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Development of models for Cytochrome P450 2A5 as well as two of its mutants

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It is known that small changes in the amino acid sequence can change the catalytic activity of cytochromes towards substrates dramatically. With the aim to broaden our knowledge about the structural properties of cytochromes and their relation with substrate specificity a model of CYP2A5 was built by homology modelling based on the crystal structure of CYP2C5. Model stability was evaluated by subjection of the model to a free molecular dynamics simulation in a waterbox under almost physiological conditions using the GROMACS program. The protein folding remains stable over 1.5 ns under these conditions. The modelling procedure was repeated for two mutated forms of CYP2A5 with known differing substrate selectivities towards corticosterone and desoxycorticosterone. A detailed analysis of the models and their behaviour in long running molecular dynamics simulations allows an understanding of the requirements for enzyme activity as well as an explanation of respective experimental data on the molecular level.

1. Introduction

Cytochromes P450 can be found in almost any living organism. They play key roles in synthesis and degradation of endogenous and exogenous substances. Synthetic cytochromes are highly specific and selective. Metabolic cytochromes however, are not highly selective and therefore able to transform a large number of different substrates. The general molecular function of the cytochromes P450s is activation of molecular oxygen. During the catalytic cycle one of the oxygen atoms is inserted into a carbon-hydrogen bond forming an hydroxyl group. The second oxygen leaves the reaction trapped in a water molecule. This requires two reduction equivalents (i.e. two electrons and two protons). The electrons are delivered by a specific electron transport system and a chain of water molecules, tunnelling from the periphery to the active site, delivers the protons.

The CYP2A5 cytochrome investigated in this study originates from the rabbit but is very homologue with the human CYP2A6. We have investigated CYP2A5 because experimental data on hydroxylation rates for this enzyme as well two of its mutants are known and can be used to validate the modelling process. CYP2A5 is active in metabolism and until now no endogenous substrate is known. So the main task probably is the degradation of xenobiotics. All therapeutic drugs are xenobiotics by definition and many of them are metabolised via the cytochrome P450 system. Since inter-individual differences in combination and activities of cytochromes can influence the metabolism of all kind of drugs it is very desirable to know more about the structural differences in cytochromes in order to explain their different behaviour towards substrates.

2. Investigations, results and discussion

2.1. Homology and sequence alignment

The publication of the first crystal structure of a mammalian cytochrome P450, CYP2C5, by Williams et al. (2000) improved the basis for homology modelling of cytochromes significantly. Especially modelling of cytochromes of the family 2 is now based on considerably higher sequence identities (40–70% compared to the rather low similarity with the previously known prokaryotic cytochromes. The identity between CYP2C5 and CYP2A5 is above 50% (sequence homology: $>76\%$). It is well known that the 3-dimensional fold of cytochromes is conserved throughout the whole cytochrome P450 family (Nelson and Strobel 1987; Hofmann et al. 1999; Nebert and Gonzales 1987; Gotoh 1997; Hasemann et al. 1995), therefore modelling of CYP enzymes in principle is possible also in case of less marked homology.

The crystal structure of CYP2C5 does not contain coordinates for two segments of the protein. First, the N-terminal end, that in common opinion serves as a membrane anchor and prohibited the crystallization of the mammalian cytochromes for a long time, was not expressed in the protein used for crystallization. Therefore the membrane-spanning N-terminal region in the CYP2A5 model is omitted

as well. The second region where coordinates are missing is a highly flexible loop between the F- and G-helix. Coordinates for the 11 amino acids in this region had to be generated employing a special loop search procedure.

2.2. Model building and relaxation

2.2.1. Static model building

For model construction the coordinates of all backbone atoms of structurally conserved regions were transferred from the 2C5 crystal structure to the model structure. Identical amino acid side chain coordinates were transferred as well (Fig. 1). For all other side chains occurring in the CYP2A5 sequence the coordinates were taken from a set of frequently occurring conformations listed in the Ponder and Richards tables (Ponder and Richards 1987). The choice of the best fitting conformation was based on the backbone angles in the model.

Coordinates for the structurally variable regions were generated using the loop search routine in the HOMOLOGY module of INSIGHT II (Molecular Simulations Inc. 2000).

Further refinement of the rough model was performed in consecutive energy minimization steps (CVFF forcefield). Firstly the areas around splice points between different structural segments was minimized. Only a few amino acids before and after the splice points were allowed to move freely. All other parts of the structure were fixed.

In the next step steric overlaps between any of the modified amino acids and the rest of the protein which arose during the replacement of the non-identical side chains as well as by insertion of loop structures were corrected.

With the help of the program SCWRL (Bower et al. 1997) a further improvement of the model was achieved. SCWRL checks all mutated side chains for steric interactions with any other part of the protein. After recognition of interdependent clusters of unfavourable side chains SCWRL tries to smoothen the interactions by optimisation of the whole cluster instead of single side chains. In the following minimizations subsequently growing parts of the protein were allowed to move freely (loop side chains ⇒ all loop atoms ⇒ mutated side chains in the conserved regions ⇒ all side chains). In order to protect the overall fold of the protein all backbone atoms in the conserved regions were fixed during this first refinement. Superimposition of the conserved backbone onto the crystal structure and copying the cofactor atoms (heme) into the model structure completed constructions of the first preliminary CYP2A5 model. All of the before mentioned calculations were performed on Silicon Graphics workstations (Indigo2, R10000).

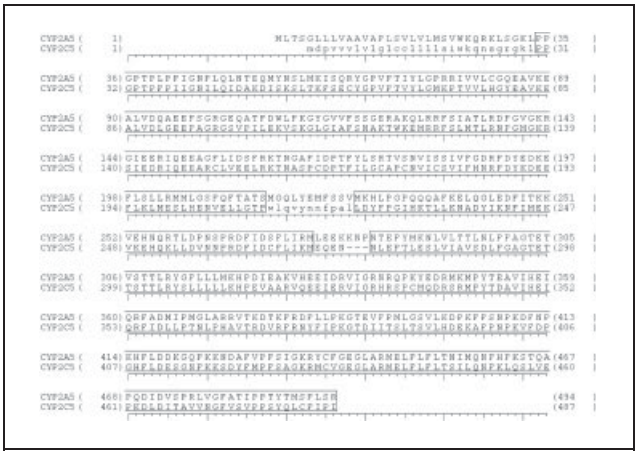


Fig. 1: Sequence alignment of CYP2A5 and crystallized CYP2C5. Boxes indicate areas of coordinate transfer. Small letters in the CYP2C5 sequence indicate that these amino acids were missing in the crystal structure. The N-terminal part of the protein was not included in the model. For the two other regions where coordinates were not transferred a loop was modelled

2.2.2. Dynamic model relaxation

After preliminary static minimization the protein was further relaxed by performing extensive molecular dynamics simulations (mds). All mds were performed using the GROMACS package with the GROMOS96 forcefield at physiological temperature of 310 K (Berendsen et al. 1995; Torda et al. 1989; Berendsen et al. 1984; Miyamoto and Kollman 1992; Hess et al. 1997; van Gunsteren et al. 1996). The model was embedded in a box with 0.5 nm add on edge length on each side of the maximum diameter of the protein and the box was filled with spc (single point charge) water molecules. In order to approach physiological conditions and to fulfil the calculational requirements of the GROMACS package for overall electrostatic neutrality ions were added to the waterbox (20 hydrogen phosphate ions as well as 37 sodium ions). Parameters for the heme moiety were chosen analogous to the work published by Wade and Schappach (Helms and Wade 1995; Schappach and Höltje 2001). The resulting system consisted of 34621 atoms (4857 protein, 157 ions, 29607 water molecules).

By inclusion of water molecules into the simulations we were able to gradually waive any artificial forces restraining the protein. In the first step all non-hydrogen atoms of the protein were restraint to their respective starting position with 1000 $kJ \cdot mol^{-1} \cdot nm^{-2}$ for 100 ps. In the following steps the restraining forces were reduced to 500, 200 and 100 $kJ \cdot mol^{-1} \cdot nm^{-2}$ in successive dynamics runs of 100 ps each. This was followed by a 1500 ps dynamics run free of any tethering forces. All relevant parameters used in the dynamics simulations are listed in Table 1.

2.3. Evaluation of protein geometry

The model was stable over a simulation period of 1.5 ns. For judging the quality of the homology model the crystal structure of cytochrome CYPeryF (pdb code: 1OXA) was subjected to identical mds conditions and the root mean square deviation (rmsd) observed were analyzed. The CYPeryF model shows slightly less movement in the backbone during dynamic treatment ($\sim$0.12 nm) compared to 0.2 nm measured for the CYP2A5 model (Fig. 2). When only the backbone atoms of the active site regions in the

Table 1: Parameters used in dynamics simulations

Length of integration step	1 fs
Temperature	310 K
Output frequency (velocity)	1000 fs (1 ps)
Output frequency (energy)	500 ps
Electrostatics calculation method	PME
Electrostatics interaction radius	0.9 nm
Fourier grid spacing	0.12 nm
PME interpolation	Cubic
FFT optimisation	Yes
VdW interaction calculation method	Cut-Off
VdW interaction radius	0.9 nm
Temperature coupling	Yes
Pressure coupling	Isotropic
Constraints	H-bonds only
Calculation (H-bond constraints)	LINCS

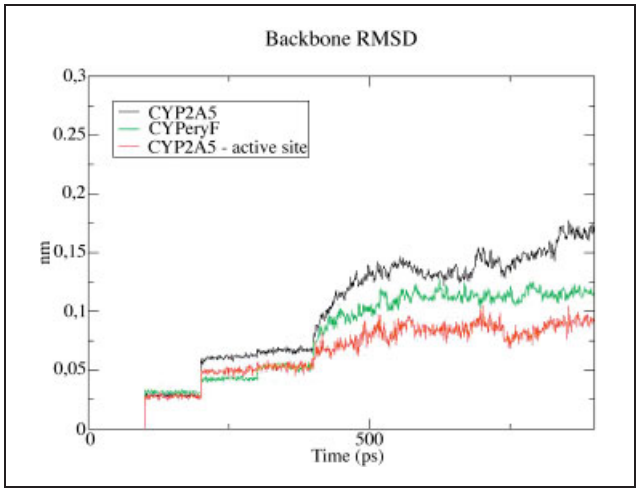


Fig. 2: RMS deviation of all backbone atoms in the CYP2A5 (black) and CYPeryF (green) models. Only backbone atoms of the active site of the CYP2A5 model are used for comparison (red). The first 400 ps were calculated with stepwise reduced position restraints followed by 500 ps of free dynamics simulation

CYP2A5 model are used for the rmsd comparison the deviation from the starting coordinates is slightly better than was found for the CYPeryF. Naturally the largest amount of flexibility is observed in the inserted loop regions, which are located at the outer bounds of the protein and show no direct interaction with the active site. The active site region in contrast does not show flexibility at all.

During the calculations the protein geometry was constantly analyzed employing PROCHECK (Laskowski et al. 1993). This program checks all important parameters characterising the quality of a protein (bond-length, bond-angle, Φ/Ψ-angle-distribution (Ramachandran-plot), χ_1/χ_2-angle-distribution, bad contacts, h-bonding energy, folding-quality) and compares the data with X-ray structures at a defined resolution level. According to PROCHECK the minimized model structure shows a good overall geometry, with most of the quality parameters being better than average or within the range of allowed tolerance. Only the values for the ω-angles are worse than average. In crystal structures these angles are found in almost all cases at perfect 180

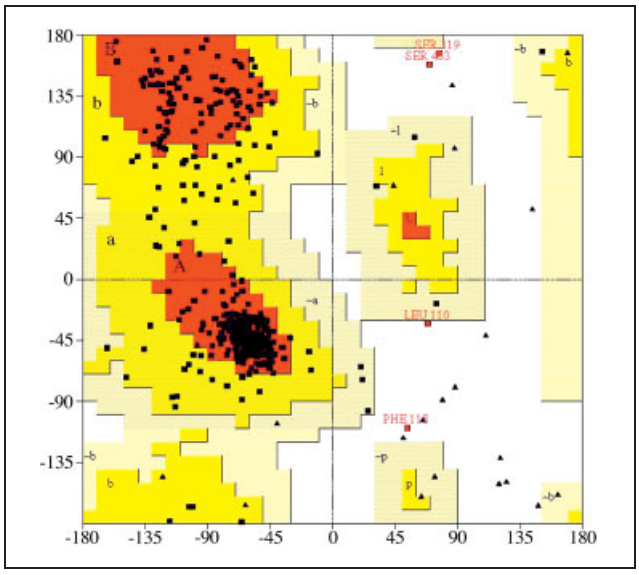


Fig. 3: Ramachandran plot of the minimized CYP2A5 model after 1500 ps of molecular dynamics

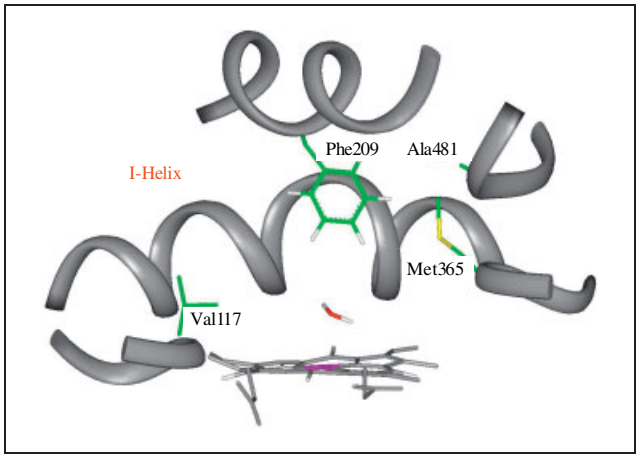


Fig. 4: Active site of the CYP2A5 model after 1400 ps of free dynamics simulation. During the whole simulation period a water molecule was found as sixth ligand to the heme iron (magenta). Only some side chains of active site amino acids are shown

degrees. The GROMACS force field, however, allows larger variability. So good values for this parameter could not be expected and were not found in any of the models.

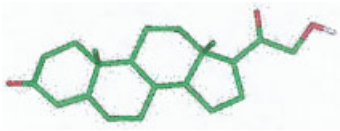
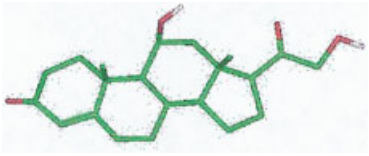
All models show a comparably good overall G-factor, which represents the sum of all individual bonding- and folding-parameters. The Φ/Ψ-angle-distribution (Ramachandran plot) is reasonable with more than 95% of the residues in the two favoured regions (Fig. 3). Only four amino acids (1%) are found in "disallowed" regions, but these amino acids were either already located in the "generously accepted" regions also in the crystal structure template and moved only slightly to the "disallowed" regions or were found to be in a beta-hairpin-turn that often shows energetically unfavourable conformations.

In all cytochromes known so far there are sets of conserved sequence motifs that result in particular 3-dimensional arrangements of the involved amino acids and salt bridges (e.g. ER/(R/H) motif, a salt bridge between G- and H-helix important for fixation of the heme). All these motifs are maintained in the model structure after the 1.5 ns dynamics. Furthermore it is known that during the substrate-free, inactive state of the cytochrome reaction cycle a water molecule is coordinated to the sixth open valence of the central iron. This water molecule was found in this position during the whole dynamics simulation of the CYP2A5 model (Fig. 4). In summary the analysis demonstrates that the generated CYP2A5 model structure shows equivalent stability and protein quality when compared to the CYPeryF and CYP2C5 crystal structures.

2.4. Validation of the model

In order to further validate the model it was used to explain the variation of activities of two closely related substrates. The transformation rates of the rather similar substrates corticosterone and deoxycorticosterone in the CYP2A5-wildtype, as well as in a F209N- and a F209L-mutant have been determined experimentally (Iwasaki et al. 1993; Juvonen et al. 1991; Iwasaki et al. 1990). Deoxycorticosterone is a substrate for CYP2A5. It is transformed by hydroxylation to the 15-alpha-product. For corticosterone this transformation occurs only in the F209N-mutant. As a test for the meaning of the models an attempt to find structural explanations for the variance in the measured activities was started.

Table 2: Transformation rates for corticosterone and deoxycorticosterone in different CYP2A5 mutants

	Transformation rate in nmol/(min · nmol [enzyme])	
	Corticosterone	Deoxycorticosterone
		
Wildtype	n.d.[1]	0.1
F209N	16	8
F209L	n.d.[1]	16

[1] n.d.: not detectable

2.4.1 *Oxygen in the active site*

The conditions in the active site prior to the hydroxylation of the substrates should be imitated as closely as possible, therefore an oxygen molecule bound to the heme had to be considered. Supposedly the increase of lipophilicity in the active site above the heme system by binding of the substrate facilitates the process of replacing the water molecule acting as the sixth heme ligand by an oxygen molecule, that is needed in the following hydroxylation reaction. The rate-limiting step in the reaction cycle of cytochromes is the then following heterolytic cleavage of the oxygen molecule leading to formation of water and an active oxygen species. This reactive oxygen species then attacks the closest hydrogen of the ligand and abstracts it. The next step is a very fast "rebound mechanism" in which the hydroxyl group is attached to the open valence of the substrate (Yoshizawa et al. 2001; Hata et al. 2001).

In 2000 Schlichting et al. published crystal structures showing the described path for cytochrome P450cam. We have used this information to position an oxygen molecule above the heme iron replacing the bound water molecule in order to prepare the model for substrate transformation. Since there are no parameters for an oxygen-iron bond in the GROMOS96 forcefield weak distance constraints had to be employed in order to fix the position of the oxygen above the heme plane. The distance between the iron and the closer oxygen atom was constraint to 0.15–0.3 nm with 50000 kJ/nm^2 · mol. Additionally in order to ensure the perpendicular position of the oxygen above the iron a distance of 0.2–0.4 nm between the oxygen atom and each of the nitrogens surrounding the iron was constrained with 25000 kJ/nm^2 · mol.

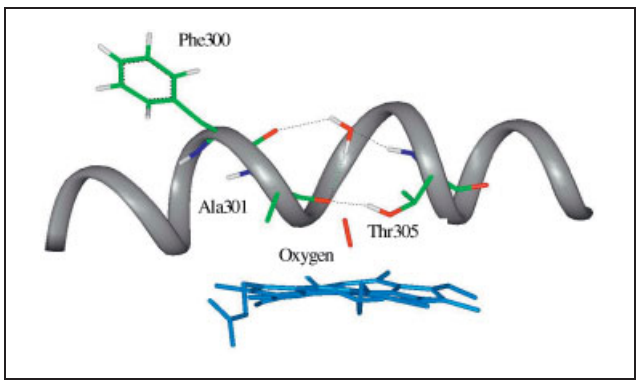


Fig. 5: Active site view with the I helix in the background (grey). Bound inside the cleft is a water molecule responsible for the delivery of protons to the active site to cleave the oxygen molecule. The oxygen molecule replaced the water molecule as sixth ligand in the docking simulations

The structures published by Schlichting et al. also revealed an explanation for the observed widening of the I-helix of cytochromes close to the heme moiety. A water molecule bound in the helix was found and it was proposed that this water molecule is the last one in a chain of water molecules used to transport protons very efficiently from the environmental solvent to the buried active site of the cytochromes. Protons are an essential part of the reaction mechanism because they are needed for formation of a water molecule after cleavage of the dioxide bond.

Applying the mentioned constraints a mds was performed for the CYP2A5 model. Shortly after starting the simulation a water molecule occupied a stable position within the I-helix and stayed in this position throughout the whole dynamics run. It forms stable hydrogen bonds to Phe 300, Ala301 and Thr305, which are conserved throughout almost the whole cytochrome P450 family (Fig. 5). This result gives an encouraging evidence for the reliability of the model building procedure.

2.4.2. *Docking into the CYP2A5 wildtype*

A representative protein geometry of CYP2A5 which is needed for the docking of corticosterone and deoxycorticosterone into the active site was chosen by extracting snapshots every 10 ps during the last 100 ps of the dynamics simulation. The individual frames were subjected to a minimization protocol (100 steps of steepest-descent minimization) to receive comparable low energy structures. The resulting frames were compared with the help of the program PROCHECK to evaluate the overall protein geometry and also by a visual inspection of the amino acids surrounding the active site. Since all structures had comparable quality the structure with fewest amino acids in the disallowed region of the Ramachandran plot was chosen as docking template.

Subsequently both ligands were docked manually into the active site furnished with an oxygen molecule bound at the heme (see 3.1). The manual docking was guided by the published cytochrome structures co-crystallized with their respective substrates. In these crystal structures it is found that the distance between heme iron and the carbon that is hydroxylized in the reaction ranges from 0.4 to 0.5 nm. Also it can be observed that the particular C–H bond which accepts the oxygen atom points directly to the activated oxygen.

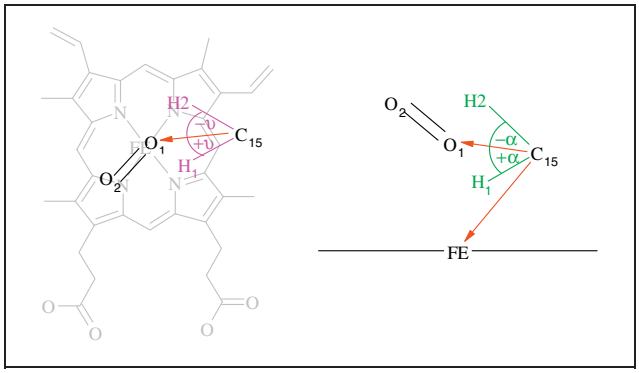


Fig. 6: Schematic view of the angles used for the analysis of the dynamics with substrate. C15 represents the carbon atom of the substrate, O1 and O2 represent the oxygen molecule bound to the heme iron (FE). Both figures show two possible positions of the hydrogen-carbon bond in which the oxygen is inserted during hydroxylation. Deviations are shown in magenta (υ) and green (α), respectively

2.4.3. Dynamics of wildtype and point mutants of CYP2A5

The point mutations of CYP2A5 were done by mutating the wildtype structure. In the F209N mutant the phenylalanine side chain was replaced with an asparagine whereas in the F209L it was replaced with a leucine. The mutated forms then were energy minimized and subsequently subjected to a molecular dynamics simulation for 700 ps each. Next the ligands corticosteron and deoxycorticosteron were docked into the active sites of both mutants and then the behaviour of all six generated ligand-enzyme-complexes during mds were analyzed.

In course of the simulations three parameters with known importance for the hydroxylation reaction were monitored.

- The distance between the heme iron and the carbon to be oxidized (range 4–5 Å)
- The position of the particular water molecule in the I-helix
- The direction of the C–H bond towards the activated oxygen atom (C–H–O angle between 170–180°, Yoshizawa et al. 2001; Hat et al. 2001). The deviation from the ideal linear geometry was measured by angles α (perpendicular to the heme plane) and υ (parallel to the heme plane). Fig. 6 illustrates the meaning and the sign of the angles.

The variation of these parameters can be utilized for illustrating the influence of mutated active site amino acids on the hydroxylation reaction.

2.4.3.1. Corticosterone and deoxycorticosterone in the wildtype

Experimental data: The measured activity of CYP2A5 against deoxycorticosterone is very low (0.1 nmol/min/nmol(P450)). Corticosterone is not a substrate at all.

Carbon iron distance: For both ligands the distance between the carbon and the heme iron increases from approx. 4.5 Å at the beginning to around 5 Å.

Water in the I helix: An important difference between the two simulations with corticosterone and deoxycorticosterone, respectively, is that for deoxycorticosterone the water molecule bound in the I helix at the beginning of the run remains in this position during the whole 300 ps of simulation time.

For corticosterone, however, this water molecule moved away from its position in the helix shortly after starting

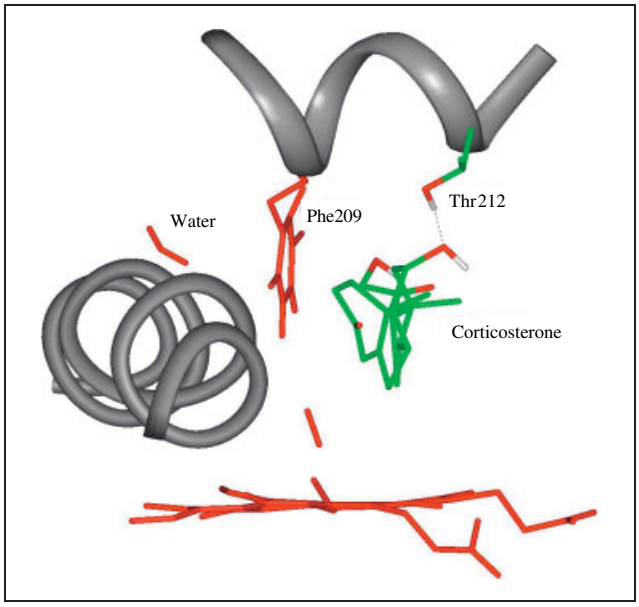


Fig. 7: Last frame of the wildtype dynamics with corticosterone. View parallel to the I-helix (left). The water molecule in the I-helix moved away from its original position. The additional hydrogen bridge between corticosterone and threonine 212 is marked

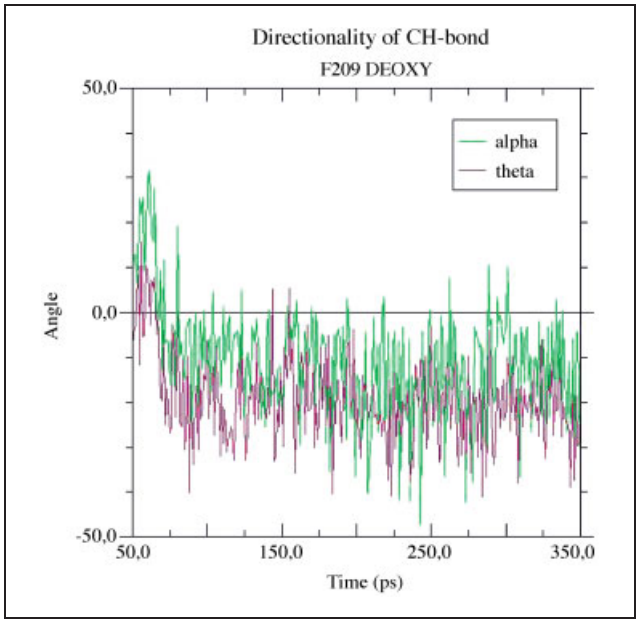


Fig. 8: Direction of the substrate CH-bond according to Fig. 6. After 50 ps of equilibration a free dynamic of 300 ps of deoxycorticosterone in the wildtype (F209) of CYP2A5 was monitored

the mds. This behaviour can be explained by the fact that corticosterone forms a hydrogen bond to the nearby Thr212. The stabilisation of the ligand in an almost perpendicular position to the heme pushes the voluminous phenylalanine side chain towards the I-helix thereby displacing the water (Fig. 7).

CHO angle: The angle between carbon, hydrogen and oxygen in the mds with corticosterone was found to be around 0° for both α and υ. Deoxycorticosterone which cannot form an additional hydrogen bond is displaced from the optimal perpendicular position above the heme so that the values for both, α and υ move into slightly negative regions (Fig. 8).

Summary: The well-positioned corticosterone can not be transformed by CYP2A5 because of displacement of the

essential water molecule in the I-helix. The position of deoxycorticosterone in the active site is not optimal but it still fulfils all prerequisites for hydroxylation.

2.4.3.2. Corticosterone and deoxycorticosterone in the F209N mutant

Experimental data: In the F209N mutant both substrates are hydrolized efficiently. The transformation rates are 16 and 8 nmol/min/nmol (P450) for corticosterone and deoxycorticosteron, respectively.
Carbon iron distance: In both simulations the distance between carbon and iron stayed well between 4 and 5 Å (0.46 ± 0.026 Å for corticosterone and 0.45 ± 0.019 Å for deoxycorticosterone).
Water in the I-helix: Due to the Phe209Asn mutation a new hydrogen bond to the water in the I-helix is formed thus stabilizing the water molecule in its important position (Fig. 9).
CHO angle: A different behaviour is observed for the two substrates. Whereas for corticosteron both angles often are found to be in optimal position for hydroxylation ($\alpha = \upsilon = 0°$), in the deoxycorticosterone complex α varies around 0° but υ oscillates between 10° and 20° (Fig. 10).
Summary: Both substrates are hydroxylized. The higher hydroxylation rate of corticosterone nicely can be explained by its more stable directionality (α- and υ-angle) towards the heme and the active oxygen species

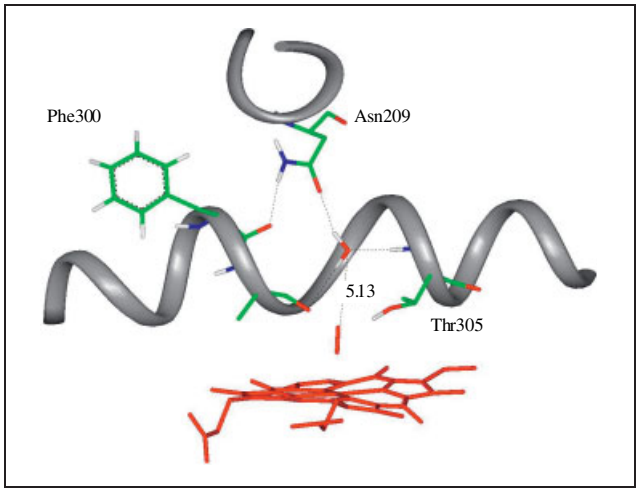


Fig. 9: Hydrogen bonding pattern in the I-helix of the F209N mutant. An additional bond to ASN 209 binds the essential water molecule

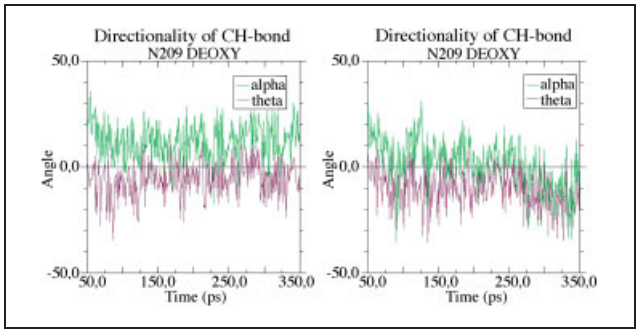


Fig. 10: Direction of the substrate CH-bond (according to Fig. 6). After 50 ps of equilibration a free dynamic of 300 ps was monitored for the N209 mutant of CYP2A5

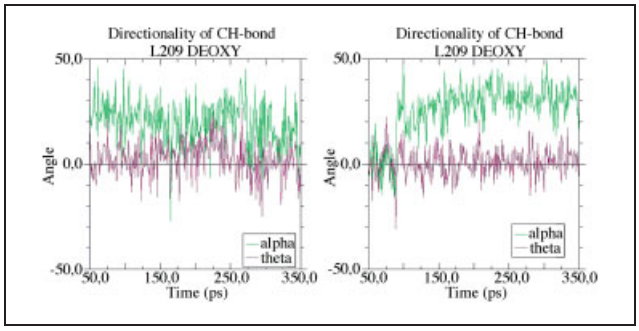


Fig. 11: Direction of the substrate CH-bond (according to Fig. 6). After 50 ps of equilibration a free dynamic of 300 ps was monitored for the L209 mutant of CYP2A5

2.4.3.3. Corticosterone and deoxycorticosterone in the F209L mutant

Experimental data: The F209L mutant shows no activity towards corticosterone whereas deoxycorticosterone was transformed well with 16 nmol/min/nmol (P450).
Carbon iron distance: In both simulations the distance remains below 5 Å for most of the 300 ps (0.46 ± 0.026 Å and 0.45 ± 0.019 Å for corticosterone and deoxycorticosterone, respectively).
Water in the I-helix: The water molecule in the I-helix keeps its position in both mds runs. The observed distance, however, is larger than in the F209N mutant because leucine is not able to form a stabilizing H-bond with the water molecule.
CHO angle: The υ angle in both runs is around 0°. Flexibility of the leucine opens space in the active site so that corticosterone can form a hydrogen bond with Thr212. This contact, however, forces the molecule in a position relative to the activated oxygen which prohibits the hydroxylation of corticosterone (α around 40°). Deoxycorticosterone in contrast shows values of angle α closer to 0° Fig. 11).
Summary: The rather large movement of the leucine side chain opens room for the formation of an H-bond between corticosterone and Thr212. The geometry in this complex does not allow the hydroxylation reaction. Deoxycorticosterone, unable to build this hydrogen bond, remains in closer vicinity and in better position towards the active oxygen species in the active site. The transformation of deoxycorticosterone in the F209L mutant is greater by a factor two than in the F209N mutant. This can be explained by the more favourable behaviour of the υ angle which shows average values of $+5°$ for the F209L and $-10°$ to $-15°$ for the F209N mutant, respectively.

2.5. Conclusions

Currently, cytochrome P450 enzymes are one of the most interesting research areas because of their broad importance in nature in the synthesis of important endogenous structures (e.g. steroid hormones) and the metabolism of all kinds of endogenous and exogenous substrates and as a consequence their pronounced influence on pharmacokinetics of drugs. In this study we offer explanations on the molecular level for substrate selectivity and the reaction mechanism of cytochromes P450 in general.
Based on extensive molecular dynamics simulations we were able to determine criteria which need to be fulfilled for hydroxylation of substrates. These are in addition to the existence of a particular water molecule in the I-helix, the correct distance between the heme iron and the carbon atom, that is going to be hydroxylized, as well as the

directionality of the C−H bond that is going to be replaced by the hydroxyl group. Whereas the importance of the first two criteria already was suggested the latter one has not been described before. Also, mds of cytochrome substrate complexes including oxygen bound to heme never have been performed so far. As already demonstrated by Stahl (2002) for the human isoform of CYP2A5 (CYP2A6) the results of the work can be transferred to other cytochrome isoforms with particular importance for biotransformation in humans thereby allowing in a predictive fashion to study the potential metabolic fate of newly designed drug molecules.

References

Berendsen HJC, Postma JPM, DiNola A, Haak JR (1984) Molecular dynamics with coupling to an external bath. J Chem Phys 81: 3684–3690.

Berendsen HJC, van der Spoel D, van Drunen R (1995) GROMACS: A message-passing parallel molecular dynamics implementation. Comp Phys Comm 91: 43–56.

Bower MJ, Cohen FE, Dunbrack Jr. RL (1997) Prediction of protein side-chain rotamers from a backbone-dependent rotamer library: A new homology modelling tool. J Mol Biol 267: 1268–1282.

Gotoh O (1997) Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analysis of amino acid and coding and nucleotide sequences. J Biol Chem 267: 83–90.

Hasemann C, Kurumbali R, Boddupalli S, Peterson J, Deisenhofer J (1995) Structure and function of cytochrome P450: a comparative analysis of three crystal structures. Structure 3: 41–62.

Hata M, Hirano Y, Hoshino T, Tsuda M (2001) Monooxygenation mechanism by cytochrome P450. J Am Chem Soc 123: 6410–6416.

Helms V, Wade R (1995) Thermodynamics of water mediating protein-ligand interactions in cytochrome P450cam: A molecular dynamics study. Biophys J 69: 810–824.

Hess B, Bekker H, Berendsen HJC, Fraaije JGEM (1997) LINCS: A Linear Constraint Solver for molecular simulations. J Comp Chem 18: 1463–1472

Hofmann K, Bucher P, Falquet L, Bairoch A (1999) The PROSITE database, ist status in 1999. Nucleic Acids Res 27: 215–219.

Iwasaki M, Juvonen RO, Lindberg RLP, Negishi M (1990) Alteration of high and low spin equilibrium by a single mutation of amino acid 209 in mouse cytochromes P450. J Biol Chem 266: 3380–3382.

Iwasaki M, Lindberg RLP, Juvonen RO, Negishi M (1993) Site-directed mutagenesis of mouse steroid 7α-hydroxylase (cytochrome P-450$_{7\alpha}$): role of residue-209 in determining steroid-cytochrome P450 interaction. Biochem J 291: 569–573.

Juvonen RO, Iwasaki M, Negishi M (1991) Structural function of residue-209 in coumarin 7-hydroxylase. J Biol Chem 266: 16431–16435.

Juvonen RO, Gynther J, Pasanen M, Alhava E, Poso A (2000) Pronounced differences in inhibition potency of lactone and non lactone compounds for mouse and human coumarin 7 hydroxylases (CYP2A5 and CYP2A6). Xenobiotica 30: 81–92.

Laskowski RA, MacArthur MW, Moss DS, Thornton JM (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Cryst 26: 283–293.

Miyamoto S, Kollman PA (1992) SETTLE: An analytical version of the SHAKE and RATTLE algorithms for rigid water models. J Comp Chem 13: 952–962.

Molecular Simulations Inc. (2000) INSIGHT II: Homology; Release 2000, March 2000.

Nebert DW, Gonzales FJ (1987) P450 genes: Structure, evolution and regulation. Annu Rev Biochem 56: 945–993.

Nelson DR, Strobel HW (1987) Evolution of cytochrome P-450 proteins. Mol Biol Evol 4: 572–593.

Ponder, JW, Richards, FM (1987) Tertiary Templates in Proteins. Use of Packing Criteria in the Enumeration of Allowed Sequences for Different Structural Classes. J Mol Biol 193: 775–791.

Poso A, Gynther J, Juvonen RO (2001) A comparative molecular field analysis of cytochrome P450 2A5 and 2A6 inhibitors. J Comp-Aid Mol Design 15: 195–202.

Schappach A, Höltje H.-D. (2001) Molecular modelling of 17α-hydroxylase-17,20-lyase. Pharmazie 56: 435–442.

Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet RM, Ringe D, Petsko GA, Sligar SG (2000) The catalytic pathway of cytochrome P450cam at atomic resolution. Science 287: 1615–1622.

Stahl GR (2002) PhD Thesis, Düsseldorf.

Torda AE, Scheek RM, van Gunsteren WF (1989) Time-dependent distance restraints in molecular dynamics simulations. Chem Phys Lett 157: 289–294.

van Gunsteren WF, Billeter SR, Eising AA, Hünenberger PH, Krüger P, Mark AE, Scott WRP, Tironi IG (1996) Biomolecular simulation: The GROMOS96 manual and user guide. Hochschulverlag AG an der ETH Zürich, Switzerland.

Williams P, Cosme J, Sridhar V, Johnson E, McRee D (2000) Mammalian microsomal cytochrome P450 monooxygenase: Structural adaptions for membrane binding and functional diversity. Molecular Cell 5: 121–131.

Yoshizawa K, Kamachi T, Shiota Y (2001) A theoretical study of the dynamic behaviour of alkane hydroxylation by Compound I model of cytochrome P450. J Am Chem Soc 123: 9806–9816.