Structural basis of the substrate specificity of the FPOD/FAOD family revealed by fructosyl peptide oxidase from *Eupenicillium terrenum*

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The FAOD/FPOD family of proteins has the potential to be useful for the longterm detection of blood glucose levels in diabetes patients. A bottleneck for this application is to find or engineer a FAOD/FPOD family enzyme that is specifically active towards α -fructosyl peptides but is inactive towards other types of glycosylated peptides. Here, the crystal structure of fructosyl peptide oxidase from *Eupenicillium terrenum* (EtFPOX) is reported at 1.9 Å resolution. In contrast to the previously reported structure of amadoriase II, EtFPOX has an open substrate entrance to accommodate the large peptide substrate. The functions of residues critical for substrate selection are discussed based on structure comparison and sequence alignment. This study reveals the first structural details of group I FPODs that prefer α -fructosyl substrates and could provide significant useful information for uncovering the mechanism of substrate specificity of FAOD/FPODs and guidance towards future enzyme engineering for diagnostic purposes.

1. Introduction

Glycated haemoglobin A1c (HbA1c), in which the N-terminal valine residue of the β subunit is modified by glucose, is the target protein for the longterm control of glycaemic levels in diabetes patients. To develop an easy and low-cost clinical diagnostic method to test for HbA1c, an enzymatic assay based on fructosyl amino-acid oxidases (FAOD or FAOX) is an attractive method. FAODs catalyze the oxidation of fructosyl amino acids to produce free amino acid, glucosone and hydrogen peroxide (Ferri *et al.*, 2009). The eukaryotic FAOD family has been divided into three groups according to the substrate specificity (Ferri *et al.*, 2009; Lin & Zheng, 2010): group I FAODs prefer α -fructosyl amino acids (*e.g.* f- α Val; Fujiwara *et al.*, 2006; Hirokawa, Gomi & Kajiyama, 2003; Kim, Ferri *et al.*, 2010; Yoshida *et al.*, 1995, 1996) and group II FAODs prefer ϵ -fructosyl amino acids (*e.g.* f- ϵ Lys; Akazawa *et al.*, 2004; Fujiwara *et al.*, 2007; Sakai *et al.*, 1995, 1996; Takahashi *et al.*, 1997a,b; Wu *et al.*, 2000), whereas group III FAODs react with both α -fructosyl and ϵ -fructosyl amino acids (Akazawa *et al.*, 2004; Collard *et al.*, 2008; Kim *et al.*, 2009; Kim, Nibe *et al.*, 2010; Takahashi *et al.*, 1997a,b; Wu *et al.*, 2002).

In practical HbA1c measurement, the direct testing target is the α -fructosyl dipeptide fVH or the α -fructosyl hexapeptide fVHLTPE (Kobold *et al.*, 1997). Unfortunately, most of the reported FAODs show low or no activity towards the fructosyl

Figure 1
Structure-guided sequence alignment of FAOD/FPODs. The secondary-structure elements in the structures of EtFPOX (above) and amadoriase II (below) are indicated. Residues are coloured according to their conservation. The sequences are classified into three groups according to their substrate specificity (Ferri *et al.*, 2009; Lin & Zheng, 2010). Sequence alignment was performed and presented using *ClustalX* (Larkin *et al.*, 2007) and *ESPr3* (Robert & Gouet, 2014). The sequences used for alignment are EtFPOX from *E. terreum* ATCC 18547 (BAD00185; Hirokawa, Gomi & Kajayima, 2003), PnFPOX from *Phaeosphaeria nodorum* SN15 (XP_001798711; Kim, Ferri *et al.*, 2010A), FPOX-C from *Coniochaeta* sp. NISL 9330 (BAD00186; Hirokawa, Gomi & Kajayima, 2003), FAOD-P from *Penicillium janthinelum* AKU 3413 (CAA70219; Yoshida *et al.*, 1995, 1996), FAOD-U from *Ulocladium* sp. JS-103 (BAE93140; Fujiwara *et al.*, 2006), N1-1 FAOD from *Pichia* sp. N1-1 (AAP83789; Ferri *et al.*, 2004), FAOD-Ao2 from *A. oryzae* (BAE58870; Akazawa *et al.*, 2004), amadoriase II from *A. fumigatus* (AAC49711; Collard *et al.*, 2008; Takahashi *et al.*, 1997a,b), FAOD-F from *Fusarium oxysporum* NBRC 9972 (Fujiwara *et al.*, 2007), amadoriase I from *A. fumigatus* (AAB88209; Takahashi *et al.*, 1997a,b) and FAOD-Ao1 from *A. oryzae* (BAD54824; Akazawa *et al.*, 2004).

peptide. Many studies have been carried out to search for enzymes that react with fructosyl peptide, referred to as fuctosyl peptide oxidases (FPODs or FPOXs; Hirokawa, Gomi, Bakke *et al.*, 2003; Hirokawa, Gomi & Kajiyama, 2003; Kim, Ferri *et al.*, 2010; Kim *et al.*, 2012). Structural biology would be helpful in engineering FPODs to optimize their activity and specificity. To date, only the crystal structure of FAOD from *Aspergillus fumigatus* (amadoriase II) has been reported (Collard *et al.*, 2008). Although amadoriase II prefers α -fructosyl amino acids as the substrate, it has also been assigned as a group III FAOD owing to its relatively high affinity for ϵ -fructosyl amino acids (Takahashi *et al.*, 1997a,b; Wu *et al.*, 2002). Therefore, to elucidate the substrate-selection mechanism of FAOD/FPODs, detailed structures of typical group I FAOD/FPODs are needed.

FPOX from *Eupenicillium terrenum* ATCC 18547 (EtFPOX) is a promising candidate for use in clinical HbA1c measurements owing to its having the highest activity ratio towards f-^εVal versus f-^εLys in the FAOD/FPOD family (Hirokawa, Gomi & Kajiyama, 2003). Here, we report the crystal structure of EtFPOX at 1.9 Å resolution. By comparing the structures of the EtFPOX and amadoriase II, together with sequence alignment and biochemical data, the structural basis of FAOD/FPOD substrate specificity is discussed.

2. Experimental procedures

The gene for EtFPOX (GenBank AB116146) was subcloned into pET-22b vector (Novagen) and then transformed into *Escherichia coli* strain BL21 (DE3). The procedures for protein purification, crystallization and data collection have been described previously (Xing *et al.*, 2013). The EtFPOX structure was determined by molecular replacement with *Phaser* (McCoy *et al.*, 2007) in the CCP4 package (Winn *et al.*, 2011; Potterton *et al.*, 2003). The search model was the crystal structure of amadoriase II (PDB entry 3dje; Collard *et al.*, 2008), which shares 35% sequence identity with EtFPOX. The FAD (flavin adenine dinucleotide) and water molecules were

Table 1

Summary of data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 65.6, b = 80.0, c = 83.4$
Resolution range (Å)	30.0–1.90 (1.97–1.90)
No. of unique reflections	35108
Data completeness (%)	99.6 (100)
$\langle I \rangle / \langle \sigma(I) \rangle$	15.2 (4.2)
$R_{\text{merge}}^\dagger$ (%)	9.9 (45.5)
R factor/ $R_{\text{free}}^\ddagger$ (%)	19.0 (26.0)/23.3 (28.7)
R.m.s.d., bond lengths (Å)	0.007
R.m.s.d., bond angles (°)	1.13
No. of atoms modelled	3725
No. of water molecules	265
Wilson B factor (Å ²)	33.9
Mean B factors (Å ²)	
Protein main-chain atoms	34.9
Protein side-chain atoms	39.6
FAD	40.8
Phosphate	34.0
Water molecules	43.0
Ramachandran plot statistics (%)	
Residues in most favoured region	94.4
Residues in additional allowed region	5.3
Residues in disallowed region	0.3

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean value of $I_i(hkl)$. $^\ddagger R$ factor = $\sum_{hkl} |F_{\text{obs}}| - |F_{\text{calc}}| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} and F_{calc} are observed and calculated structure factors, respectively. The free R factor was calculated using 5% of reflections which were omitted from the refinement.

deleted from the model. Initial trials of molecular replacement failed to find a correct solution, which indicates that the target structure might have major differences from the search model. Based on sequence alignment, residues Ser57–Asn72, Val113–Glu123 and the C-terminal fragment starting from Asp398, which are located in regions with sequence insertions or deletions, were deleted. All of the residues which differed between EtFPOX and amadoriase II were mutated to Ala. The B factors were all assigned as 30 Å² as indicated by the Wilson plot. The modified search model resulted in a

reasonable molecular-replacement solution. Structure refinement was carried out with alternate cycles of manual fitting and building into $2F_o - F_c$ and $F_o - F_c$ composite OMIT electron-density maps using *Coot* (Emsley *et al.*, 2010) and calculations with *REFMAC* (Murshudov *et al.*, 2011). FAD and water molecules were added in the later stages of the refinement according to the $F_o - F_c$ map at the 3σ level. The last cycle of refinement was performed using *PHENIX* (Adams *et al.*, 2010). One FAD, one phosphate from the crystallization solution and 265 water molecules were included in the final model. Structural figures were prepared using *PyMOL* (DeLano, 2002).

3. Results

3.1. Structural overview of EtFPOX

The structure of EtFPOX was refined to an R factor of 0.190 ($R_{\text{free}} = 0.233$) at 1.9 Å resolution. There is one EtFPOX model in the asymmetric unit and all of the 436 residues except the N-terminal first methionine are present with clear electron density. Analysis by *PROCHECK* (Laskowski *et al.*, 1993) shows that the final model has reasonable geometry except for Thr316, which fits very well into the electron density. One FAD molecule is bound in the active site of EtFPOX. A phosphate molecule forms hydrogen bonds to Arg288, Lys290, Arg307, Arg311 and His312. The data-collection and refinement statistics are summarized in Table 1. As in the amino-acid oxidase FAD-binding protein family, EtFPOX also contains two domains: a FAD-binding domain and a catalytic domain. Superimposing the EtFPOX structure onto amadoriase II, it is found that the main-chain folds of these two structures are similar, with an atomic r.m.s.d. value of 2.20 Å (Fig. 1). Detailed analysis shows that half of the catalytic domain (Asp96–Asp156) moves outwards from the active site by about 4 Å compared with that of amadoriase II (Fig. 2). On

Figure 2

Stereoview of structure comparison of EtFPOX (green) and amadoriase II (magenta). The fragments Gln91–Gly157 in EtFPOX and Asp93–Ala155 in amadoriase II (loop A1 and loop A2), the conformations of which differ in these two enzymes, are shown in cartoon mode. The other peptide chains, which superimpose well, are shown as lines. FAD and FSA molecules are shown as sticks.

excluding this part, the r.m.s.d. value on superimposition decreases to 1.33 Å.

3.2. The substrate entrance of EtFPOX is wider

In EtFPOX, the substrate entrance is gated by loop A1 (active loop 1; residues Leu63–Gly66), helix 3 (residues Lys101–Asp116) and loop A2 (active loop 2; residues Arg412–Ala426) (Fig. 1). In the free structure of amadoriase II, loop A1 and part of helix 3 are disordered (Collard *et al.*, 2008). On binding to FSA (fructosyl thioacetate), which mimics the fructosyl glycine substrate, loop A1, helix 3 and loop A2 close the active entrance, leaving no space for other residues of a peptide substrate (Figs. 3*b* and 3*d*). Tyr60 from loop A1, Arg108 and Arg112 from helix 3, the protruding Gly410 and the aromatic ring of Phe412 in loop A2 act as lids, completely covering the FSA molecule and leaving no space for the second residue if a peptide substrate were present. Arg108 in helix 3 appears to be more critical. It forms hydrogen bonds to the carbonyl O atoms of Tyr60 (in loop A1) and Gly410 (in loop A2). Even in the free structure of amadoriase II, the ordered parts of helix 3 and loop A1 partially cover the active entrance. Although the remaining residues in loop A1 and helix 3 are disordered in the absence of substrate, they still

occupy some space and would make the substrate entrance narrower than that seen in Fig. 3(*c*).

On comparing the structures of EtFPOX and amadoriase II, it is obvious that the entrance to the active site in EtFPOX is wider (Fig. 3*a*). In EtFPOX, loops A1 and A2 are shorter than those in amadoriase II by six and seven residues, respectively. Correspondingly, helix 2 is one turn shorter and loop A2 is a short and sharp turn in EtFPOX. Although helix 3 in EtFPOX is longer, the position of helix 3 is more distant from the active site. Together with the conformations of loop A1, loop A2 and helix 3, the substrate entrance in EtFPOX is much wider (Fig. 3*a*). Asp96, which is conserved in group I but is substituted by a hydrophobic residue in groups II and III, is repelled by the conserved hydrophobic bottom (Met94 and Tyr261) of the active site. Another group I-specific residue, Cys97, which backs against the active site, is closer to a hydrophobic core formed by Phe141, Trp149, Val260 and Phe332 than the equivalent residue Ser97 in amadoriase II. Starting from these residues, the fragment of Asp96–Asp156 is arranged differently from that in amadoriase II.

In addition, unlike in amadoriase II, in which Arg108 and Arg112 stretch their side chains to cover the active site, Arg109 in EtFPOX helix 3 bends the side chain almost along helix 3, forming a hydrogen bond to Asn106 in the previous

Figure 3

Electrostatic surface diagrams of (*a*) EtFPOX, (*b*) the FSA-bound form of amadoriase II and (*c*) free amadoriase II viewed from the top of the substrate entrance. An FSA molecule shown in stick representation is placed into the active site of EtFPOX and free amadoriase II in the same position as in the FSA-bound structure of amadoriase II, in which the active pocket is completely covered by loop A1, loop A2 and helix 3. (*d*) The same as in (*b*) but the surface is shown with transparency to show FSA; loop A1, loop A2 and helix 3 are coloured green, magenta and yellow, respectively.

turn of the same helix. This, combined with the backwards relocation of helix 3 and the shortness of loops A1 and A2, results in the lids covering the active site disappearing and leaving a space for the peptide substrate (Fig. 3*a*).

3.3. FAD binding

FAD is bound by EtFPOX in a manner similar to that of amadoriase II. A covalent bond is formed between FAD and Cys347 in EtFPOX. Three hydrogen bonds are formed between the FAD O4 atom and the amide group of Ile58, between the FAD N3 atom and the carbonyl group of Ile58 and between the FAD O2 and the N^ε atom and the amide group of Lys380. Thr18 and Ser50 contribute to stabilize the phosphate group in the middle of FAD. The pentose ring of FAD is fixed mainly by the formation of hydrogen bonds from O2' and O3' of the pentose to the O^δ atom of Asp41 and the O^γ atom of Ser47. Furthermore, Lys57 and Lys78 in EtFPOX approach the *si* side of the flavin aromatic ring in the same orientation as in amadoriase II. Remarkably, residues Thr18, Asp41, Ser47, Ser50, Lys57, Ile58, Cys347 and Lys278, which are involved in interactions with FAD, are highly conserved in all three groups of FAOD/FPODs. This suggests that the FAD-binding mode would be conserved in the FAOD/FPOD family and that the substrate specificity does not result from FAD binding.

3.4. Fructose binding

The C—N reaction site of the substrate, which forms the intermediate ketoamine bond, is positioned directly above N₍₅₎—C_(4a) of the flavin ring to trigger a proton relay (Zhao *et al.*, 2002). Thus, the correct orientation of the substrate in the catalytic site is vitally important for the reaction to proceed. According to the structure of the amadoriase II—FSA complex, the fructosyl portion of the ligand is stabilized by a series of hydrogen bonds involving the side chains of Glu280 and Arg411 and the main-chain carbonyl group of Gly364. The side chain of Trp236 is parallel to the pyranose ring of FSA. In EtFPOX, Trp239, Glu282 and Gly376 are in the same positions as the equivalent residues (Trp239, Glu280 and Gly364) in amadoriase II. The role of Arg411 in amadoriase II

is played by Arg418 in EtFPOX. Arg418 is located in loop A2. Although the C^α-atom positions of these two arginines are different owing to the varying conformation of loop A2, the guanine group of Arg418 in EtFPOX is superimposable with that of Arg411 in amadoriase II (Fig. 4). Considering the high conservation of these residues (Arg418 is conserved in group I and Arg411 is conserved in group II and group III), this suggests that the fructose-binding model is also similar in the whole FPOD/FAOD family.

4. Discussion

4.1. Group I differs from the other groups in the substrate entrance

Both EtFPOX and amadoriase II bind FAD in the same way, sharing almost identical interactions between the protein and the FAD molecule. The residues forming the fructose-binding pocket and their conformations are also similar. This suggests that a common catalytic chemistry occurs in the FPOD/FAOD family. The substrate specificity should depend on the substrate-binding environment.

The structure of EtFPOX shows an open channel in which the peptide substrate could be accommodated. The substrate cave in EtFPOX is obviously wider than that in amadoriase II. Based on sequence alignment, the group I members EtFPOX, PnFPOX, FPOX-C, FAOD-P and FAOD-U share three similar motifs in their sequence and it is likely that the open substrate cave is a common characteristic of group I. Consistently, EtFPOX, PnFPOX and FPOX-C have been reported to be active with fVH as a substrate (Hirokawa, Gomi & Kajayima, 2003; Kim, Ferri *et al.*, 2010). We suggest that FAOD-P and FAOD-U may also be able to act as FPODs. If this were true, the group I FPOD/FAODs which prefer α-fructosyl substrates might also have the potential to adopt peptide substrates because of the wide-open entrance. The architectures of helix 3 and two shorter active loops distinguish group I from group II and III.

Interestingly, in the FPOD/FAOD family the lengths of loop A1 and loop A2 are shortest in group I, longest in group II and intermediate in group III, while the length of helix 3 plus the

Figure 4

Comparison of the active-site residues of (a) EtFPOX, in which an FSA molecule is present as in amadoriase II, and (b) amadoriase II bound to FSA. Residues involved in substrate binding and critical for activity are labelled. FSA and FAD are shown as stick models coloured cyan and grey, respectively.

following loop is longest in group I and shortest in group II. These phenomena are consistent with the specificity changes from group I *via* group III to group II. Since the shape of the f- α Val substrate is relatively wide compared with f- ϵ Lys, it is reasonable that group I enzymes have a more open substrate entrance while group II enzymes have a narrow entrance.

4.2. The active site is more charged in group I

In the structure of amadoriase II, a hydrophobic channel is formed by the side chains of four residues, Phe264, Phe258, Met98 and Val54, which interact with the amino-acid part of the substrate. Val54 and Phe264 are conserved in all three groups of FPOD/FAODs. Met98 and Phe258 are conserved in group II and group III but change to Asp96 and Tyr261 in group I. Lys110 from helix 3 inserts to form a hydrogen bond to Asp96. These substitutions make this pocket less hydrophobic in group I.

In addition, the other side of the entrance in EtFPOX is rich in positive charge provided by four arginines, Arg62, Arg64, Arg417 and Arg418, which are conserved in group I but absent in groups II and III. Elucidation of whether these positive charges play a critical role in binding the peptide substrate, such as holding the second or the third carbonyl group of the peptide, must await determination of the enzyme–substrate complex structure.

4.3. Other possibly critical residues for substrate selection

In *Pichia* N1-1 FAOD (group III), it was reported that Asn354 (equivalent to His377 in EtFPOX) plays an important role in the recognition of f- α Val and f- ϵ Lys (Kim *et al.*, 2009; Miura *et al.*, 2006, 2008). Compared with the wild-type enzyme, the N354H mutant displays a higher activity towards f- α Val and a lower activity towards f- ϵ Lys (Kim *et al.*, 2009). In EtFPOX, the imidazole ring of His377 and the guanine ring of Arg62 stack on each other. A similar stacking, with Arg365 pairing with Tyr60, is also found in amadoriase II. These stacking residue pair is strictly conserved as Arg–His in group I. In group II, most of these pairing residues are Tyr–Arg. In group III, the pair is Leu/Val–Asn. Whether this residue pairing plays an important role in substrate specificity requires further investigation.

Checking all of the residues around the active pocket, it is found that Lys380 is the only residue that is conserved in groups I and III, but it changes to a methionine in group II. Therefore, this residue might be critical to substrate specificity. In amadoriase II, the equivalent residue Lys368 binds to the carboxylate group of the inhibitor FSA, suggesting that this lysine might be responsible for binding of the carboxylate of f- α Val. Supporting data come from the observation that the substitution K368M increases the K_m value for f- α Val but little affects the V_{max} (Collard *et al.*, 2008), implying that the function of this position is mainly in substrate selection.

Also worth mentioning is the phosphate-binding region. Besides Arg288, Lys290, Arg307, Arg311 and His312 that hydrogen-bond to the phosphate, His292 and Lys432 are also clustered in this region, which is therefore very rich in positive

charge. All seven of these residues are conserved in group I but they are either missing or nonpositively charged residues in the other groups. In particular, the fragment Arg311–Asp315 is only present in group I and forms an extra loop compared with amadoriase II. Whether this positively charged region is significant to any function is of interest for investigation in the future.

In conclusion, according to structural analysis of EtFPOX and amadoriase II, eukaryotic FPOD/FAODs bind to FAD and the fructose portion of the substrate with a similar mode. The substrate specificity may come from the conformations of loop A1, loop A2 and helix 3, which form a wider substrate entrance in group I. Unlike the hydrophobic channel in group II and group III, one side of the channel in group I is more hydrophilic, involving Asp96 and four arginines. The polar distribution at the positions of Arg62, His377 and Lys380 might also contribute to substrate selection. These results could provide useful information for studies of the substrate preference of eukaryotic FPOD/FAODs at the molecular level and their potential applications in protein engineering.

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