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Crystal structures of two *Bacillus* carboxylesterases with different enantioselectivities

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ABSTRACT

Naproxen esterase (NP) from *Bacillus subtilis* Thai I-8 is a carboxylesterase that catalyzes the enantioselective hydrolysis of naproxenmethylester to produce *S*-naproxen (E > 200). It is a homolog of CesA (98% sequence identity) and CesB (64% identity), both produced by *B. subtilis strain* 168. CesB can be used for the enantioselective hydrolysis of 1,2-*O*-isopropylideneglycerol (solketal) esters (E > 200 for IPG-caprylate). Crystal structures of NP and CesB, determined to a resolution of 1.75 Å and 2.04 Å, respectively, showed that both proteins have a canonical α/β hydrolase fold with an extra N-terminal helix stabilizing the cap subdomain. The active site in both enzymes is located in a deep hydrophobic groove and includes the catalytic triad residues Ser130, His274, and Glu245. A product analog, presumably 2-(2-hydroxyethoxy)acetic acid, was bound in the NP active site. The enzymes have different enantioselectivities, which previously were shown to result from only a few amino acid substitutions in the cap domain. Modeling of a substrate in the active site of NP allowed explaining the different enantioselectivities. In addition, Ala156 may be a determinant of enantioselectivity as well, since its side chain appears to interfere with the binding of certain *R*-enantiomers in the active site of NP. However, the exchange route for substrate and product between the active site and the solvent is not obvious from the structures. Flexibility of the cap domain might facilitate such exchange. Interestingly, both carboxylesterases show higher structural similarity to *meta*-cleavage compound (MCP) hydrolases than to other α/β hydrolase fold esterases.

1. Introduction

Nature has evolved a diverse range of esterases with high regio- and stereoselectivity to convert a broad variety of chemical compounds in various metabolic processes [1]. Because of their specificity and selectivity, they are frequently used in biotechnological applications, such as in the food industry, for the production of perfumes, and for the synthesis of pharmaceuticals [2].

Carboxylesterases (carboxylic acid ester hydrolases, E.C. 3.1.1.1.) are a subset of esterases that hydrolyze small, water-soluble ester containing molecules resulting in the formation of an alcohol and a carboxylic acid. The microorganism *B. subtilis* strain Thai I-8 produces an enantiospecific carboxylesterase, called naproxen esterase or carboxylesterase NP (NP), which can be applied as a biocatalyst for the enantioselective resolution of racemic 2-arylpropionates, such as the important non-steroidal anti-inflammatory drugs naproxen, ibuprofen, indomethacin, and nabumetone. NP has a product ee of 96% based on the substrate *S*-naproxenmethylester (Fig. 1) and 99% on *S*-naproxenethylester (E > 200) [3,4]. In contrast, the enzyme's enantioselectivity towards 1,2-*O*-isopropylideneglycerol esters (IPG esters) (Fig. 1) is not sufficient for industrial processes (E values of ~1.2 for C4 and 1.3 for C8 IPG esters, respectively) [5]. (*S*)-IPG (D-(+)-solketal) is an important building block for the synthesis of the biologically active forms of several well-known pharmaceuticals and endogenous compounds such as β-blockers, prostaglandins, and leukotrienes [5,6].

B. subtilis strain 168 contains two homologs of NP called CesA (98% identical to NP; also known as carboxylesterase NA) and CesB (64% identical to NP; also known as YbfK). Unlike NP, CesB does convert (*S*)-IPG-caprylate ester with high enantioselectivity to (*S*)-IPG (ee of 99.9%, E > 200), but not (*R*)-IPG-caprylate ester (Fig. 1) [5]. No marked differences in activity and selectivity were observed between NP and CesA [7].

All carboxylesterases of which 3D-structures have been elucidated to date share high structural similarity; all contain the characteristic α/β hydrolase fold (see the ESTHER database) [8]. This fold is composed of a central β-sheet flanked on both sides by α-helices and serves as a stable protein core where, during evolution, amino acid substitutions, loop insertions or deletions have led to enzymes with diverse catalytic functions [9–13].

Abbreviations: ee, enantiomeric excess; CesA, carboxylesterase CesA; CesB, carboxylesterase CesB; MCP, *meta*-cleavage compound; IPG, 1,2-*O*-isopropylideneglycerol; NP, carboxylesterase NP

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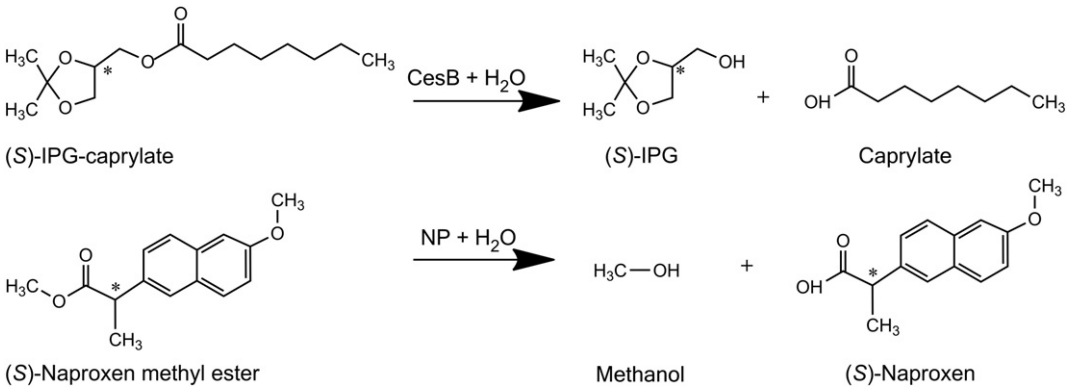

Fig. 1. Two-dimensional depiction of the reaction catalyzed by the carboxylesterases. The * indicates the chiral carbon atom.

Members of the α/β hydrolase fold have a nucleophile-His-acid catalytic triad with a sequence order of nucleophile, acid, and histidine [14]. In carboxylesterases the nucleophile is a serine residue, while the acid can be an aspartate or a glutamate residue. The serine residue is the central residue of the conserved pentapeptide sequence motif Gly–X1–Ser–*X*2–Gly [1], which defines a 'nucleophilic elbow' characterized by a very sharp turn between strand β5 and the following α-helix. In the discussed carboxylesterases this motif is Gly–Leu–Ser–Leu–Gly.

The catalytic mechanism proposed for carboxylesterases starts with nucleophilic attack by the serine hydroxyl group on the substrate carbonyl carbon atom of the scissile bond. The serine hydroxyl group is activated by the catalytic histidine/acid pair, which takes up the proton from the Ser OH group. A transient tetrahedral intermediate is formed, which is stabilized by two peptide nitrogen atoms, usually from residues on strands β5 and β3, forming the so-called "oxyanion hole". The proton is then transferred from the histidine to the leaving alcohol group, while the acid group of the substrate becomes covalently bound to the serine forming the covalent intermediate. Next, the histidine activates a water molecule, which hydrolyzes the covalent intermediate via nucleophilic attack on the carbonyl carbon of the intermediate. After the hydrolysis, the histidine donates its proton back to the serine, and the acyl component of the substrate is released [11,15].

Although the catalytic machinery of α/β hydrolases is very similar, the means by which they bind substrates varies from protein to protein. In most cases, the "cap" or "lid" domain, which shields the catalytic triad, contributes to substrate binding [12]. This domain is usually inserted at the C-terminal ends of strands β4, β6, β7, or β8, and may differ considerably in size in the various enzymes [12].

Here, we report the crystal structure of NP at 1.75 Å resolution, with a product analog in the active site, as well as the crystal structure of CesB at 2.0 Å resolution. Surprisingly, the NP and CesB structures resemble more those of the C–C bond cleaving (MCP) hydrolases than the α/β hydrolase fold esterases. Modeling of a substrate in the active site of NP allowed to defining the residues that determine their different enantiopecificities, in agreement with previous site-directed mutagenesis results [6].

2. Materials and methods

2.1. Expression, purification, crystallization and structure determination of NP

The *nap* gene coding for NP from *B. subtilis* Thai I-8 (CBS 679.85) was cloned into *B. subtilis* I-85. Cells were grown in 2× YT medium and harvested by centrifugation. After treatment of the cells with lysozyme and DNAse, the soluble fraction was harvested by centrifugation. Proteins were precipitated with 60% ammonium sulfate and redissolved in 0.02 M MOPS buffer, pH 7.5, and ultrafiltrated with an Amicon YM filter. The resulting solution was applied to an analytical HPLC-SEC column (TSK 2000 SW, 2 times 300 × 7.5 mm), and the protein was eluted with 0.01 M MES (2-(N-morpholino)ethane sulfonic acid), pH 5.6, and 0.1 M NaCl with a flow rate of 1 ml/min. The purified enzyme was lyophilized [16].

Crystallization of naproxen esterase was performed as described earlier [17]. In short, hexagonal plate-like crystals were grown by liquid–liquid diffusion by filling glass capillaries with 5–10 μl of 80% (w/v) PEG 6000 in 0.1 M Tris–HCl, pH 8.0, and closing them on one side. The equivalent amount of the protein solution (10 mg/ml) in 0.1 M Tris–HCl was added on top of the PEG column and then the capillaries were closed. The temperature was gradually increased over a four-week period from 4 °C to room temperature.

Crystals were mounted in a cryoloop and flash-cooled, without adding any cryoprotectant, prior to data collection. Data were collected on the BW7B beamline of the EMBL outstation at DESY (Hamburg, Germany) at 100 K. Intensity data were processed using DENZO and SCALEPACK [18]. The crystal diffracted to 1.75 Å and belonged to the trigonal space group $P3_221$ (Table 1), with one monomer of 33.8 kDa in the asymmetric unit (corresponding to a calculated V_M of 2.1 Å3/Da [19], and an estimated solvent content of 40%).

The crystal structure of NP was solved by molecular replacement with PHASER [20], using a search model composed of three homologous proteins, which were suggested by the Fold and Function Assignment System (FFAS) server [21]. The search model was composed of the structures of *Pseudomonas putida* esterase (PDB ID: 1ZOI) [22], the *meta*-cleavage product hydrolase (CumD) from *Pseudomonas fluorescens* IP01 (PDB ID: 1IUN) [23], and the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD) from *Rhodococcus* sp. strain RHA1 (PDB ID: 1C4X) [24], superimposed on each other.

Several cycles of manual re-building with Coot [25], automatic model building with ARP/wARP [26], and refinement with REFMAC5 [27] were performed in order to improve and to expand the original model of 125 amino acid residues distributed in 6 β-strands and 5 α-helices. Subsequently, a strategy to avoid model bias was applied, employing density modification, solvent flattening, statistical phase improvement, and automated model building performed in cycles using RESOLVE [28]. The best partial model generated by RESOLVE contained 180 amino acid residues with additional interpretable electron density, suitable for manual building. The model was completed by a few cycles with ARP/wARP and was further refined with REFMAC5 using TLS refinement [29]. Water molecules were automatically added by Coot, and manually checked. A 2-(2-hydroxyethoxy)acetic acid molecule was included at the last stage of refinement in the proximity of the nucleophile Ser130. The final model consists of 283 amino acid residues (9–293), 220 water molecules and one 2-(2-hydroxyethoxy)acetic acid molecule and has a *R* factor of 22.4% and R_{free} of 26.4% (Table 1). The first 8 and last 7 residues are not visible in the electron density and therefore not included in the final model. The stereochemical