Structure and function studies on flavoproteins degrading xenobiotics

PhD thesis

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Introduction

Widespread contamination of the environment by high explosives, resulting from the manufacture, disposal and testing of munitions is becoming a matter of increasing concern. Most explosives are considered to be a major hazard to biological systems due to their toxic and mutagenic effects. Bioremediation is an attractive means by which such sites can be decontaminated. A strain of *Enterobacter cloacae*, designated PB2, was isolated from explosive contaminated land on the basis of its ability to use nitrate esters, such as pentaerythritol tetranitrate (PETN) and glycerol trinitrate (GTN, nitroglycerin), as its sole nitrogen source. This ability is attributed to a NADPH-dependent flavoenzyme PETN reductase. The enzyme is also active, at a lower level, against 2,4,6-trinitrotoluoene (TNT), which is the most persistent pollutant in military sites. The broad substrate range makes the enzyme attractive in the phytoremediation of explosive contaminated land. The *onr* gene encoding PETN reductase has been engineered into tobacco plant seeds and the transgenic plant seedlings expressing PETN reductase have been shown to germinate and grow in the presence of GTN and TNT, at concentrations that normally inhibit the germination and growth of wild-type seedlings.

Morphinone reductase (MR) from *Pseudomonas putida* M10, plays a key role in a unique opiate biotransforming pathway. *Pseudomonas putida* M10 was isolated from opiate factory waste liquor, was demonstrated to transform morphine and codeine to hydromorphone and hydrocodone via two consecutive steps. The second reaction is the reduction of the unsaturated bond between C7-C8 of morphinone and codeinone by NADH-dependent morphinone reductase, yielding hydromorphone and hydrocodone respectively. The production of hydromorphone and hydrocodone are of industrial interest owing to their application as analgesics and cough suppressants. The limitation, in the application, of the two *P. putatida* enzymes driven biotransformations is the two different nicotinamide cofactors the enzymes use. It would be desirable to switch the nicotinamide specificity of MR, from NADH to NADPH, using rational protein engineering methods, in order to recycle of NADPH/$NADP^+$ between MR and morphine dehydrogenase catalyzed reactions.

Both enzymes are the members of nicotinamide cofactor dependent FMN containing oxido-reductases. PETN reductase is specific for NADPH, whereas MR is highly specific for NADH. They share a 53 % sequence identity . MR and PETN reductase show high sequence similarity with the membres of of Old Yellow Enzyme (OYE) family.

The main objectives of this research

The work described in this thesis aims to elucidate the factors controlling the activity and specificity of PETN reductase, towards xenobiotic compounds, with a view to engineering more active and specific enzymes. On a more fundamental level, such an understanding could provide further insight into the successful application of rational protein design, which requires a good understanding of the structure and mechanism of the target enzyme.

Results and conclusion

The molecular structure of PETN reductase when complexed with substrates and inhibitors of different classes, such as nitroaromatics (TNT, picric acid, DNP), cyclic enones (3-oxo-steroids, 2-cyclohexen-1-one) and monovalent anions as well as the structure of the reduced form of PETN reductase, were solved at atomic resolution by X-ray diffraction. The crystal structure of morphinon reductase in the presence of codeinone substrate was also solved at high resolution. The overall structure of PETN reductase and morphinone reductase shows a typical eight stranded β/α barrel with a non-covalently bound FMN as prosthetic group. The pattern of the hydrogen bond network, that the flavin is involved in, is conserved throughout the β/α barrel of FMN dependent oxidoreductases.

Two further supersecondary structures have been found to confer certain characteristic ligand binding patterns on PETN reductase and MR. An external helix between strand β8 and helix8 has the function of stabilizing the phosphoryl moiety of FMN. The other supersecondary structure consists of an extra barrel and a pair of β-strands (β-loop excursion) that run antiparallel in front of the access channel to the active site between barrel (β_3/α_3). The β-loop excursion in PETN reductase is comprised of several positively charged amino acid residues. In contrast, in MR negatively charged amino acids dominate this β-loop. In PETN reductase, steroid binding has induced large conformational changes in two arginines residues Arg^{142} and Arg^{130} in the β-loop excursion. The position of the two arginines is optimal to form interactions with the phosphate group of NADPH, therefore they are potential candidates to confer specificity for NADPH. The corresponding arginines in MR are replaced by acidic residues. Structural data suggests that the β-loop excursion of PETN reductase and MR can be the motif of nicotinamide coenzyme recognition and differentiation.

The *si*-face of the flavin is open to a well-defined solvent-filled access channel (20 Å in length) of the active centre. Two juxtaposed tyrosines in the access channel provide a gating mechanism for controlling ligand binding. All ligands in PETN reductase and MR binds above the *si*-face of the flavin. Ligands such as phenolic derivatives (picric acid, DNP, benzaldehide) and cyclic enones (3-oxo steroids and codeinone) through their O-donor atoms are hydrogen bonded to the conserved His^{181} and His^{184} in PETN reductase and with the corresponding His^{186} and Asn^{189} in MR. Comparing the active site of PETN reductase with that of MR, the striking difference is the presence of a cysteine (Cys^{191}) residue in MR, which replaces the conserved Tyr^{186} of PETN reductase. The position of Tyr^{186} of PETN reductase is the putative proton donor in cyclic enones reduction in OYE homologs. In contrast, Cys^{191} is not involved in the mechanism of cyclic enones reduction, as mutagenesis studies have shown. The function of Cys^{191} is to create an appropriate space in the active centre for a bulky and conformationally restrained opiate binding.

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PETN reductase catalyses the hydrogenation of C1-C2 olefinic bond of 3-oxo-1,4-diene steroids. PETN reductase shows multiple substrate binding modes in different redox states of the enzyme. In the oxidized form of PETN reductase, steroids bind with their β face above the *si* face of FMN. In this binding geometry the reductive olefinic bond is not positioned in close proximity to the flavin N5, which can be compatible with hydride transfer. Deuterium labeling methods have been developed to elucidate the stereochemistry of bond reduction. The two electron reduced form of PETN reductase binds the steroid substrate with its opposite face above the *si* face of FMN to realign the C1-C2 double bond favourable for hydride transfer. Hydride addition from flavin N5 occurs to the 1a steroid position followed by proton uptake from the putative acid, Tyr^{186}, to the 2β position of the steroid. In the reduced enzyme the flavins' "butterfly"configuration may induce an alternative substrate binding mode, to avoid an unfavourable steric interaction between the C19 β-methyl group of the steroid and the flavin.

The reductive transformation of TNT catalysed by PETN reductase, proceeds via parallel running reaction pathways, which consist of the direct hydrogenation of the aromatic ring (i) and the successive two-electron reductions of the nitro groups (nitroreductase pathway, ii). The molecular structure of the enzyme-TNT and enzyme-picric complexes provides the rational of direct hydrogenation of the aromatic ring. The π-π interactions between the isoalloxazine ring of FMN and the nitroaromatics places the ligands above the flavin. The position of the nitro groups are also influenced by the two active site histidines through either hydrogen bonds (in case of picric acid and DNP) or van der Waals forces (in case of TNT). All these interactions contribute for ideal binding geometry of the nitroaromatic substrate for hydride attack from flavin N5 to C5 and

C3 positions of TNT and picric acid, resulting in hydride-Meisenheimer and dihidro-Meisenheimer complexes. In contrast with *Mycobacterium sp*. strain HL 4NT-1 and Xenobiotic reductase, B from *Pseudomonas fluorescens* I-C, the dihidro-Meisenheimer complex is not the dead end of the enzymatic reduction. PETN reductase is able to reduce dihidro-Meisenheimer complex into an unknown product in the presence of nitrite liberation. The importance of nitrite liberation in the PETN catalysed TNT biotransformation is that the bacteria strain, *E. Cloacea,* which produces the enzyme, can utilize TNT as a sole nitrogen source.

The two active site histidines, His^{181} and His^{184}, were investigated to establish their function in nitroaromatics degradation. The two histidines were changed to aniline by site-directed mutagenesis and created H181A and H184A PETN reductase mutants. The change of histidine to alanine has dramatically altered the chemical activity of the enzyme. The mutations either of 181 or 184 positions have completely eliminated the aromatic ring hydrogenation ability of the two electron reduced PETN reductase on TNT. The mutant enzymes have shown only nitroreductase activity, like its close horologes such as MR and OYE. Solution studies indicate that the presence of both histidines, His^{181} and His^{184}, is strictly necessary to maintain the active site geometry, which positions the electrophilic C5 C3 of the nitroaromatic ring optimally for direct hydride transfer from flavin N5.

The crystal structure of PETN reductase, in complex with picric acid, at 0.9 Å resolution, has revealed that Trp^{102} can be a role in nitroaromatics degradation. Picric acid binding induces conformational change in Trp^{102} to avoid steric clash. Since the binding of picric acid is partial (the calculated occupancy is 34%), Trp^{102} exists in multiple conformations. The change in the Trp^{102} conformation generates a series of multiple conformations in the main chain and side chains. Besides the substrate binding effect there are region of the structure where multiple conformations are found due to the disordered nature of these amino acids. Most frequently the side chains of arginine, glutamate, valine and serine show mixed conformational populations. The structure at subatomic resolution provides further information about the protein dynamics.

Based on structural data Trp^{102} in PETN reductase, was mutated into phenylalanine (W102F) and tyrosine (W102Y). The crystal structure of W102F and W102Y PETN reductase mutants in complexes with picric acid show that ligand is bound at full occupancy in the active centre without steric hindrance of the Tyr^{102} and Phe^{102} side chains. The alignment of C5 of picric acid with the flavin N5 is more optimal for hydride transfer in the mutants enzymes. In the absence of structural data with TNT-PETN reductase mutants (W102F and W102Y) complexes, it is assumed that TNT is likely to bind in a similar manner as can be seen in the wild type enzyme. The consequence of optimal nitroaromatics binding for hydride transfer, in the mutants enzymes is that the observed rate of Meisenheimer complex formation has increased three times in the presence of W102Y PETN reductase mutant compared with that measured in the presence of wild type enzyme. Mutation of Trp^{102} has minor effects on the overall active site structure of PETN reductase, but the small structural changes in the active centre has a direct effect on nitroaromatics binding, accelerating the hydrogenation of the aromatic ring.

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