Enzymology

Flavin-containing monooxygenases: new structures from 'old' proteins

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A recent study reports the structures of reconstructed ancestral mammalian flavin-containing monooxygenases (FMOs), which are the first to be solved for membrane-bound FMOs. The resulting models provide a structural basis for the mechanism of action of these enzymes and greatly enhance our understanding of how the proteins interact with membranes and how they permit access of substrates to their active site.

FMOs catalyze the oxidative metabolism of a range of chemicals, which include drugs, pesticides and compounds derived from the diet by the action of gut bacteria[1-4]. The enzymes act at the interface between an organism and its chemical environment to play an important role in protection against potentially harmful foreign chemicals. Some FMOs are also involved in endogenous metabolic processes: in the regulation of energy balance[5], metabolic ageing[6] and glucose homeostasis[7]. In vertebrates FMOs are located in the membranes of the endoplasmic reticulum and have proved, to date, impossible to crystallize. Recently, based on the reconstruction of ancestral mammalian FMO sequences, Nicoll et al.[8] reported the crystallization and structures of three membrane-bound FMOs. This major advance provides a structural basis for the catalytic mechanism of FMOs and gives novel insights into how the enzymes bind to membranes and control access to their catalytic site.

Early during the evolution of tetrapods duplications of a single *FMO* gene gave rise to a family of genes. In humans the *FMO* gene family consists of five functional genes (*FMO1*, *2*, *3*, *4* and *5*) and six pseudogenes[9], one of which is *FMO6*. *FMO3* and *FMO6* arose from a more recent duplication that took place early during mammalian evolution[9]. Based on a phylogenetic analysis, Nicoll et al.[8] reconstructed ancestral sequences for three mammalian FMOs: FMO2, FMO5 and the precursor of FMO3/6. The ancestral FMOs, which have 83-92% amino-acid sequence identity with the corresponding extant human FMO, were expressed in *E. coli* as holoenzymes, bound to their prosthetic group FAD. The ancestral FMOs were catalytically active, with kinetic parameters similar to those of the corresponding human FMO, and ancestral FMOs 2 and 3/6 reacted rapidly with oxygen to form a stable C4a hydroperoxyflavin intermediate, a key feature of the catalytic mechanism (Fig. 1A).

Remarkably, despite numerous unsuccessful attempts to crystallize human FMO3 and FMO5, all three of the ancestral proteins crystallized, possibly because of their higher melting temperatures. The crystal structures of the ancestral FMOs provide valuable insights into how FMOs bind to membranes, interact with FAD and the cofactor $NADP^+$ and control access to their catalytic site. The three ancestral proteins, each of which crystallized as a dimer, have very similar structures. Each monomer is anchored in the membrane by a 30 Å-long C-terminal trans-membrane α-helix that spans the membrane bilayer, with the final few C-terminal residues exiting the other side of the membrane. In addition, each monomer features a large hydrophobic strip, which is lined by a ring of positively charged residues. These structural features facilitate binding of FMOs to the membrane, via hydrophobic interactions with the phospholipid bilayer and ionic interactions between the positively charged amino-acid side chains and negative charges on the polar head group of membrane phospholipids. This unexpected feature of FMO structure explains why human FMO2.2, which is encoded by the variant *FMO2*2* allele and lacks the C-terminal 64 amino-acid residues of FMO2.1,

retains the ability to bind to the membranes of the endoplasmic reticulum[14]. However, a relatively short truncation of the C-terminal domain of FMO3 was found to be sufficient to solubilize the polypeptide[15].

Comparison of the structures of the ancestral mammalian FMOs with that of a soluble FMO from a *Methylophaga* bacterium[16] shows that the FAD- and NADP(H)-binding domains of FMOs have been well conserved throughout evolution. However, there are some important differences. In the soluble protein FAD is exposed to the solvent, allowing easy access of substrates. In contrast, membrane-bound FMOs have an 80-residue-long insertion, which forms a subdomain that shields the active site, creating a closed substrate-binding cavity.

The structures of the ancestral FMOs reveal a number of conserved amino-acid residues within the active site that are likely to play important roles, via H-bonding and steric interactions, in orienting the nicotinamide and ribose moieties of $NADP^+$ and the isoalloxazone ring of FAD, or in the stabilization of the C4a-hydroperoxyflavin intermediate. Mutations in the human *FMO3* gene of two of the residues conserved in the active site, Asn61 and Arg223, gives rise to the inherited disorder trimethylaminuria[17,18], confirming the importance of the residues for enzyme function.

FMO5 has been shown to be an effective Baeyer-Villiger monooxygenase[13] (Fig.1B). Nicoll et al.[8] postulate that a His at position 282 of ancestral FMO5, instead of a Glu at an equivalent position in ancestral FMO2 and FMO3/6, has the potential to stabilize a Criegee intermediate and, thus, may account for the Baeyer-Villiger activity of FMO5. Consistent with this hypothesis, a His282Glu mutation in ancestral FMO5 inactivated the enzyme. However, substitution of His for native Glu at this position in FMO2 and FMO3/6 was insufficient to confer Baeyer-Villiger activity to these proteins.

In each of the ancestral FMOs substrates access the buried active site via a conserved tunnel that extends outwards from the face of the isoalloxazone ring of FAD. A leucine

residue that is conserved in all three ancestral FMOs and in extant human FMOs (Leu375 in ancestral FMO3/6) acts as a 'gate keeper' to the active site. The tunnel bifurcates, in ancestral FMO2 and FMO3/6 one branch leads to the membrane, the other to the aqueous environment (Fig. 2), thus allowing entry of hydrophobic and hydrophilic substrates by diffusion from the membrane or cytosol, respectively, and the exit of products. In the case of ancestral FMO5 both branches apparently lead to the membrane.

The high degree of structural similarity among the active sites of the three ancestral FMOs indicate that substrate selectivity is determined by the ease with which a molecule can gain access to the active site, in line with results of earlier biochemical experiments[10,11,19]. Although structural differences at the tunnel entrances may contribute to selectivity, the molecular architecture of the tunnels does not fully explain the differential substrate selectivity of individual FMO isoforms.

The availability of structures of ancestral mammalian FMOs will enable more accurate modeling of structures of extant human FMOs. For instance, a structure of human FMO3 would contribute to understanding the effect on enzyme activity of known causative mutations of trimethylaminuria and help predict the likely consequences of novel mutations. Such structures would also provide a basis for structure-based design of drugs that are substrates of FMOs. In comparison with cytochromes P450 (CYPs), the major drug-metabolizing enzyme system, FMOs are not generally inducible and are less likely to produce toxic intermediates[20]. Consequently, the rational design of drugs that are metabolized by FMOs and not by CYPs would have the advantage of decreasing the likelihood of undesirable drug-drug interactions and avoiding the production of potentially harmful metabolites.

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Figure legends

Fig. 1. **The catalytic cycle of mammalian FMOs.** For catalysis FMOs require FAD as a prosthetic group, NADPH as a cofactor and oxygen as a co-substrate. The catalytic mechanism differs from those of other monooxygenases because FMOs can bind and activate oxygen, in the form of C4a-hydroperoxyflavin (the structure of which is shown in the Figure), before the substrate binds[10,11]. A consequence of this unusual mechanism is that FMOs are present in an activated form capable of oxygenating any appropriate substrate that can gain access to the active site. FMOs can also act as NADPH oxidases, producing hydrogen peroxide, as shown by the dashed lines[12]. (A) Oxygenation of trimethylamine catalyzed by FMO3. (B) Baeyer-Villiger monooxygenation of heptan-2-one catalyzed by FMO5, showing the Criegee intermediate[8,13].

Fig.2. **Schematic representation of tunnels within FMOs leading to the active site.** In ancestral FMO2 and FMO3/6 one branch of the tunnel leads to the membrane and the other to the cytosol. In FMO5, however, both branches lead to the membrane. The leucine ‘gate keeper’ at the entrance to the active site is shown. Curved line indicates boundary of the active site.