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The role of active-site Phe87 in modulating the organic co-solvent tolerance of cytochrome P450 BM3 monooxygenase

Understanding the effects of organic co-solvents on protein structure and function is pivotal to engineering enzymes for biotransformation in non-aqueous solvents. The effects of DMSO on the catalytic activity of cytochrome P450 BM3 have previously been investigated and the importance of Phe87 in its organic co-solvent tolerance was identified. To probe the DMSO inactivation mechanism and the functional role of Phe87 in modulating the organic co-solvent tolerance of P450 BM3, the haem domain (Thr1–Leu455) of the F87A variant was cocrystallized in the presence of 14%(v/v) and 28%(v/v) DMSO. At both DMSO concentrations the protein retained the canonical structure of the P450 haem domain without any sign of partial or global unfolding. Interestingly, a DMSO molecule was found in the active site of both structures, with its O atom pointing towards the haem iron. The orientation of the DMSO molecule indicated a dynamic coordination process that was in competition with the active-site water molecule. The ability of the DMSO molecule to coordinate the haem iron is plausibly the main reason why P450 BM3 is inactivated at elevated DMSO concentrations. The data allowed an interesting comparison with the wild-type structures reported previously. A DMSO molecule was found when the wild-type protein was placed in 28%(v/v) DMSO, in which the DMSO molecule coordinated the haem iron directly *via* its S atom. Intriguingly, no DMSO molecule was observed at 14%(v/v) DMSO for the wild-type structure. These results suggested that the bulky phenyl side chain of Phe87 protects the haem from being accessed by the DMSO molecule and explains the higher tolerance of the wild-type enzyme towards organic co-solvents compared with its F87A variant.

1. Introduction

Taming the effects of non-aqueous solvents on protein structure and function is one of the most challenging quests in protein engineering. However, understanding protein stability in and tolerance of non-aqueous solvents (*e.g.* neat organic solvents, organic co-solvents, ionic liquids, supercritical fluids *etc.*) is of paramount significance to extend the practical application of proteins in biotransformations.

We selected cytochrome P450 BM3 monooxygenase (CYP102A1, 118 kDa; denoted BM3 in the following; Munro *et al.*, 2002) as a model protein to explore the effects of organic co-solvents. BM3, which was isolated from *Bacillus megaterium*, catalyzes a broad array of reactions including hydrocarbon hydroxylation, alkene epoxidation, alkyne oxygenation, arene epoxidation, aromatic hydroxylation, dealkylation *etc.* (Munro *et al.*, 2002). Many of these reactions involve stereospecific and regioselective oxygen insertion, rendering BM3 an attractive candidate for use in the chemical and pharmaceutical industries. The caveat is the notoriously low solubility of most of the substrates of BM3 in aqueous solution; therefore, organic co-solvents (for instance DMSO) are often supplemented to enhance substrate solubility and to increase the enzyme-turnover rate. This leads to a concomitant need to improve the organic co-solvent tolerance of BM3 and to understand the underlying mechanism of organic co-solvent effects on BM3.

We have previously used the method of directed evolution to tackle this problem and have successfully identified a BM3 variant (W5F5) that is more tolerant towards a series of commonly used

Table 1

Summary of crystallization conditions.

	2x7y	2x80
Method	Sitting-drop vapour diffusion in 96-well microtitre plate (CrystalQuick LBR; Greiner Bio-One GmbH)	Hanging-drop vapour diffusion in 24-well plate (EasyXtal Tool; Qiagen)
Protein sample	30 mg ml ⁻¹ protein in phosphate buffer (50 mM NaH ₂ PO ₄ pH 7.5) to which 14%(v/v) DMSO was added	30 mg ml ⁻¹ protein in phosphate buffer (50 mM NaH ₂ PO ₄ pH 7.5) to which 14%(v/v) DMSO was added
Mother liquor	0.05 M zinc acetate, 20%(w/v) PEG 3350	0.03 M zinc acetate, 13%(w/v) PEG 3350, 14%(v/v) DMSO
Drop size	600 nl (300 nl protein sample + 300 nl mother liquor)	4 µl (2 µl protein sample + 2 µl mother liquor)
Crystallization temperature (K)	278	278
Diffraction	Crystal subjected to data collection after one week at 278 K	Crystal subjected to data collection after one week at 278 K

water-miscible organic co-solvents (DMSO, THF, ethanol, DMF, acetone and acetonitrile; Wong *et al.*, 2004). During the course of directed evolution, an active-site amino acid (Phe87) was identified to be crucial in modulating the activity of BM3 in organic co-solvents. Substitution of Phe87 by an alanine (F87A) resulted in a drastic loss of organic co-solvent tolerance (Wong *et al.*, 2004). In this study, we aimed at obtaining insight into the role of Phe87 by measuring the effects of DMSO on the kinetic parameters of BM3 and cocrystallizing the F87A haem domain in the presence of DMSO.

2. Materials and methods

2.1. Cloning and expression of cytochrome P450 BM3 F87A

A gene encoding the P450 BM3 F87A haem domain (Thr1–Leu455) was cloned into pETM-11 vector using *NcoI* and *EcoRI* restriction sites (Kuper *et al.*, 2007). The protein was expressed as described previously (Kuper *et al.*, 2007).

2.2. Photometric enzyme assay

The activity of P450 BM3 F87A was measured using the 12-pNCA assay as described previously (Kuper *et al.*, 2007; Schwaneberg *et al.*, 1999, 2001).

2.3. Crystallization conditions

The protein was purified as described previously (Kuper *et al.*, 2007). A protein sample was prepared by adding 14%(v/v) DMSO to the F87A haem domain protein solution (~30 mg ml⁻¹ in 50 mM NaH₂PO₄ pH 7.5). Crystals were prepared under two conditions and are referred to by the PDB codes corresponding to their structures. For 2x7y, crystallization trials were carried out using sitting-drop vapour diffusion in a 96-well microtitre plate (CrystalQuick LBR; Greiner Bio-One GmbH, Frickenhausen, Germany) with a drop size of 600 nl (300 nl protein sample + 300 nl mother liquor). Needle-shaped crystals were obtained in 0.05 M zinc acetate, 20%(w/v) PEG 3350 at 278 K. For 2x80, crystallization trials were carried out using vapour diffusion in a hanging-drop plate (EasyXtal Tool; Qiagen) with a drop size of 4 µl (2 µl protein sample + 2 µl mother liquor). Needle-shaped crystals were obtained in 0.03 M zinc acetate, 13%(w/v) PEG 3350, 14%(v/v) DMSO at 278 K. All crystals were subjected to data collection after one week at 278 K. Crystallization conditions are summarized in Table 1.

2.4. Data collection and refinement

X-ray data were collected from flash-cooled crystals to 2.1 Å (2x7y) and 2.3 Å (2x80) resolution on beamline X12 at EMBL/DESY. Mother liquor to which 25%(v/v) glycerol had been added was used as a cryoprotectant. Data were indexed with *MOSFLM* (Leslie, 2006) and processed with *SCALA* (Evans, 1997) from the *CCP4* suite (Winn *et al.*, 2011). The initial phases were obtained by molecular

Table 2

Summary of the refinement statistics of the cytochrome P450 BM3 F87A structures 2x7y and 2x80.

Values in parentheses are for the last shell.

	2x7y	2x80
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å)	<i>a</i> = 83.0, <i>b</i> = 87.9, <i>c</i> = 159.6	<i>a</i> = 82.2, <i>b</i> = 96.6, <i>c</i> = 164.2
No. of independent reflections	68898	58691
Resolution range (Å)	57.54–2.10	47.62–2.30
<i>R</i> _{merge} (%)	12 (55)	11 (49)
Data completeness (%)	100.0 (100.0)	99.7 (99.6)
<i>I</i> / <i>σ</i> (<i>I</i>)	10.47 (2.41)	12.42 (3.17)
Multiplicity	4.10 (4.13)	6.49 (6.42)
No. of reflections used in refinement	65322	58625
No. of non-H protein atoms	7031	7330
No. of water molecules	552	406
<i>R</i> _{work} (%)	19.8	20.3
<i>R</i> _{free} (%)	24.3	25.0
R.m.s.d. in bond lengths (Å)	0.014	0.011
R.m.s.d. in bond angles (°)	1.528	1.289
Mean <i>B</i> value (Å ²)		
Protein	24.2	44.0
Heteroatoms		
HEM	12.8	30.5
DMS	57.7	68.1
ZN	34.0	62.3
Missing residues	189–202 and 227–230	None
R.m.s.d. C ^α , molecule <i>A</i> versus molecule <i>B</i> (Å)	0.414	0.604

replacement with *Phaser* (Read, 2001) using PDB entry 1bu7 (Sevrioukova *et al.*, 1999) as a search model. The structure was refined by alternating rounds of manual model building in *Coot* (Emsley & Cowtan, 2004) and refinement in *REFMAC* (Murshudov *et al.*, 2011). Atomic coordinates and structure factors have been deposited in the PDB as entries 2x7y [F87A, 14%(v/v) DMSO] and 2x80 [F87A, 28%(v/v) DMSO]. Data-collection and refinement statistics are summarized in Table 2.

3. Results and discussion

3.1. Kinetic parameters

In the presence of 14%(v/v) DMSO, the enzymatic activity of the wild-type enzyme (WT; Phe87) was fully retained, while the F87A variant showed a residual activity of only ~33% (Supplementary Fig. S1;¹ Wong *et al.*, 2004). When the DMSO concentration was raised further to 28%(v/v), a residual activity of ~27% was observed for the wild-type enzyme. F87A showed almost no enzymatic activity (~2%) at this DMSO concentration (Supplementary Fig. S1;¹ Wong *et al.*, 2004). Comparing the kinetic parameters of both enzymes in the absence and presence of 5%(v/v) DMSO, the *K_m* of WT for a surrogate substrate, 12-(4-nitrophenoxy)-dodecanoic acid (12-pNCA;

¹ Supplementary material has been deposited in the IUCr electronic archive (Reference: GX5207).

Table 3

Crystal structures of cytochrome P450 BM3 monooxygenase and its F87A variant in the absence and presence of DMSO.

P450 BM3	PDB code	Residue 87	DMSO concentration	Reference
WT	1bu7	Phe	0%(v/v)	Sevrioukova <i>et al.</i> (1999)
WT	2j4s	Phe	14%(v/v)	Kuper <i>et al.</i> (2007)
WT	2j1m	Phe	28%(v/v)	Kuper <i>et al.</i> (2007)
F87A	2x7y	Ala	14%(v/v)	This study
F87A	2x80	Ala	28%(v/v)	This study

Schwaneberg *et al.*, 1999, 2001), was increased threefold [from $7.2 \pm 2.7 \mu\text{M}$ (Lentz *et al.*, 2001) to $20.7 \pm 2.5 \mu\text{M}$], while the k_{cat} showed a slight increase [from $102 \pm 18 \text{ min}^{-1}$ (Lentz *et al.*, 2001) to $130 \pm 13 \text{ min}^{-1}$]. The K_{m} of F87A was increased similarly by threefold [from $4.4 \pm 1.5 \mu\text{M}$ (Lentz *et al.*, 2001) to $12.6 \pm 2.6 \mu\text{M}$], but the k_{cat} decreased from $210 \pm 24 \text{ min}^{-1}$ (Lentz *et al.*, 2001) to $179 \pm 25 \text{ min}^{-1}$. We selected 5%(v/v) DMSO to study the enzyme kinetics because both WT and its F87A counterpart showed reasonably high activity at this concentration for accurate quantification of K_{m} and k_{cat} . The increase in the K_{m} values supports the idea of DMSO molecules entering the substrate channel and competing with 12-pNCA. The decrease in the k_{cat} value in F87A could possibly be explained by protein inactivation by DMSO molecules. As the crystal structure of full-length P450 BM3 (haem and reductase domains) has not yet been reported, we can only base our discussion on the crystallized haem domain.

Similar observations were obtained when we investigated the tolerance of the WT and the F87A variant towards various ionic liquids (EMIM Cl, Bpy Cl, BMPyr Cl, IM11CN Cl, HMIM Cl, OMIM Cl *etc.*), in which the k_{cat} of F87A was more severely affected by these co-solvents compared with that of the WT (Tee *et al.*, 2008). Interestingly, Phe87 has also been demonstrated to modulate the reaction rate, stereoselectivity and regioselectivity as well as the substrate specificity of BM3 (Brenner *et al.*, 2007; Carmichael & Wong, 2001; Feenstra *et al.*, 2007; Graham-Lorence *et al.*, 1997; Noble *et al.*, 1999; Schwaneberg *et al.*, 1999).

3.2. Overall structures

To probe the DMSO inactivation mechanism and the functional role of Phe87, we cocrystallized the F87A haem domain (Thr1–Leu455) in the presence of 14%(v/v) and 28%(v/v) DMSO (Table 1). The coordinates of both crystal structures have been deposited in the PDB as entries 2x7y and 2x80, respectively (Table 2). Previously, our group has solved the WT structures obtained using identical DMSO concentrations (PDB entries 2j4s and 2j1m, respectively; Kuper *et al.*, 2007) and Table 3 summarizes the structures used for comparison in this study. At both DMSO concentrations the F87A haem domain shared the canonical structure of the P450 haem domain. There was no sign of partial or global unfolding, corroborating the UV–Vis

measurements. F87A displayed a typical Soret peak at 418 nm in phosphate buffer and in buffer supplemented with up to 30%(v/v) DMSO. An almost identical peak height at 418 nm was observed for all samples measured (Supplementary Fig. S2).

A pairwise comparison between different crystallographic structures of BM3 (Table 3) showed a small overall r.m.s.d. (in the range 0.84–1.43 Å; Table 4 and Supplementary Table S1) and a small backbone r.m.s.d. (in the range 0.33–1.39 Å; Table 4 and Supplementary Table S1). This clearly indicated that neither the addition of DMSO nor the Phe→Ala substitution at position 87 induced substantial differences in protein structure.

3.3. DMSO–haem interaction

A comparison of all of the crystallographic structures (Table 3) revealed a striking difference in the haem coordination. In PDB entry 1bu7 a water molecule was coordinated to haem at its sixth position, with an average distance of 2.63 Å (Table 5; Sevrioukova *et al.*, 1999). When the WT was placed in 14%(v/v) DMSO (PDB entry 2j4s), this active-site water molecule was displaced to a farther average distance of 3.77 Å from the haem iron (Fig. 1; Kuper *et al.*, 2007). There was also a slight nonplanar distortion of the haem, in which the iron was displaced distally by ~ 0.16 Å (Kuper *et al.*, 2007). In the presence of 28%(v/v) DMSO (PDB entry 2j1m), a DMSO molecule was found to coordinate the haem iron at its sixth position in the WT structure *via*

Figure 1

Active sites of the P450 BM3 haem domain at various DMSO concentrations. (a) F87A in 14%(v/v) DMSO (PDB entry 2x7y). (b) F87A in 28%(v/v) DMSO (PDB entry 2x80). (c) WT in 14%(v/v) DMSO (PDB entry 2j4s). (d) WT in 28%(v/v) DMSO (PDB entry 2j1m).

Table 4
Total r.m.s.d. and backbone r.m.s.d. (in parentheses) (Å) between different crystallographic structures of cytochrome P450 BM3 monooxygenase.
In this pairwise comparison, residues 1, 189–202, 227–230 and 243–247 were not considered as they are missing in some of the structures.

		1bu7		2j1m		2j4s		2x80		2x7y†	
		A	B	A	B	A	B	A	B	A	B
1bu7	A	—	1.18 (0.89)	0.94 (0.49)	0.89 (0.33)	0.91 (0.66)	1.00 (0.78)	0.96 (0.68)	1.30 (1.03)	(0.99)	(0.87)
	B		—	1.38 (1.02)	1.14 (0.93)	1.02 (0.56)	0.99 (0.53)	1.05 (0.49)	0.98 (0.42)	(1.34)	(1.29)
2j1m	A			—	0.91 (0.41)	1.22 (0.87)	1.33 (0.95)	1.12 (0.73)	1.43 (1.11)	(1.05)	(0.91)
	B				—	1.33 (0.95)	1.20 (0.87)	1.08 (0.69)	1.35 (1.03)	(0.96)	(0.87)
2j4s	A					—	0.84 (0.35)	0.92 (0.54)	1.06 (0.74)	(1.23)	(1.14)
	B						—	0.89 (0.60)	1.00 (0.68)	(1.28)	(1.22)
2x80	A							—	0.94 (0.56)	(1.23)	(1.15)
	B								—	(1.39)	(1.37)
2x7y	A									—	(0.40)
	B										—

† The total r.m.s.d.s are not reported owing to missing residues in 2x7y.

Table 5
Summary of haem-coordination geometry.

Monomer A	Monomer B	Average
Fe _{haem} —S _{Cys400} distance (Å)		
1bu7	2.38	2.33
2j4s	2.31	2.30
2j1m	2.30	2.31
2x7y	2.32	2.32
2x80	2.29	2.29
Fe _{haem} —O _{water} distance (Å)		
1bu7	2.57	2.63
2j4s	3.56	3.77
2j1m	N/A	N/A
2x7y	3.12 (O _{water} —Fe _{haem} —O _{DMSO} angle 45.20°)	3.66 (O _{water} —Fe _{haem} —O _{DMSO} angle 39.31°)
2x80	N/A	N/A
Fe _{haem} —S _{DMSO} distance (Å)		
1bu7	N/A	N/A
2j4s	N/A	N/A
2j1m	2.35	2.37
2x7y	N/A	N/A
2x80	N/A	N/A
Fe _{haem} —O _{DMSO} distance (Å)		
1bu7	N/A	N/A
2j4s	N/A	N/A
2j1m	N/A	N/A
2x7y	3.60	3.25
2x80	3.03	3.03
O _{DMSO} —Fe _{haem} —S _{Cys400} angle (°)		
1bu7	N/A	N/A
2j4s	N/A	N/A
2j1m	N/A	N/A
2x7y	165.01	168.47
2x80	166.08	165.66

an S atom (Fig. 1; Kuper *et al.*, 2007). The covalent Fe—S_{DMSO} bond had a length of 2.37 Å, similar to the fifth cysteine ligand (Fe_{haem}—S_{Cys400} = 2.33 Å; Kuper *et al.*, 2007).

In contrast to the WT, we observed a DMSO molecule in the active sites of both F87A structures [14%(v/v) and 28%(v/v)], with its O atom pointing towards the haem iron (Fig. 1 and Supplementary Fig. S3). The distances between O_{DMSO} and Fe_{haem} were 3.25 Å (PDB entry 2x7y) and 3.03 Å (PDB entry 2x80). At the lower DMSO concentration [14%(v/v); PDB entry 2x7y], a water molecule appeared to compete with the DMSO for possible haem coordination. When the DMSO concentration was raised to 28%(v/v) (PDB entry 2x80) no water molecule was found in the proximity of Fe_{haem}, while the DMSO molecule occupied the same position with similar orientation. Even though the DMSO molecule did not coordinate the haem iron directly in both F87A structures, the orientation of the DMSO molecule indicated a dynamic coordination process that was in competition with an active-site water molecule. This dynamic equilibrium coordination between a water molecule and a DMSO

Table 6
Distance (Å) between residues defining the opening of the substrate-access channel (Pro45 C^α–Ala191 C^α).

Monomer A	Monomer B	Average
1bu7	8.42	9.83
2j4s	9.52	9.63
2j1m	11.00	10.48
2x7y†	N/A	N/A
2x80	12.72	12.47

† Values not reported owing to missing residues in 2x7y.

molecule would keep the iron in a low-spin state. This explains the Soret band at 418 nm in both phosphate buffer and in buffer/DMSO mixtures. Moreover, this DMSO orientation was in line with the Pearson theorem of hard/soft acid and base (HSAB), in which the ‘harder’ O atom of DMSO is expected to coordinate the ‘hard’ acid (Fe^{III} in this case) instead of sulfur. Judged by the DMSO–haem interaction, one could conclude that F87A was less tolerant to DMSO than the WT, which is in good agreement with results of the enzymatic assay (Supplementary Fig. S1). Therefore, the phenyl side chain at position 87 plays a key role in modulating the accessibility of the DMSO molecule to the haem iron. In both F87A structures the DMSO methyl groups were at van der Waals contact distances to the methyl groups of Ala87 (3.85 Å in 2x7y; 4.09 Å in 2x80) and Ala328 (3.88 Å in 2x7y; 4.50 Å in 2x80).

3.4. DMSO channelling

In P450s, the active sites are situated deep inside the proteins and are often completely isolated from the surrounding solvent. Wade and coworkers have analyzed the channels (those that allow the passage of a sphere with radius greater than 1.2 Å) between the active site and the protein surface in P450 crystal structures (Cojocaru *et al.*, 2007). In view of the size of a DMSO molecule (probe radius 2.41 Å), we postulate that the DMSO molecule is likely to enter BM3 *via* the substrate-access/egress pathways channel 2a and channel 2d identified by Wade and coworkers (Cojocaru *et al.*, 2007). This hypothesis is supported by the increase in the K_m value in the presence of DMSO, as mentioned previously. BM3 is known to undergo substantial conformation change upon substrate binding, specifically in the regions of helix *F* (His171–Arg190), helix *G* (Ala197–Ser226), the *F/G* loop and the *G/H* loop (Li & Poulos, 1997). In the palmitoleic acid-bound BM3 haem structure (PDB entry 1fag) the substrate channel was closed (Li & Poulos, 1997). The distance between Pro45 and Ala191 (which define the mouth of the substrate-access channel) was 7.94 Å (Li & Poulos, 1997), in contrast to 9.83 Å

in the substrate-free structure (PDB entry 1bu7; Sevrioukova *et al.*, 1999). In the DMSO-bound WT structures (PDB entries 2j4s and 2j1m), the Pro45 C α –Ala191 C α distances were 9.65 and 10.48 Å (Table 6), respectively (Kuper *et al.*, 2007). Therefore, the substrate-access channel remained open upon DMSO entry. In the case of F87A, the Pro45 C α –Ala191 C α distance was significantly larger (12.47 Å in PDB entry 2x80). It remains unknown whether this enlargement was induced by the addition of DMSO or by the Phe→Ala substitution at position 87.

4. Conclusion

It is interesting that BM3 retains its folded structure even at a high DMSO concentration [28%(v/v)], as demonstrated by our crystallographic data. The reduced catalytic activity of BM3 in DMSO is instead attributed to at least two other factors. Firstly, the DMSO molecule competes with the substrate molecule to enter the substrate-access channel (as reflected by an increase in the K_m value). Secondly, DMSO can coordinate the haem iron *via* its O atom (F87A variant) or its S atom (wild type), thereby inhibiting BM3 (a decrease in the k_{cat} value).

By comparing the crystal structures of the F87A variant with those of its wild-type counterpart, this work provided direct evidence that phenylalanine at position 87 limits the access of the DMSO molecule to the haem iron. This 'protective mechanism' of Phe87 explains the higher organic co-solvent tolerance of wild-type BM3. Our findings add to the understanding of organic co-solvent inactivation of enzymes and provide a possible engineering principle for tailoring haem enzymes to function in organic co-solvents. Currently, we are extending our work to other organic co-solvents and enzyme systems using a combination of protein-engineering, biochemistry and structural biology approaches.

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