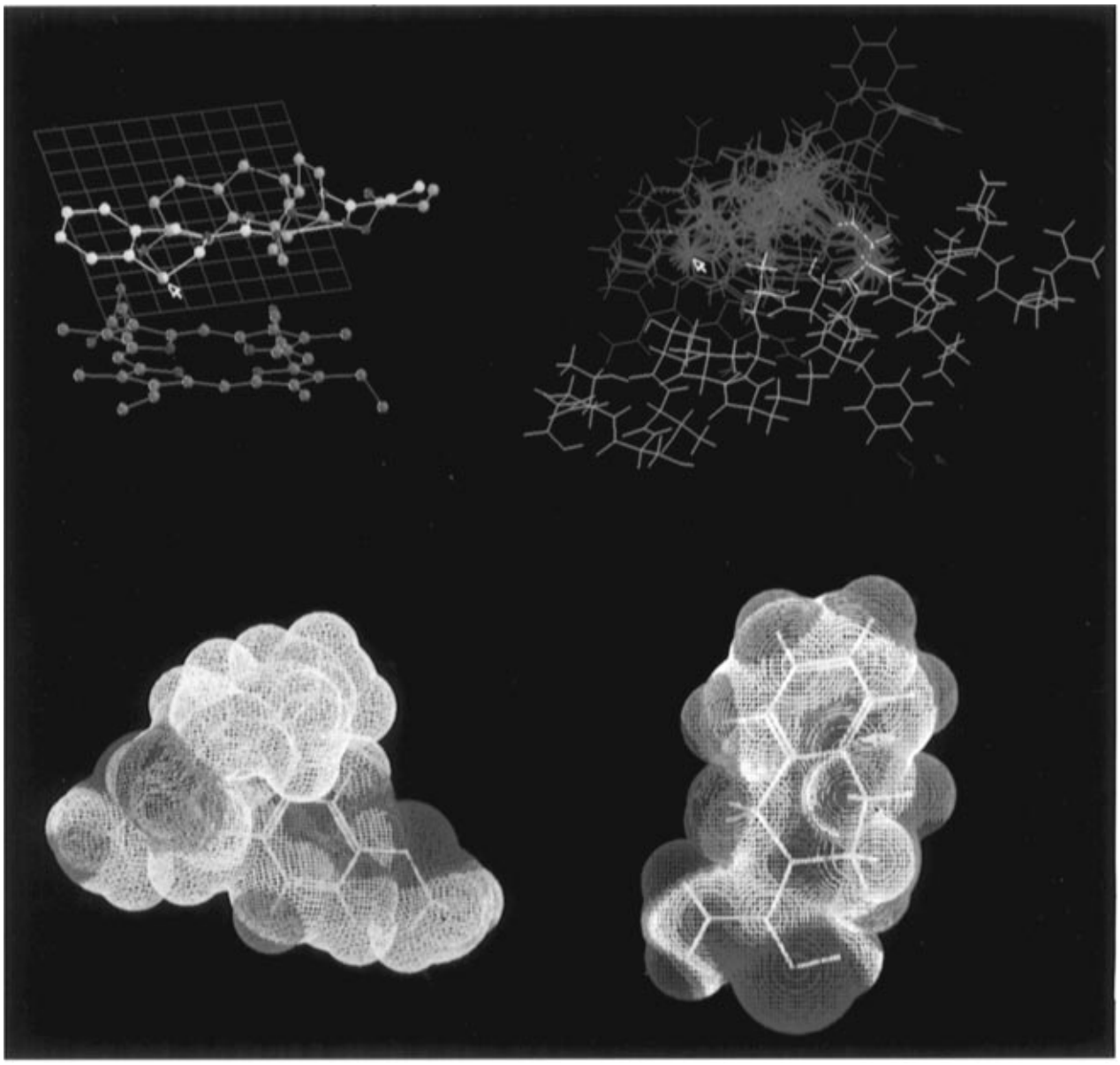

Figure 4. Refined substrate model for P450 2D6 and MEPs. (a, top left) The refined substrate model for P450 2D6 containing the template molecules **1** and **2**. The heme moiety is shown in pink, **1** in green, and **2** in yellow, and the grid, used for matching planar regions, in blue. Nitrogen atoms are colored blue, and oxygen atoms are colored red, while hydrogen atoms have been omitted for clarity. The site of oxidation is indicated by an arrow. (b, top right) Superposition of all substrates used (red), the I-helix (green) containing Asp^{301} (superposition highlighted in yellow), and the heme moiety (blue). Both protein elements were obtained from a protein model for P450 2D6 (*21*). The site of oxidation is indicated by an arrow. (c and d) MEPs based on Coulomb point charge interactions between a substrate and a positive point charge using DMA derived charges (c, bottom left) dextromethorphan (**1**) and (d, bottom right) debrisoquine (**2**). MEP colors: red < −20 kcal/mol < orange < −10 kcal/mol < yellow < 0 kcal/mol < white < 10 kcal/mol < green < 20 kcal/mol < blue.

semiempirical methods (*12*) resulted in larger energy differences when calculated with density functional methods, as used for the present refined substrate model.[3] The absolute limit for ΔE probably depends on the overall number of favorable interactions between protein and substrate. This limit can be different for each substrate, although it is generally expected that a larger substrate (e.g., **4**) can have more favorable interactions with the protein than a smaller substrate (e.g., **2**), allowing for a higher ΔE value. Since the number and kind of interactions between protein and substrate are mostly unknown, we generally used a ΔE limit of 10 kcal/mol, although deviations were allowed for, depending on the size and nature of the substrate. Experimentally determined binding energies for protein−ligand complexes have been compared with binding energies for a wide variety of compounds, predicted on the basis of the functional groups present in the ligand (*44*). The observed binding energies ranged from 3.1 to 20.5 kcal/mol, with the majority of observed binding energies between 8 and 14 kcal/mol (*44*). In case this binding energy can be fully used to compensate the conformational energy increase, a ΔE limit up to 20−25 kcal/mol is probably justified for the P450 enzyme system.

As can be seen in Figure 2 and Table 1, a variety of known P450 2D6 substrates could be accommodated well in the present refined substrate model. Fits for several

[3] For example, the energy difference between the fitted conformation and the semiempirically optimized geometries of (*R*)-metoprolol (benzylic hydroxylation) was calculated to be *8.9 kcal/mol* (*12*). The corresponding density functional energy difference turned out to be *196.7 kcal/mol* (ADF, double zeta basis set *without* gradient corrections).

Table 1. Type of Substrate, Results of Rigid and Flexible Fits, and Energy Differences between Fitted Conformation and Minimal Energy Conformation

			rigid fit	flexible fit		
substrate	(oxidation site)[a]	type	$\Delta r_{(ox-ox)}$[b] (10^{-3} Å)	ΔC_α[c] (Å)	ΔC_β[c] (Å)	$\Delta E_{(opt-fit)}$ (kcal/mol)
1	(O-dem)	7 Å	0.0	0.49	0.24	0.0
2	(Arom-OH)	5 Å	10.0	0.01	1.00[d]	0.0
3a	(O-dem)	7 Å	0.4	0.15	0.43	1.2
3b	(O-dem)	7 Å	0.0	0.06	0.25	10.3
4a	(O-dem)	7 Å	0.4	0.24	0.27	23.5
	(Benz-OH)	7 Å	0.2	0.12	0.49	23.8
4b	(O-dem)	7 Å	0.2	0.12	0.23	43.5[e]
	(Benz-OH)	7 Å	0.1	0.08	0.20	38.3[e]
5a	(O-dem)	5 Å	0.4	0.43	1.07[d]	9.6
	(O-dem)	7 Å	0.0	0.04	0.27	5.0
	(Arom-OH)	5 Å	9.0	0.15	1.11[d]	12.8
5b	(O-dem)	7 Å	7.0	0.11	0.14	9.0
	(Arom-OH)	5 Å	0.7	0.08	0.74[d]	2.2
6	(O-dem)	7 Å	0.0	0.64	1.05	48.0[e]
7	(Arom-OH)	7 Å	0.0	0.42	0.31	6.4
	(Benz-OH)	7 Å	0.8	0.35	0.30	36.9[e]
8a	(Arom-OH)	7 Å	0.0	0.18	0.36	12.4
8b	(Arom-OH)	7 Å	0.0	0.20	0.21	18.5

[a] In some substrates multiple oxidation sites are present. Abbreviations are as follows: (Arom-OH) = oxidation at the aromatic ring; (Benz-OH) = oxidation at the benzylic carbon atom; (O-dem) = oxidation at the methoxy carbon atom, resulting in O-demethylation. [b] $\Delta r_{(ox-ox)}$ = distance between site of oxidation above heme moiety (ring B) and site of oxidation in the substrate after rigid fitting procedure. [c] $\Delta C_\alpha/\Delta C_\beta$ = distance between C_α/C_β atoms of the aspartic acid residue attached to the substrate and corresponding atoms in Asp^{301} after flexible fitting procedure. [d] For the 5 Å substrates, the C_α-atoms and sites of oxidation could be superimposed nicely, whereas the C_β-atoms could not be superimposed without sacrificing the match of the flat hydrophobic region with the grid and/or the hydrogen bond interaction(s) between the basic nitrogen atom(s) and the carboxylate moiety of Asp^{301} (see text). [e] Large energy differences (compared to ref 44), possibly related to relative minor metabolic routes.

substrates revealed ΔE values above the limit of 10 kcal/mol. For **4** the ΔE values in both the present and the original substrate model[2] were larger than 10 kcal/mol for both metabolic pathways. The ΔE values for the O-demethylation and benzylic hydroxylation pathways of the *S*-enantiomer (**4b**) were respectively 20.0 and 14.5 kcal/mol higher than the corresponding ΔE values for the *R*-enantiomer (**4a**). With respect to the O-demethylation reaction, the *R*-enantiomer of **4** (**4a**) is known to be the preferred enantiomer (*R*/*S* ratio = 1.79 ± 0.33 (*45*), or 1.7 ± 0.1 (*46*)). With respect to the benzylic hydroxylation reaction, however, no real preference for either **4a** or **4b** has been observed experimentally (*R*/*S* ratio = 0.98 ± 0.05 (*45*), or 0.9 ± 0.1 (*46*)). It is therefore tempting to suggest that ΔE values contain information on relative rates of metabolism. For the O-demethylation reaction of **5a**, two possible conformations were found which could be accommodated into the substrate model, one resembling the 7 Å conformation of **1** and the other resembling the 5 Å conformation of **2** (both fitted on the site labeled "7 Å" in Figure 1). For the O-demethylation reaction of **5b** only one conformation (the conformation resembling the 5 Å conformation of **2**, fitted on the site labeled "7 Å" in Figure 1) is included in Table 1. The fits and the ΔE values for both the 5 Å and the 7 Å orientations of **5a** and **5b** were within the accepted criteria. For **6** the quality of the fit was poor and the energy difference relatively large. This observation might corroborate with the observation that O-demethylation of **6** is only a minor metabolic route (*47*). For the benzylic oxidation of **7** a ΔE value of 36.9 kcal/mol and for the aromatic hydroxylation of **7** a ΔE value of 6.4 kcal/mol were found, respectively (Table 1). Interestingly, this rank order also corresponds with the relative turnover rate of both metabolic reactions, observed in microsomal fractions from a human lymphoblastoid AHH-1 cell line expressing P450 2D6 (13 and 83 pmol/(min·mg of protein), respectively (*17*)).

All substrates could be fitted into the refined substrate model with their sites of oxidation fitted onto the "7 Å" oxidation site above ring B of the heme moiety (Figure 1). The presence of a second site of oxidation (above ring A of the heme moiety) was not necessary for the incorporation of substrates in the refined substrate model. The results obtained for the 7 Å substrates were generally more satisfactory than the results obtained for the 5 Å substrates. In the latter case, the C_β-atoms of the aspartic acid residues attached to the basic nitrogen atoms of the substrate could not be matched onto the corresponding atom in Asp^{301}. The C_β-atoms of the aspartic acid residues attached to the basic nitrogen atoms of all examined 5 Å substrates, however, were superimposed onto each other. This suggests that the substrate model actually should allow for even more flexibility in Asp^{301}.

Previous docking experiments with various substrates into a recent protein model for P450 2D6 (*21*) revealed slightly different orientations for Asp^{301}. We have re-examined these results and it appeared that Asp^{301} indeed possesses the required orientational freedom (data not shown). Another recently derived set of 13 protein models for P450 2D6 using homology modeling techniques augmented with NMR distance restraints showed a similar conformational freedom for Asp^{301} (*20*). Based on the results described above, only one site of oxidation is probably present in the enzyme. However, by incorporating more conformational freedom in the hydrogen bond between Asp^{301} and the basic nitrogen atoms in the substrates, and assuming additional compensation from other interactions between protein and substrate, for 5 Å substrates an orientation fitted onto the "5 Å" dummy above ring A in Figure 1 *might* also be possible. The conclusion of the present study is that only one site of oxidation is present in the protein, while different orientations of Asp^{301} allow incorporation of both 5 Å and 7 Å substrates in the refined substrate model. Further modeling studies are currently in progress.

In summary, the presence of several steric restraints and orientational preferences in the refined substrate model enables a more precise and therefore a more reliable prediction of possible sites of oxidation in the case of potential P450 2D6 substrates. The heme moiety clearly limits the model in one direction. The I-helix, also included in the small molecule model, is limiting the model in another direction. The introduction of hydrogen bond interactions between substrates and Asp^{301} in the protein further limits the conformational freedom of the substrates in the active site of P450 2D6. Figure 4b shows a superposition of 8 substrates (representing 17 metabolic pathways) investigated in the present refined substrate model for P450 2D6 together with the heme moiety and the I-helix.

Molecular Electrostatic Potentials (MEPs). MEPs generated on the van der Waals surface of all substrates qualitatively support the proposed refined substrate model. Evaluation of the MEPs of the various substrates revealed a highly negative region in the flat area near the site of oxidation for all compounds, which is in close

agreement with the results obtained with the original substrate model (*12*).[2] The MEPs of **1** and **2** are shown in Figures 4c and 4d, respectively.

Comparison of the Present Refined Substrate Model with Previous Substrate Models. The present refined substrate model, the substrate model developed by Koymans *et al.* (*12*) and the substrate model described by Islam *et al.* (*16*), all contain a single site of oxidation for both 5 Å and 7 Å substrates, a site interacting with the (protonated) basic nitrogen atom(s), and a flat hydrophobic region. In this flat region negative MEPs are present both in the substrate model developed by Koymans *et al.* (*12*) and in the present refined model (Figures 4c and 4d). All three substrate models can accommodate a wide variety of chemically diverse P450 2D6 substrates. Both the substrate model of Islam *et al.* (*16*) and the present refined substrate model incorporate the heme moiety and define the positions of the site of oxidation, the heme moiety, and a negatively charged site in the protein interacting with the (protonated) basic nitrogen atom(s) in the substrate.

The previous substrate models and the present refined substrate model for P450 2D6 differ in the size of the model, the position of the site of oxidation relative to the heme moiety, the nature and/or orientation of the residue interacting with the (protonated) basic nitrogen atom(s) in the substrates, and the orientation of the various substrates relative to each other. The present model is the most extended small molecule model developed so far, and it includes the heme moiety and the Asp^{301} containing I-helix, both derived from a protein model for P450 2D6 (*21*).

In the present substrate model for P450 2D6 one site of oxidation is located above ring B of the heme moiety (Figure 1). The substrate model of Islam *et al.* (*16*), on the contrary, contains a site of oxidation above ring A (Figure 1) as derived from the site of oxidation of camphor as found in the crystal structure of P450 101. In the P450 2D6 substrate model of Islam *et al.*, the basic nitrogen atom present in the substrates was proposed to interact with an "anion" (*16*), while in the substrate model of Koymans *et al.* (*12*) and in the present refined substrate model Asp^{301} is used explicitly. A possible role for Asp^{301} was initially predicted (using homology modeling (*18*)) and recently confirmed (using site-specific mutation and expression experiments) to be important for the activity of P450 2D6 (*22*).

Although all three P450 2D6 substrate models contain a flat hydrophobic region, the orientation of the substrates in the present refined substrate model is different from the orientation in the other two substrate models (*12*, *16*). In the model of Islam *et al.*, merely the sites of oxidation were fitted onto each other, and the substrates were further solely orientated with a protonated nitrogen atom in a similar orientation (*16*). Koymans *et al.* superimposed the respective sites of oxidation of the substrates and fitted the basic nitrogen atoms of the different substrates either onto the basic nitrogen atom of debrisoquine, a 5 Å substrate, or onto the basic nitrogen atom of dextromethorphan, a 7 Å substrate (*12*). In the present refined substrate model for P450 2D6, the sites of oxidation are superimposed and the C_α- and C_β-atoms of an attached aspartic acid residue are fitted onto the corresponding atoms of Asp^{301}, ensuring an appropriate direction for a hydrogen bond. The use of a grid in the present refined substrate model facilitates the parallel fitting of the hydrophobic regions, while the presence of the I-helix adds additional steric and orientational restraints compared to the earlier substrate models (*12*, *16*).

Conclusions

A refined small molecule model for P450 2D6 substrates has been derived. Compared to the original model from our group (*12*, *17*),[2] the heme moiety and the I-helix have been incorporated into the small molecule model. The template molecules from the original small molecule model (*12*) have been reoriented in order to comply with the added steric restraints due to the heme moiety and the I-helix, and to ensure an appropriate directionality of a hydrogen bond between the basic nitrogen atoms in substrates and the negatively charged aspartic acid residue (Asp^{301}) in the P450 2D6 protein. Substrates were fitted into the refined model by superimposing their sites of oxidation onto one site of oxidation above ring B of the heme moiety, and fitting the C_α- and C_β-atoms of an aspartic acid residue attached to the (protonated) basic nitrogen atom(s) of the substrates onto the corresponding atoms of Asp^{301}. A variety of 8 P450 2D6 substrates comprising 17 metabolic pathways present in the original substrate model for P450 2D6 (*12*, *17*)[2] have been successfully fitted into the present refined substrate model, indicating that the refined model (with additional sterical restraints and directional preferences) can accommodate the same variety in molecular structures as the original model. It may be concluded that the present substrate model for P450 2D6 is more selective due to the incorporation of steric and directional restraints. As the refined substrate model represents a more realistic representation of the active site region of P450 2D6, predictions based on this substrate model will most likely be more reliable than the previous substrate models. Work is in progress to include more substrates in the present model and to test the predictive value of this refined substrate model compared to the original substrate model.

Acknowledgment. NCF (Nederlandse Computer Faciliteiten) is gratefully acknowledged for providing supercomputer facilities. The authors wish to thank Dr. G. M. Donné-Op den Kelder for her interest in the described research and for careful reading of the manuscript.

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