Structural insights into the mechanism of the drastic changes in enzymatic activity of the cytochrome P450 vitamin D₃ hydroxylase (CYP107BR1) caused by a mutation distant from the active site

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Cytochromes P450 (P450s) are haem-containing enzymes that catalyze medically and industrially important oxidative reactions, and many P450s have been subjected to directed evolution and site-directed mutagenesis to improve their activity and substrate specificity. Nonetheless, in most cases the mechanism that leads to drastic changes in specific activity after the introduction of an amino-acid substitution distant from the active-site pocket is unclear. Here, two crystal structures of inactive mutants of the P450 vitamin D₃ hydroxylase (Vdh), Vdh-F106V and Vdh-L348M, which were obtained in the course of protein-engineering experiments on Vdh, are reported. The overall structures of these mutants show an open conformation similar to that of wild-type Vdh (Vdh-WT), whereas a rearrangement of the common main-chain hydrogen bonds is observed in the CD-loop (residues 102–106), resulting in a more compactly folded CD-loop relative to that of Vdh-WT. The previously reported structures of Vdh-WT and of the highly active Vdh-T107A and Vdh-K1 mutants have a more stretched CD-loop, with partial formation of ₃₁₀-helix-type hydrogen bonds, both in the open and closed states. Molecular-dynamics simulations also showed that the frequency of the ₃₁₀-helix is significantly reduced in Vdh-F106V and Vdh-L348M. The closed conformation is crucial for substrate and ferredoxin binding to initiate the catalytic reaction of Vdh. Therefore, it is implied that the small local structural changes observed in this study might disrupt the conformational transition from the open to the closed state, thereby leading to a complete loss of vitamin D₃ hydroxylase activity.

1. Introduction

Cytochromes P450 (P450s) are a large family of haem-containing proteins that are ubiquitously present in organisms from bacteria to humans (Coon *et al.*, 1992). They typically catalyze the regioselective and stereoselective hydroxylation (monooxygenation) of an inert C–H bond of a substrate compound using molecular oxygen and two electrons sequentially transferred from redox-partner proteins. Even though P450s form a well conserved three-dimensional structure, *i.e.* the P450 fold, they show a wide variety of substrate specificity and are involved in diverse biological functions, such as the biosynthesis of various secondary metabolites, steroid and lipid metabolism, and xenobiotic degradation (Coon *et al.*, 1992; Lamb *et al.*, 2007; Podust & Sherman, 2012). To date, many crystal structures of P450s have been reported and have helped to understand the mechanism of substrate binding, electron transfer and formation of a high-energy intermediate (Denisov *et al.*, 2005). In addition, P450s are of special interest as a biocatalyst for the production of

valuable chemicals such as pharmaceuticals because regioselective and stereoselective C—H bond oxidation is a challenging step in synthetic chemistry. Functional P450 expression libraries have been constructed to find new P450 species that show useful enzymatic activities (Girhard *et al.*, 2009; Nazir *et al.*, 2011). Directed-evolution and structure-guided mutagenesis studies have also been applied to increase the catalytic activity, change the substrate specificity and improve the organic cosolvent tolerance of potentially useful P450 enzymes (Girvan & Munro, 2016; Kuper *et al.*, 2012).

Vitamin D₃ (VD₃) hydroxylase (Vdh) from the actinomycete *Pseudonocardia autotrophica* is a cytochrome P450 (CYP107BR1) that catalyzes the regioselective and stereoselective two-step hydroxylation of VD₃ to produce 25-hydroxyvitamin D₃ [25(OH)VD₃] and 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂VD₃] (Fujii *et al.*, 2009). In the human body, 25(OH)VD₃ and 1 α ,25(OH)₂VD₃ play a critical role as hormones that regulate calcium homeostasis and control the proliferation and differentiation of various cell types. Hydroxylated VD₃ and its derivatives are thus used as pharmaceutical agents for the treatment of symptoms caused by VD₃ deficiency and/or VD₃ metabolic disorders, such as osteoporosis, psoriasis, hypoparathyroidism and rickets (Jones *et al.*, 1998). Microbial conversion using *P. autotrophica* cells is currently in practical use for the production of 25(OH)VD₃ and 1 α ,25(OH)₂VD₃ (Sasaki *et al.*, 1992; Takeda *et al.*, 1994). To enhance the biocatalytic production of 25(OH)VD₃, we have developed a recombinant system based on *Rhodococcus erythropolis* (Nakashima & Tamura, 2004), overexpressing P450 Vdh and the redox-partner proteins ferredoxin (Fdx) and Fdx reductase (Fujii *et al.*, 2009). Furthermore, protein-engineering studies have also been conducted to increase the specific activity of Vdh. We have previously employed directed evolution of this enzyme for the first time, and a highly active quadruple mutant, Vdh-K1, was obtained. Vdh-K1 contains four amino-acid substitutions, T70R, V156L, E216M and E384R, all of which are distant from the active site (Fujii *et al.*, 2009). We have also performed site-directed mutagenesis at the Fdx-binding site to improve the electron-transfer efficiency; as a result, a single substitution, T107A (Vdh-T107A), that confers higher VD₃-hydroxylating activity, was identified (Yasutake *et al.*, 2013).

Enzymatic assays revealed that both Vdh-K1 and Vdh-T107A have the same kinetic properties, *i.e.* both mutants have approximately 25-fold higher k_{cat} values and approximately fourfold lower K_{m} values towards Fdx than does wild-type Vdh (Vdh-WT), thus resulting in an approximately 100-fold better catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$). Crystallographic studies have also revealed that Vdh-WT has an open conformation that extensively exposes the hydrophobic substrate-binding pocket to the solvent, whereas both Vdh-K1 and Vdh-T107A form a closed conformation accommodating VD₃ or 25(OH)VD₃ in the pocket (Yasutake *et al.*, 2010, 2013). Recent crystallographic, NMR and computational studies indicate that P450s exist in a conformational equilibrium between typically open and closed states, in contrast to an induced-fit mechanism (Sherman *et al.*, 2006; Savino *et al.*,

2009; Lee *et al.*, 2010; Yasutake *et al.*, 2010; Hollingsworth *et al.*, 2016). In the case of Vdh, both the four substitutions in Vdh-K1 and the single T107A substitution are likely to be responsible for the shift of the conformational equilibrium towards the closed state, thereby enabling better Fdx-binding and substrate-binding affinity and catalytic activity. In contrast, we have also obtained two notable mutants, Vdh-F106V and Vdh-L348M, that completely disable the VD₃ hydroxylase activity (Yasutake *et al.*, 2013). Phe106 is a neighbouring residue of Thr107, located in the connecting loop between α -helix C (α C) and α -helix D (α D) (the CD-loop). Leu348 also lies in the vicinity of the CD-loop, and its side chain forms hydrophobic interactions with the side chain of Phe106. In this study, to provide insights into the structural mechanism of the drastic changes in Vdh activity on the introduction of a seemingly trivial F106V or L348M substitution distant from the active-site pocket, we undertook crystallographic analyses of Vdh-F106V and Vdh-L348M. Together with previously reported structures of Vdh-WT and the highly active Vdh-T107A, a possible mechanism that affects the conformational dynamics of Vdh is discussed.

2. Materials and methods

2.1. Protein expression and purification

Plasmids expressing the Vdh mutants Vdh-F106V and Vdh-L348M were prepared by the inverse PCR method as described previously (Yasutake *et al.*, 2013). The recombinant proteins were overexpressed in *Escherichia coli* BL21-CodonPlus(DE3)-RIL cells and were purified using the same procedure as used for Vdh-WT (Yasutake *et al.*, 2010). The purified sample was dialyzed against a buffer consisting of 20 mM Tris–HCl pH 7.5, 50 mM NaCl, 1.0 mM dithiothreitol (DTT), 20% glycerol and was stored at -22°C until use in crystallization experiments. A carbon monoxide difference spectral assay was carried out to verify the concentration of Vdh (Omura & Sato, 1964).

2.2. Crystallization

The samples were thawed on ice, dialyzed against a buffer consisting of 20 mM Tris–HCl pH 7.5, 20 mM NaCl and concentrated to 20 mg ml⁻¹ using a centrifugal filtration device (molecular-weight cutoff 30 kDa; Millipore).

All crystallization experiments were conducted using the vapour-diffusion method at 20°C. An initial search for crystallization conditions was performed using the sparse-matrix screening kits Crystal Screen, Crystal Screen 2, Index, PEG/Ion, PEG/Ion 2, PEGRx (Hampton Research) and Wizard Classic 1 and 2 (Rigaku Reagents). No crystals were obtained using these commercial screening kits. We also attempted to crystallize the samples using the conditions previously identified for Vdh-WT, Vdh-K1 and Vdh-T107A with varying buffer pH levels, concentrations of salts and molecular weights of polyethylene glycol (PEG). Drops were comprised of 1.0 μ l sample solution and an equal volume of reservoir solution and were equilibrated against 500 μ l reservoir solution in a 24-well

Table 1

X-ray diffraction data-collection and refinement statistics.

Values in parentheses are for the outermost resolution shell.

	Vdh-F106V	Vdh-L348M
PDB code	5gnl	5gnm
Data collection		
Beamline	BL-17A, PF	BL-17A, PF
Wavelength (Å)	0.98000	0.98000
Temperature (K)	100	100
Space group	$P3_1$	$P2_1$
Unit-cell parameters (Å, °)	$a = b = 61.7$, $c = 98.2$	$a = 81.2$, $b = 107.7$, $c = 88.4$, $\beta = 90.2$
Resolution (Å)	50–1.95 (1.98–1.95)	50–2.70 (2.85–2.70)
Unique reflections	30377	41314
$R_{\text{merge}}^\dagger$	0.075 (0.949)	0.142 (0.482)
Mean $I/\sigma(I)$	46.0 (3.7)	8.4 (3.0)
Completeness (%)	99.6 (99.5)	98.8 (99.0)
Multiplicity	8.6 (8.5)	3.1 (3.0)
Overall B factor from Wilson plot (Å ²)	48.5	23.6
Refinement		
$R_{\text{work}}/R_{\text{free}}^{\ddagger}$	0.195/0.233	0.223/0.275
Twinning operator	—	$-h, -k, l$
Twinning fraction	—	0.359
No. of atoms		
Total	3227	12690
Water	117	132
Average B factor (Å ²)		
Overall	50.5	28.5
Protein (chain A/B/C/D)	51.2	29.5/26.0/27.5/32.3
Haem (chain A/B/C/D)	29.0	13.6/14.5/16.8/14.4
R.m.s.d. from ideal		
Bond lengths (Å)	0.012	0.008
Bond angles (°)	1.55	1.33
Ramachandran plot		
Favoured (%)	90.5	90.9
Allowed (%)	9.5	9.1
Outliers (%)	0.0	0.0

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean intensity of a set of equivalent reflections. [‡] $R_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$ for 95% of the reflection data used in the refinement. F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes, respectively. R_{free} is the equivalent of R_{work} , except that it was calculated for a randomly chosen 5% test set of reflections that were excluded from refinement. The same test-set reflections as for the original Vdh-WT structure were used for R_{free} calculation of Vdh-F106V.

hanging-drop plate. All of the crystals used in this work were crystallized under conditions that were somewhat similar to those used for Vdh-WT [0.1 M bis-tris pH 7.5, 50 mM CaCl₂, 60 mM NaCl, 38%(v/v) PEG 400; Yasutake *et al.*, 2009]. Rod-shaped crystals of Vdh-F106V appeared within 3 d and grew to full size (20 × 20 × 500 µm) using a reservoir solution consisting of 0.1 M bis-tris pH 7.5, 50 mM CaCl₂, 25–75 mM NaCl, 38–42%(v/v) PEG 400. Small plate-shaped crystals (100 × 100 × 10 µm) of Vdh-L348M were also obtained using a reservoir solution consisting of 0.1 M bis-tris pH 7.5, 50 mM CaCl₂, 100 mM NaCl, 22–24%(w/v) PEG 2000 monomethyl ether (MME).

2.3. Crystal structure determination and model refinement

For X-ray diffraction experiments, the crystals were flash-cooled in a liquid-nitrogen vapour stream at 100 K. Prior to flash-cooling, the crystals of Vdh-L348M were transferred stepwise into a cryoprotectant solution consisting of 0.1 M bis-tris pH 7.5, 50 mM CaCl₂, 100 mM NaCl, 25%(w/v) PEG 2000

MME with an increasing concentration of glycerol to 20%(v/v). All X-ray diffraction data were collected on beamline BL-17A of the Photon Factory (PF), Tsukuba, Japan at a radiation wavelength of 0.9800 Å, and the raw image data were processed using the *HKL-2000* software package (Otwinowski & Minor, 1997). The X-ray diffraction data set for Vdh-F106V was collected to a resolution of 1.95 Å using an ADSC Quantum 270 CCD detector. The crystals of Vdh-F106V belonged to the trigonal space group $P3_1$, with unit-cell parameters $a = b = 62$, $c = 98$ Å; the space group and unit-cell parameters are the same as those for Vdh-WT. Model refinement was performed using *REFMAC5* (Murshudov *et al.*, 2011) in the *CCP4* suite (Winn *et al.*, 2011), with the atomic coordinates of Vdh-WT (PDB entry 3a4g; Yasutake *et al.*, 2010) as a starting model. Manual model fitting was carried out in *Coot* (Emsley *et al.*, 2010). The X-ray diffraction data set for Vdh-L348M was acquired at 2.7 Å resolution using a Dectris PILATUS3 6M area detector. The data were initially processed in an orthorhombic system with unit-cell parameters $a = 81$, $b = 88$, $c = 108$ Å. Molecular replacement was conducted using *Phaser* (McCoy *et al.*, 2007) with the atomic coordinates of Vdh-WT (PDB entry 3a4g) as a search model. High correlation peaks were obtained for both the rotation and translation search, and two Vdh monomers could be successfully placed in the asymmetric unit of the $P2_22_1$ lattice. Model refinement was performed in *REFMAC5* (Murshudov *et al.*, 2011). However, the R_{work} and R_{free} factors were stuck at ~40%. An *L*-test analysis (Padilla & Yeates, 2003) suggested that the crystal of Vdh-L348M might be twinned (Supplementary Fig. S1). We thus reduced the symmetry to a monoclinic space group, and correct molecular-replacement solutions were obtained in *Phaser* (McCoy *et al.*, 2007) using Vdh-WT as a search model. Model refinement was performed in *REFMAC5*, incorporating the twinning operator ($-h, -k, l$) and a twinning fraction of 0.36. Noncrystallographic symmetry restraints were not applied to the model refinement. Manual model fitting was conducted using *Coot* (Emsley *et al.*, 2010). For both data sets, TLS parameters were computed and used in the final stage of model refinement (Painter & Merritt, 2006). The stereochemical quality of the final refined models was assessed using *PROCHECK* (Laskowski *et al.*, 1993). The data-collection and refinement statistics are summarized in Table 1. Molecular drawings were prepared in *PyMOL* (<http://www.pymol.org>). Atomic coordinates and structure-factor amplitudes of Vdh-F106V and Vdh-L348M have been deposited in the Protein Data Bank with accession codes 5gnl and 5gnm, respectively.

2.4. Molecular-dynamics (MD) simulations

MD simulations of 100 ns were performed three times for each system using the *GROMACS* 4.6.4 simulator (Berendsen *et al.*, 1995). The system contained a cytochrome P450 (Vdh-WT, PDB entry 3a4g; Vdh-T107A, PDB entry 3a4g with a T107A substitution; Vdh-F106V, PDB entry 5gnl; Vdh-L348M, PDB entry 5gnm), 26 sodium ions and ~25 000 water molecules. AMBER99SB (Hornak *et al.*, 2006) was used for

the protein and the TIP3P model was used for waters (Jorgensen *et al.*, 1983). Potential parameters for haem and

chloride ion have been described in the literature (Shahrokh *et al.*, 2012; Joung & Cheatham, 2008). MD simulations were

Figure 1

Overall structures of the Vdh-F106V and Vdh-L348M mutants. (a, b) Ribbon diagrams of Vdh-F106V (a) and Vdh-L348M (b). The right-hand panels show the view from the proximal side, while the left panels show a view from the distal side of the haem cofactor. The two views are approximately 130° apart. The haem cofactor, the side chains of the mutated residue and Cys347 forming a coordinate bond to the haem iron are shown in stick representation. The α -helices are labelled as per the established conventions for the P450 fold (αA – αK , $\alpha K'$ and αL ; Poulos *et al.*, 1985). (c) Main-chain superimposition of Vdh-F106V (green), Vdh-L348M (cyan) and Vdh-WT (yellow). The CD-loop moiety, where the main-chain conformational change is observed, is indicated by a red box.

Table 2

Main-chain r.m.s.d. values between Vdh structures reported to date (Å).

Parentheses refer to maximum displacement values. All superimposition analyses were performed using residues 9–401.

	Vdh-F106V	Vdh-L348M				Vdh-WT	Vdh-K1					Vdh-T107A
Conformation	Open	Open				Open	Closed					Closed
Resolution (Å)	1.95	2.7				1.75	2.0					2.57
Space group	$P3_1$	$P2_1$				$P3_1$	$P2_12_12_1$					$C222_1$
PDB code	5gnl	5gnm				3a4g	3a5l					4vrn
Chain ID	A	A	B	C	D	A	A	B	C	D	E	A
Vdh-F106V												
A	0.00 (0.00)	0.90 (4.72)	0.84 (4.65)	0.82 (4.29)	0.72 (4.16)	0.41 (2.83)	2.29 (12.1)	2.43 (13.2)	2.45 (12.8)	2.42 (12.7)	2.53 (12.9)	2.53 (13.4)
Vdh-L348M												
A		0.00 (0.00)	0.45 (3.60)	0.62 (4.01)	0.77 (4.73)	0.99 (5.84)	2.17 (12.6)	2.23 (13.7)	2.30 (13.4)	2.23 (13.3)	2.33 (13.5)	2.33 (13.9)
B			0.00 (0.00)	0.67 (4.19)	0.77 (4.88)	0.92 (4.35)	2.08 (12.8)	2.22 (13.4)	2.21 (13.0)	2.15 (12.9)	2.24 (13.1)	2.23 (13.6)
C				0.00 (0.00)	0.52 (3.08)	0.92 (4.87)	2.27 (12.7)	2.41 (13.1)	2.40 (13.5)	2.33 (13.4)	2.43 (13.6)	2.43 (14.0)
D					0.00 (0.00)	0.84 (4.23)	2.25 (12.3)	2.39 (13.9)	2.39 (13.0)	2.35 (12.9)	2.45 (13.1)	2.45 (13.6)
Vdh-WT												
A						0.00 (0.00)	2.29 (12.1)	2.44 (13.2)	2.47 (12.8)	2.42 (12.7)	2.53 (12.9)	2.53 (13.4)
Vdh-K1												
A							0.00 (0.00)	0.47 (3.22)	0.49 (3.99)	0.57 (3.88)	0.55 (3.81)	0.69 (4.01)
B								0.00 (0.00)	0.37 (3.02)	0.54 (5.15)	0.47 (4.46)	0.61 (4.35)
C									0.00 (0.00)	0.45 (3.43)	0.36 (2.31)	0.56 (3.56)
D										0.00 (0.00)	0.35 (3.14)	0.51 (3.14)
E											0.00 (0.00)	0.52 (3.47)
Vdh-T107A												
A												0.00 (0.00)

conducted with the NPT ensemble (300 K, 100 kPa) in a truncated dodecahedral box with dimensions of 104.3 Å. Temperature was controlled using a Langevin thermostat with a viscosity of 0.5 ps⁻¹. Pressure was controlled by a Parrinello–Rahman barostat with a relaxation time of 2.0 ps (Parrinello & Rahman, 1981). The electrostatics were analyzed by the particle mesh Ewald (PME) method with a 10.0 Å cutoff distance (Darden *et al.*, 1993). The van der Waals interactions were expressed using the cutoff method with 10.0 Å cutoff distances. Covalent bonds for H atoms in the polypeptide were constrained using the linear constraint solver (*LINCS*; Hess *et al.*, 1997). Covalent bonds in the waters were constrained by the *SETTLE* algorithm (Miyamoto & Kollman, 1992). The integration time step was 2 fs.

3. Results and discussion

3.1. Determination of the structures of inactive mutants

Vdh-F106V was crystallized using a reservoir solution similar to that used for Vdh-WT, and the crystal was identical to the trigonal crystal of Vdh-WT (Yasutake *et al.*, 2010). The atomic model of Vdh-F106V was refined to a resolution of 1.95 Å, with R_{work} and R_{free} factors of 20.0 and 23.4%, respectively. There is one monomer of Vdh-F106V in the asymmetric unit, with a solvent content of 48.1% and a V_M value of 2.37 Å³ Da⁻¹. The clear electron density enabled us to build a continuous model (residues 9–403) except for eight disordered N-terminal residues and the eight C-terminal residues, including the His tag. The crystal of Vdh-L348M was also obtained using a similar reservoir solution but with PEG 2000 MME as a precipitant instead of PEG 400. Initially, we analyzed the data as an orthorhombic system in space group $P22_12_1$, but model refinement failed, with the R_{work} and R_{free}

factors stuck at ~40%. On the basis of the results of *L*-test analysis, which showed that the data might be twinned, we reduced the symmetry from $P22_12_1$ to the monoclinic $P2$ or $P2_1$, with reindexing as (h, k, l) , (k, l, h) and (l, h, k) . Finally, we identified the true space group and lattice orientation, as judged by the lowest R_{free} factor value at the model-refinement stage. The atomic model of Vdh-L348M was refined to a resolution of 2.7 Å, with R_{work} and R_{free} factors of 22.3 and 27.5%, respectively. There are four Vdh-L348M monomers in the asymmetric unit, chains *A* (residues 3–401), *B* (residues 3–401), *C* (residues 3–403) and *D* (residues 3–403), with a solvent content of 42.0% and a V_M of 2.12 Å³ Da⁻¹.

3.2. Overall structure description

The structures of Vdh show a typical P450 fold, primarily composed of 13 α -helices (αA – αK , $\alpha K'$ and αL) and eight β -strands (Fig. 1). The haem cofactor is embedded into the core of the molecule, sandwiched by two α -helices: αI and αL . The haem iron is coordinated by the side-chain thiol of Cys347 as a fifth (proximal) ligand. The electron density shows a water molecule bound to the haem iron as a sixth coordinated ligand in the structure of Vdh-F106V, whereas the corresponding water molecule is not visible in the structure of Vdh-L348M, probably because of the relatively low resolution limit of Vdh-L348M (2.7 Å). The UV–visible absorption spectra of both Vdh-F106V and Vdh-L348M show a Soret peak at 420 nm as in Vdh-WT, indicating that the haem iron of both inactive mutants is present in the water-bound low-spin state, which is typical for the substrate-free, resting state of bacterial P450s (data not shown).

A pairwise comparison of the main-chain structures of Vdh-F106V, Vdh-L348M and the previously reported Vdh-WT, Vdh-K1 and Vdh-T107A is summarized in Table 2. Evidently,

both Vdh-F106V and Vdh-L348M have an open conformation similar to that of Vdh-WT, with a main-chain root-mean-square deviation (r.m.s.d.) of ~ 1.0 Å (Table 2 and Fig. 1). Maximal displacements of 2.8–4.7 Å are observed, all of which correspond to the main-chain atoms of Asp174. The FG-loop including Asp174 is known to typically be a mobile moiety of the P450 fold and is also found to be flexible within the same open/closed conformational state of the Vdh structures (Table 2 and Fig. 1).

3.3. The F106V and L348M mutations cause the same local structural change

Although the overall structures of the Vdh-F106V and Vdh-L348M mutants superimpose very well on that of Vdh-WT, we found that there is a small but notable local structural change which could be caused by the F106V or L348M mutation. In the structure of Vdh-WT residues 104–106 correspond to a connecting loop between α C and α D (the CD-loop) which is partly formed of a 3_{10} -helix structure: the main-chain N atoms of Phe106 and Ser105 form a hydrogen bond to the three-residue upstream main-chain O atoms of Gly103 and Val102, respectively. In contrast, the main-chain N atom of Phe106 in Vdh-L348M forms a hydrogen bond to the main-chain O atom of Val102 in a normal α -helix formation. This hydrogen-bond rearrangement causes the CD-loop to be folded more compactly than in Vdh-WT (Table 3, Fig. 2 and Supplementary Fig. S2). It is likely that the introduction of a bulky thioether group at Met348 causes a relocation of the side chain of Phe106 to keep appropriate interatomic distances between them, and consequently the main chain of residues 101–113 deviates progressively by up to 2.5 Å, thereby creating a hydrogen bond between the Phe106 N atom and the Val102 O

Table 3

Interatomic distances between main-chain N, O and C $^{\alpha}$ atoms in the CD-loop (Å).

Bold values represent hydrogen-bonding interactions (~ 3.2 Å).

	Phe/Val106 N– Val102 O (distance <i>X</i>)	Phe/Val106 N– Gly103 O (distance <i>Y</i>)	Ser105 N– Leu101 O	Ser105 N– Val102 O	Phe/Val106 C $^{\alpha}$ – Val102 C $^{\alpha}$
Vdh-F106V	A 3.07	4.15	4.14	2.93	6.05
Vdh-L348M	A 2.64	4.17	4.26	2.87	5.75
<i>B</i>	2.69	4.69	4.21	3.06	5.74
<i>C</i>	2.73	4.62	3.90	3.33	5.83
<i>D</i>	2.72	4.19	4.28	3.03	5.82
Vdh-WT	<i>A</i> 3.74	2.89	4.55	3.01	7.18
Vdh-K1	<i>A</i> 3.64	3.15	4.45	2.95	7.05
<i>B</i>	3.54	3.25	5.11	2.87	6.81
<i>C</i>	3.74	3.09	5.60	3.07	7.11
<i>D</i>	3.89	3.09	5.63	3.13	7.19
<i>E</i>	3.67	3.08	5.40	3.20	6.99
Vdh-T107A	<i>A</i> 3.56	3.00	5.44	3.05	7.03

atom. This main-chain deviation and hydrogen-bond rearrangement is observed in all four monomers of Vdh-L348M in the asymmetric unit, suggesting that it is independent of the intermolecular crystal contacts. An identical local structural change is observed in the structure of Vdh-F106V: the main-chain deviation of residues 101–113 and the hydrogen-bonding rearrangement at Val106 from a 3_{10} -helix to a normal α -helix also occur (Table 3, Fig. 2 and Supplementary Fig. S2). The F106V mutation replaces the bulky aromatic side chain with a smaller isopropyl group. It is therefore likely that the main-chain deviation and the hydrogen-bond rearrangement take place *via* the weakened hydrophobic interactions between the

Figure 2

A comparison of the hydrogen-bonding patterns at the main-chain N atoms of Phe/Val106 among Vdh-F106V, Vdh-L348M, Vdh-WT and Vdh-T107A. Residues 102–106 (CD-loop) and residue 348 are shown as stick models. Hydrogen bonds are indicated by dashed lines in grey. The interatomic distances for all chains are summarized in Table 3. The $mF_o - DF_c$ OMIT maps for residues 102–106 are shown in Supplementary Fig. S2.

side chains of Val106 and Leu348. It should be noted that both the Vdh-WT and the Vdh-F106V structures were determined using an identical crystal form in the trigonal space group $P3_1$, and in the crystal lattice the residues of α C, α D and the connecting CD-loop (the CD helices) make elaborate intermolecular crystal contacts with residues 69–72 and 301–307 of the neighbouring molecule. Of note, the main chain of residues 69–72 and 301–307 of Vdh-F106V bends slightly and compensates for the deviations of residues 101–113, resulting in complete retention of the intermolecular crystal contacts observed in the structure of Vdh-WT.

To confirm and quantify this hydrogen-bond rearrangement at Phe/Val106, MD simulations were carried out on the open conformations of Vdh-WT, Vdh-T107A, Vdh-F106V and Vdh-L348M. Crystal structures are available for Vdh-WT, Vdh-F106V and Vdh-L348M, whereas the open state of Vdh-T107A was created from Vdh-WT (PDB entry 3a4g) by introducing the T107A mutation. Fig. 3(a) shows the frequency of a 3_{10} -helix/ α -helix at the main-chain N atom of Phe/Val106 during MD simulations. The results clearly indicated that although the normal α -helix is predominant in all structures, the frequency of 3_{10} -helix formation is reduced for the inactive mutants Vdh-F106V and Vdh-L348M. In

particular, the 3_{10} -helix appeared with a frequency of only 3% in Vdh-F106V. The lower frequencies of the 3_{10} -helix for Vdh-F106V and Vdh-L348M are consistent with the crystallographic data described above. It should also be noted that the T107A mutation promotes the destruction of both hydrogen bonds and significantly reduces the frequency of α -helix formation. A hydrogen bond between the side-chain OH of Thr107 and the main-chain N atom of Arg110 was observed in all of the open states of Vdh structures. Vdh-T107A lacks this hydrogen bond and therefore the T107A mutation may increase the flexibility of Ala107 or Arg110 and their neighbouring residues, and thus trigger a conformational transition from the open to the closed state.

3.4. A possible activation or inactivation mechanism behind the open and closed conformational dynamics

The closed conformation of Vdh observed in the crystal structures of Vdh-T107A and Vdh-K1 is essential for initiation of the catalytic cycle involving substrate and Fdx binding (Yasutake *et al.*, 2013). The most apparent structural feature of the closed state is α F and α G (the FG helices), which move by up to 8 Å, thus covering the substrate-binding pocket; the

Figure 3

Results of MD simulations. (a) The frequencies of formation of two hydrogen bonds at the main-chain N atom of Phe/Val106 during MD simulations. The distances X and Y correspond to those defined in Table 3. The threshold value defining a hydrogen bond is set to <3.2 Å. The frequencies are average values calculated from three independent MD simulation runs. The last 50 ns of the trajectories were used in the analyses. (b) R.m.s.f. of main-chain C^α atoms during MD simulations. The average r.m.s.f. values were calculated from the three independent MD simulation runs. The last 50 ns of the trajectories were used in the analyses. Mutation points are labelled, and the residue ranges representing the reduction in fluctuation of Vdh-F106V and Vdh-L348M and the increase in fluctuation of Vdh-T107A are indicated by red and blue arrows, respectively. The secondary-structure elements are also shown.

movement of the FG helices is accompanied by the breakage of a couple of hydrogen bonds between secondary-structure units. The closed form of Vdh maintains the 3_{10} -helix at Phe106 as in the open form of Vdh-WT. We theorized that the newly created hydrogen bond between the Phe/Val106 N atom and the Gly102 O atom observed in the present study might hinder the stretching of the CD-loop to form the 3_{10} -helix and could disallow the gliding of FG helices, possibly resulting in complete inactivation of Vdh. We reasoned that MD simulations could illuminate the differences in dynamic properties between the highly active Vdh-T107A, the active Vdh-WT and the completely inactive Vdh-V106F and Vdh-L348M. Fig. 3(b) depicts the root-mean-square fluctuations (r.m.s.f.s) of C α atoms for all of the variants. Slight but significant reductions in r.m.s.f. are observed in both inactive mutants within some restricted regions of the DEFG helices. These four helices are layered and associated with each other, and the conformational change between the open and closed state involves gliding of the FG helices (Fig. 4). Within these regions, two specific inter-side-chain salt bridges and hydrogen bonds

(Arg116–Asp152 and Ser175–Asp179) are observed in the open state, both of which are severed in the closed state (Fig. 4). Although the relation between the hydrogen-bond rearrangement at Phe/Val106 and the reduction of fluctuation in the DEFG helices is still unclear, it seems likely that these common structural and dynamic properties of Vdh-F106V and Vdh-L348M are related to the inertness in the conformational transition from the open to the closed state. In contrast, an increase in fluctuation in the C-terminal part of the α C helix and HI-loop is observed only for Vdh-T107A. These regions also include a couple of inter-side-chain hydrogen bonds and salt bridges in the open state, *e.g.* Ser105–Ser209 and Lys100–Asp215, both of which are also absent in the closed conformation (Fig. 4). These observations suggest that the increase of mobility in the CHI helices induced by the T107A mutation may contribute to activation of the conformational dynamics of Vdh. As shown in Fig. 4, the specific inter-side-chain interactions are required to be broken for the conformational transition from open to the closed state. However, no significant changes in the frequencies of those interactions were

Figure 4

Structural comparison between the open and closed states of Vdh. A stereoview ribbon diagram of the superimposition of Vdh-F106V in the open state (green) and Vdh-T107A in the closed state (grey) is presented in two perpendicular views. The residues corresponding to the range indicated by the red and blue arrows in Fig. 3(b) are also coloured in red and blue, respectively, in the Vdh-F106V model. The residues forming specific inter-side-chain hydrogen bonds or salt bridges in the open state (Ser105, Lys100, Asp152, Arg161, Ser175, Asp179, Ser209 and Asp215) are shown as stick models and are labelled. Primes indicate residues in the closed state (Vdh-T107A). Arrows in purple represent the motion of the FG helices and the HI-loop between the open and closed state. The hydrogen bonds and salt bridges are shown as grey dashed lines. The secondary-structure elements (CDEFGHI helices) are also labelled.

observed in the MD simulations. The results indicate that the increase in fluctuation in the CHI helices is simply a dynamic property caused by the T107A mutation within the open state, while such dynamic behaviour might be a prerequisite to break the interactions and leave the open state. Although it is challenging to simulate the complete conformational transition of the 45 kDa haemoprotein, longer time-scale MD simulations are certainly required in future studies in order to more clearly understand the mechanism required to leave the open state for activation.

Directed-evolution experiments on P450s using random mutagenesis often yield a mutation that is distant from the active-site pocket that significantly improves the specific activity (Kumar *et al.*, 2005; Kabumoto *et al.*, 2009; Fujii *et al.*, 2009; Pengpai *et al.*, 2013; Sideri *et al.*, 2013). These remote mutations may somehow affect the conformational dynamics of P450s, and furthermore may indirectly contribute to the enhancement of substrate binding and/or electron transfer. It is interesting that drug-resistant mutations of *Candida albicans* sterol 14 α -demethylating P450 (CYP51; Lepesheva & Waterman, 2007) are located in a loop on the molecular surface distant from the active site (Podust *et al.*, 2001). We presumed that the mutations also affect the conformational equilibrium of CYP51, thereby helping to escape drug binding, just as in the case of the inactive Vdh mutants described in this study. Many P450s are known to have broad substrate specificity for the degradation of xenobiotics, and this phenomenon involves an open–closed conformational transition (Poulos, 2003). Accordingly, it seems that manipulation of the conformational dynamics by remote mutations is a possible mechanism that rapidly adapts P450s to ever-emerging new compounds in nature.

Finally, this study provides a glimpse into the mechanism of a drastic activity change relating to P450 conformational dynamics. Exhaustive experimental and/or *in silico* mutations together with crystallographic and MD simulation studies may facilitate the discovery and prediction of intrinsic hot-spot residues that control the conformational equilibrium of P450s.

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References

- Berendsen, H. J. C., van der Spoel, D. & van Drunen, R. (1995). *Comput. Phys. Commun.* **91**, 43–56.
 Coon, M. J., Ding, X. X., Pernecky, S. J. & Vaz, A. D. (1992). *FASEB J.* **6**, 669–673.
 Darden, T., York, D. & Pedersen, L. (1993). *J. Chem. Phys.* **98**, 10089–10092.
 Denisov, I. G., Makris, T. M., Sligar, S. G. & Schlichting, I. (2005). *Chem. Rev.* **105**, 2253–2277.

- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
 Fujii, Y., Kabumoto, H., Nishimura, K., Fujii, T., Yanai, S., Takeda, K., Tamura, N., Arisawa, A. & Tamura, T. (2009). *Biochem. Biophys. Res. Commun.* **385**, 170–175.
 Girhard, M., Machida, K., Itoh, M., Schmid, R. D., Arisawa, A. & Urlacher, V. B. (2009). *Microb. Cell Fact.* **8**, 36.
 Girvan, H. M. & Munro, A. W. (2016). *Curr. Opin. Chem. Biol.* **31**, 136–145.
 Hess, B., Bekker, H., Berendsen, H. J. C. & Fraaije, J. G. E. M. (1997). *J. Comput. Chem.* **18**, 1463–1472.
 Hollingsworth, S. A., Batabyal, D., Nguyen, B. D. & Poulos, T. M. (2016). *Proc. Natl Acad. Sci. USA*, **113**, 8723–8728.
 Hornak, V., Abel, R., Okur, A., Strockbine, B., Roitberg, A. & Simmerling, C. (2006). *Proteins*, **65**, 712–725.
 Jones, G., Strugnell, S. A. & DeLuca, H. F. (1998). *Physiol. Rev.* **78**, 1193–1231.
 Jorgensen, W. L., Chandrasekhar, J., Madura, J., Impey, R. W. & Klein, M. L. (1983). *J. Chem. Phys.* **79**, 926–935.
 Joung, S. & Cheatham, T. E. III (2008). *J. Phys. Chem. B*, **112**, 9020–9041.
 Kabumoto, H., Miyazaki, K. & Arisawa, A. (2009). *Biosci. Biotechnol. Biochem.* **73**, 1922–1927.
 Kumar, S., Chen, C. S., Waxman, D. J. & Halpert, J. R. (2005). *J. Biol. Chem.* **280**, 19569–19575.
 Kuper, J., Tee, K. L., Wilmanns, M., Roccatano, D., Schwaneberg, U. & Wong, T. S. (2012). *Acta Cryst.* **F68**, 1013–1017.
 Lamb, D. C., Waterman, M. R., Kelly, S. L. & Guengerich, F. P. (2007). *Curr. Opin. Biotechnol.* **18**, 504–512.
 Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). *J. Appl. Cryst.* **26**, 283–291.
 Lee, Y.-T., Wilson, R. F., Rupniewski, I. & Goodin, D. B. (2010). *Biochemistry*, **49**, 3412–3419.
 Lepesheva, G. I. & Waterman, M. R. (2007). *Biochim. Biophys. Acta*, **1770**, 467–477.
 McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
 Miyamoto, S. & Kollman, P. A. (1992). *J. Comput. Chem.* **13**, 952–962.
 Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
 Nakashima, N. & Tamura, T. (2004). *Appl. Environ. Microbiol.* **70**, 5557–5568.
 Nazir, K. H. M., Ichinose, H. & Wariishi, H. (2011). *Appl. Environ. Microbiol.* **77**, 3147–3150.
 Omura, T. & Sato, R. (1964). *J. Biol. Chem.* **239**, 2370–2378.
 Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
 Padilla, J. E. & Yeates, T. O. (2003). *Acta Cryst.* **D59**, 1124–1130.
 Painter, J. & Merritt, E. A. (2006). *Acta Cryst.* **D62**, 439–450.
 Parrinello, M. & Rahman, A. (1981). *J. Appl. Phys.* **52**, 7182–7190.
 Pengpai, Z., Sheng, H., Lehe, M., Yinlin, L., Zhihua, J. & Guixiang, H. (2013). *Appl. Biochem. Biotechnol.* **171**, 93–103.
 Podust, L. M., Poulos, T. L. & Waterman, M. R. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 3068–3073.
 Podust, L. M. & Sherman, D. H. (2012). *Nat. Prod. Rep.* **29**, 1251–1266.
 Poulos, T. L. (2003). *Proc. Natl Acad. Sci. USA*, **100**, 13121–13122.
 Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C. & Kraut, J. (1985). *J. Biol. Chem.* **260**, 16122–16130.
 Sasaki, J., Miyazaki, A., Saito, M., Adachi, T., Mizoue, K., Hanada, K. & Omura, S. (1992). *Appl. Microbiol. Biotechnol.* **38**, 152–157.
 Savino, C., Montemiglio, L. C., Sciara, G., Miele, A. E., Kendrew, S. G., Jemth, P., Gianni, S. & Vallone, B. (2009). *J. Biol. Chem.* **284**, 29170–29179.
 Shahrokhi, K., Orendt, A., Yost, G. S. & Cheatham, T. E. III (2012). *J. Comput. Chem.* **33**, 119–133.

- Sherman, D. H., Li, S., Yermalitskaya, L. V., Kim, Y., Smith, J. A., Waterman, M. R. & Podust, L. M. (2006). *J. Biol. Chem.* **281**, 26289–26297.
- Sideri, A., Goyal, A., Di Nardo, G., Tsotsou, G. E. & Gilardi, G. (2013). *J. Inorg. Biochem.* **120**, 1–7.
- Takeda, K., Asou, T., Matsuda, A., Kimura, K., Okamura, K., Okamoto, R., Sasaki, J., Adachi, T. & Omura, S. (1994). *J. Ferment. Bioeng.* **78**, 380–382.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Yasutake, Y., Fujii, Y., Cheon, W.-K., Arisawa, A. & Tamura, T. (2009). *Acta Cryst.* **F65**, 372–375.
- Yasutake, Y., Fujii, Y., Nishioka, T., Cheon, W.-K., Arisawa, A. & Tamura, T. (2010). *J. Biol. Chem.* **285**, 31193–31201.
- Yasutake, Y., Nishioka, T., Imoto, N. & Tamura, T. (2013). *Chembiochem*, **14**, 2284–2291.