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Roles of tryptophan residue and disulfide bond in the variable lid region of oxidized polyvinyl alcohol hydrolase

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ABSTRACT

Oxidized polyvinyl alcohol hydrolase (OPH) catalyzes the cleavage of C–C bond in β-diketone. It belongs to the α/β-hydrolase family and contains a unique lid region that covers the active site. The lid is the most variable region when pOPH from *Pseudomonas* sp. VM15C and sOPH from *Sphingopyxis* sp. 113P3 are compared. The wild-type enzymes and the pOPH mutants W255A, W255Y and W255F were analyzed for lipase activity by using p-nitrophenyl (pNP) esters as the substrates. The wild-type enzymes showed increased K_m and decreased k_{cat}/K_m with the acyl chain length, and the mutants showed reduced k_{cat}/K_m for pNP acetate, indicating the importance of Trp255 in sequestering the active site from solvent. The significantly lower activity for pNP butyrate can be a result of product inhibition, as suggested by the complex crystal structures, in which butyric acid, DMSO or PEG occupied the same substrate-binding cleft. The mutant activity was retained with pNP caprylate and pNP laurate as the substrates, reflecting the amphipathic nature of the cleft. Moreover, the disulfide bond formation of Cys257/267 is important for the activity of pOPH, but it is not essential for sOPH, which has a shorter lid structure.

1. Introduction

Polyvinyl alcohol (PVA) has been widely used in the industry and may cause environmental problems. However, PVA is biodegradable by oxidation and hydrolysis. Oxidized PVA hydrolase (OPH) was identified in hydrolyzing oxidized PVA (OPA) [1,2], which is a product of PVA dehydrogenase or oxidase [3]. Previously we obtained the crystal structures of pOPH from *Pseudomonas* sp. VM15C and sOPH from *Sphingopyxis* sp. 113P3 [4]. Analyses of the crystal structures and amino-acid sequences of related proteins confirmed that OPH belongs to the α/β-hydrolase family.

The α/β-hydrolase family is constantly expanding. They are mainly involved in breaking C–O bond (as in esterase and lipase), C–N bond (protease), C–S bond (thioester hydrolase), C–C bond (β-diketone hydrolase), and so on [5]. The core of α/β-hydrolase contains a highly conserved catalytic triad [6]: a nucleophile residue (Ser or Cys), a His residue, and an acidic residue (Asp or Glu). The nucleophile residue is located in a sharp turn, so called the "nucleophile elbow", which has a consensus sequence Sm-X-Nu-X-Sm (Sm = small residue, mostly Gly, X = any residue, and Nu = nucleophile residue). The oxyanion hole is always formed by the backbone amides to stabilize the transition state during the catalytic reactions [6]. Interestingly, in our previous study, a double oxyanion hole was identified in OPH.

The two OPH structures also showed a lid region with β-ribbon topology. The lid, covering the active site, is seen in most lipases as well but is always an amphipathic helix. When lipases hydrolyze nonpolar substrates, it is essential to expose the hydrophobic binding pocket by changing the lid region from "close" form to "open" form [7]. In addition, the lid plays a key role in substrate specificity of lipases [8]. Xu et al. reported a structure of lipase from *Malassezia globosa* (SMG1; PDB 3UUE), which is strictly specific for mono- and diacylglycerol [9]. Trp229 and Phe278 were considered as key residues to hinder triacylglycerol binding. The mutants of W299L and W116A altered the substrate preference and improved the thermostability of SMG1 [10]. Other lid modifications of lipase increased

Abbreviations: PVA, polyvinyl alcohol; OPA, oxidized PVA; OPH, OPA hydrolase; pNP, p-nitrophenyl; pNPA, pNP acetate; pNPB, pNP butyrate; pNPC, pNP caprylate; pNPL, pNP laurate; pNPP, pNP palmitate; DMSO, dimethyl sulfoxide; PEG, polyethylene glycol; DTT, dithiothreitol.

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the catalytic activity [11] and changed the substrate specificity and enantioselectivity [12].

The lid is the most variable region when the sOPH and pOPH structures are compared. The lid-mutant W255Y of pOPH showed the highest activity towards OPA but not pNP caprylate (pNPC). The mutants R264A and Y270F significantly improved catalytic efficiency towards both substrates, whereas the double mutant C257A/C267A showed decreased activity [4]. Trp255, located at the entrance of the active site tunnel, is essential for substrate specificity. In this study we measured the activity by using different chain-length pNP esters and compared the results of wild-type sOPH and pOPH as well as three pOPH mutants of the lid residue Trp255. The pNP esters were also used in complex crystallization, which however showed only the acid part as a hydrolyzed product. Possible roles of the lid disulfide bond were also investigated.

2. Materials and methods

2.1. Expression and purification of the wild-type and mutant enzymes

The wild-type sOPH and pOPH were expressed and purified as before, and so were the pOPH mutants of S172A, S172C, W255A, W255Y and W255F [4]. Here we also used hosts of *Escherichia coli* BL21 *trxB* (DE3) to produce the fusion proteins of pOPH with TEV cutting site and His-tagged thioredoxin in the vector pET32a. After 48 h cultivation in Luria-Bertani (LB) medium at 20 °C, the *E. coli* cells were collected by centrifugation at 7000×*g* and lysed with a French Press instrument in a buffer containing 25 mM Tris, pH 8.0, 150 mM NaCl, and 20 mM imidazole. After centrifugation at 17,000×*g* to remove cell debris, the supernatant containing pOPH was purified using a Ni–NTA column (GE Health, Uppsala, Sweden). The fusing protein was eluted and digested by TEV protease to remove the His-tagged thioredoxin. The untagged pOPH was eluted from a second Ni–NTA column, and further purified by using a Q column. Each purified protein was concentrated by a 60 mL Amicon Ultracentrifugal stirred cell with a 10,000 molecular weight cutoff membrane (Millipore, Bedford, MA, USA). The purity (>95%) was checked by using SDS–PAGE.

Construction of the sOPH mutant C241A/C248A used pET20b (+)-sOPH as the template with the forward primer 5′-GGTGAAAAA-GATCTGTGGGATGCTGGTCCACCTCTGGGTCTGGCTTCTGAT-TACCGTCCAACCACC-3′. After 72 h cultivation at 25 °C in Terrific Broth (TB) medium, the supernatant containing His-tagged sOPH was harvested by centrifugation at 7000×*g*, and loaded on a Ni–NTA column. The sOPH was eluted with about 60 mM imidazole in the above buffer and, after checking for purity, concentrated for activity test.

2.2. Analysis of enzymatic activity

OPH activity toward different pNP esters, including pNP acetate (pNPA; C2), pNP butyrate (pNPB; C4), pNPC (C8), pNPL (C12) and pNP palmitate (pNPP; C16), was analyzed in a reaction mixture (0.1% gum Arabic, 0.2% sodium deoxycholate and 50 mM Tris–HCl, pH 8.0) as previously described [4]. The increment of absorbance at 405 nm was recorded for 3 min at 37 °C. The kinetic parameters (K_m and k_{cat}/K_m) of sOPH, pOPH and its mutants were calculated by Michaelis–Menten curve fitting. Each value represents the mean of triplicate experiments by varying substrate concentrations (1.0 mM, 1.5 mM, 2.0 mM and 3.0 mM). The enzyme concentrations were determined by the Bradford method [13] using bovine serum albumin (BSA) as the standard.

2.3. Crystallization, data collection and structure determination

The wild-type (WT) pOPH was crystallized by using Crystal Screen II kit (Hampton Research, Laguna Niguel, CA) and sitting-drop method. The reservoir solution (No. 26) contained 30% PEG5000 MME, 0.1 M MES pH 6.5 and 0.2 M $(NH_4)_2SO_4$. No cryoprotectant was necessary for data collection of the WT crystal. The mutant pOPH crystals of S172A and S172C were obtained by using a reservoir solution of 0.1 M tri-sodium citrate, pH 5.6, 30% w/v PEG4000, and 0.3% w/v n-octyl-β-D-glucoside, similar to the previous conditions [4].

The complex crystals were prepared by soaking, in which a cryoprotectant solution (0.15 M tri-sodium citrate, pH 5.6, 35% w/v PEG4000, 0.8% w/v n-octyl-β-D-glucoside) was employed. To obtain pNPB and nonanedione complexes, the S172A and S172C crystals were soaked in the above cryoprotectant solution containing 20 mM pNPB and nonanedione (dissolved in DMSO), respectively, for at least 3 h before data collection. Later these crystals were found to contain butyrate and DMSO.

The X-ray diffraction datasets were collected at beam line BL13B1 and BL15A1 of the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan). The data were processed and scaled using the program HKL2000 [14]. Each asymmetric unit had one pOPH molecule. The structures were solved by molecular replacement, using PDB 3WL6 (chain A) as a search model. The models were refined by employing the programs CNS [15] and Coot [16]. PyMOL (DeLano Scientific, http://pymol.sourceforge.net/) was used in making figures.

2.4. PDB accession numbers

The coordinates of the pOPH crystals S172A/pNPB, S172C/DMSO, and WT/PEG have been deposited in the PDB with the accession codes of 3WWC, 3WWD, 3WWE, respectively.

3. Results

3.1. Characteristics of sOPH and pOPH with pNP esters as the substrates

As shown in Table 1, pOPH had lower K_m and higher k_{cat}/K_m values than sOPH for most pNP esters. The difference is most prominent in catalyzing the hydrolysis of the longer-chain substrates pNPC (C8) and pNPL (C12). It indicates that pOPH is endowed with the better catalytic properties in general. Moreover, the K_m values of both OPHs for pNP esters increased with the chain length of the fatty acyl substrates, whereas the k_{cat}/K_m values tended to decrease with the chain length, except for pNPB (C4). The k_{cat}/K_m for pNPB is significantly lower than those for all other substrates, no matter whether the enzyme is sOPH, pOPH or a lid mutant of Trp255.

To probe its function, Trp255 of pOPH was mutated to Ala, Tyr or Phe, making three separate mutants. All three mutants showed reduced catalytic activity for pNPA (C2). W255A and W255Y had improved k_{cat}/K_m values for pNPC (C8) and pNPL (C12). The activity of both mutants for pNPP (C16) was also enhanced, with a higher k_{cat}/K_m of W255A and a lower K_m of W255Y. The activity of W255F for pNPC, pNPL and pNPP all remained about the same as WT.

3.2. X-ray structures of pOPH complexes with butyrate, PEG and DMSO

Because pNPB showed significantly slower rate of hydrolysis than the other substrates, we tried to crystallize the OPH/pNPB complex. In the end a complex was obtained by soaking the pOPH mutant S172A crystal. Besides, the wild-type pOPH and the mutant