Archives of Biochemistry and Biophysics 573 (2015) 59–68

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Altering the substrate specificity of methyl parathion hydrolase with directed evolution

Tee-Kheang Ng [a], Lawrence R. Gahan [b], Gerhard Schenk [b], David L. Ollis [a,*]

[a] Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia
[b] School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, Brisbane, QLD 4072, Australia

ARTICLE INFO

Article history:
Received 2 December 2014
and in revised form 10 March 2015
Available online 19 March 2015

Keywords:
Organophosphate pesticides
Bioremediation
Methyl parathion hydrolase
Substrate specificity
Directed evolution

ABSTRACT

Many organophosphates (OPs) are used as pesticides in agriculture. They pose a severe health hazard due to their inhibitory effect on acetylcholinesterase. Therefore, detoxification of water and soil contaminated by OPs is important. Metalloenzymes such as methyl parathion hydrolase (MPH) from *Pseudomonas* sp. WBC-3 hold great promise as bioremediators as they are able to hydrolyze a wide range of OPs. MPH is highly efficient towards methyl parathion ($1 \times 10^6\ s^{-1}\ M^{-1}$), but its activity towards other OPs is more modest. Thus, site saturation mutagenesis (SSM) and DNA shuffling were performed to find mutants with improved activities on ethyl paraxon ($6.1 \times 10^3\ s^{-1}\ M^{-1}$). SSM was performed on nine residues lining the active site. Several mutants with modest activity enhancement towards ethyl paraoxon were isolated and used as templates for DNA shuffling. Ultimately, 14 multiple-site mutants with enhanced activity were isolated. One mutant, R2F3, exhibited a nearly 100-fold increase in the k_{cat}/K_m value for ethyl paraoxon ($5.9 \times 10^5\ s^{-1}\ M^{-1}$). These studies highlight the 'plasticity' of the MPH active site that facilitates the fine-tuning of its active site towards specific substrates with only minor changes required. MPH is thus an ideal candidate for the development of an enzyme-based bioremediation system.

Introduction

Organophosphates (OPs)[1] constitute the most commonly used pesticides in agriculture and account for approximately 40% of total pesticide usage [1]. OP are generally toxic as they inhibit acetylcholinesterase, a serine hydrolase that catalyzes the breakdown of acetylcholine at cholinergic synapses, leading to hyperstimulation of nerve cells and death [2]. It is estimated that OPs are responsible for up to 3,000,000 poisonings and 220,000 deaths annually [3]. Oximes, a group of strong nucleophiles, were shown to be capable to reactivate inhibited acetylcholinesterase [4,5], and current treatments for OP poisoning rely on chemical antidotes such as cholinolytics, oximes, atropine and anticonvulsants to minimize toxic manifestations [6]. However, these antidotes have to be administered soon after exposure, as they are impotent against aged inhibited acetylcholinesterase. Furthermore, the effectiveness of oxime reactivation appears to depend on the identity of the OP compounds [7]. Excess OPs in the bloodstream will also re-inhibit acetylcholinesterase if they are not removed. Therefore, there is an urgent need to develop (i) methods to remove OPs from contaminated soils, as well as (ii) therapies to treat OP poisoning and (iii) biosensors for OP detection.

The use of OP-degrading enzymes is an effective approach for OP decontamination. Methyl parathion hydrolase (MPH; E.C. 3.1.8.1), a metal ion-dependent enzyme isolated from *Pseudomonas* sp. WBC-3, has attracted considerable attention due to its ability to hydrolyze a broad range of OPs [8,9]. The crystal structure of MPH reveals an αββα fold, characteristic for metallohydrolases [10]. Interestingly, MPH is structurally similar to metallo-β-lactamases (MBLs) from the B3 subgroup, including the enzyme L1 from *Stenotrophomonas maltophilia* and AIM-1 from *Pseudomonas aeruginosa*. MBLs are Zn^{2+}-dependent enzymes that have emerged as major threat to global health since they are capable of inactivating most of the commonly used antibiotics [11,12].

MPH is able to hydrolyze methyl parathion (MPS) at a rapid rate, with a k_{cat}/K_m ratio of $>10^6\ M^{-1}\ s^{-1}$ [10]. Limited information about the substrate preference and reaction mechanism of MPH is currently available. Residues Leu65, Leu67, Phe119, Trp179,

* Corresponding author.
E-mail address: ollis@rsc.anu.edu.au (D.L. Ollis).

[1] *Abbreviations used:* OP, organophosphate; MPH, methyl parathion hydrolase; SSM, site saturation mutagenesis; MBls, metallo-β-lactamases; EPO, ethyl paraoxon; LB, Luria–Bertani; StEP, staggered extension process; MCO, methyl chlorpyrifos oxon; ECO, ethyl chlorpyrifos oxon; TCPy, 3,5,6-trichloro-2-pyridinol.

http://dx.doi.org/10.1016/j.abb.2015.03.012
0003-9861/

Phe196, Leu258 and Leu273 are part of the substrate binding pocket [10]; while the substitutions of Phe196 and Leu273 by alanines enhanced enzymatic activity towards the substrate *p*-nitrophenyl diphenylphosphate [13], replacing Phe119, Trp179 and Phe196 by alanines is detrimental for the catalytic activity towards MPS [10].

An ideal bioremediator exhibits significant levels of activity for a broad range of OPs. Considering the close structural similarity between MPH and enzymes from the MBL superfamily, we hypothesized that the substrate specificity of MPH can be altered easily. Here, site saturation mutagenesis (SSM) was performed to generate mutant libraries that were screened for improved activity towards ethyl paraoxon (EPO), a substrate that is approximately 100-fold less efficient than MPS in the wild-type enzyme. Mutants isolated from each library were used as parents for DNA shuffling to obtain further enhancement towards EPO. Several mutants with significant improvement also towards "poor" substrates (*e.g.* chlorpyrifos) were thus found. The results highlight the active site 'plasticity' of MPH and establish this enzyme as a promising agent to be employed in bioremediation applications.

Materials and methods

Materials, bacterial strains, growth conditions and plasmids

Pesticides were purchased from either Chem Service (West Chester, PA, USA) or Sigma Aldrich (St. Louis, MO, USA). Molecular biology reagents and enzymes were obtained from New England Biolabs (Ipswich, MA, USA), Thermo Scientific (Waltham, MA, USA) or Sigma–Aldrich (St. Louis, MO, USA). DNA primers (Table S1 in Supplemental material) were synthesized by Geneworks (Hindmarsh, SA, Australia) or Integrated DNA Technologies (Coralville, IA, USA). Plasmid extraction and purification kits were obtained from Qiagen (Limburg, Netherlands) or Promega (Madison, WI, USA). Protein purification columns were purchased from GE Healthcare (Buckinghamshire, UK). *Escherichia coli* strain DH5α was used for all aspects of the work described. Cells harboring pJWL1030 plasmids were grown at 37 °C on Luria–Bertani (LB) broth or agar plates supplemented with 50 μg/mL kanamycin. Construction of pJWL1030 plasmid has been described previously [14]. *mpd*, the gene coding for MPH, was synthesized by DNA 2.0 (Menlo Park, CA, USA). The recombinant plasmid containing the *mpd* gene (MPH-pJWL1030) was constructed by amplification from the MPH-pET47b plasmid using primers P1 and P2. The nucleotides that encode the first 35 amino acid residues that constitute the signal peptide were excluded in this amplification. The PCR product was digested with *Ase*I and *Pst*I, and ligated into the *Nde*I and *Pst*I sites, and transformed into *E. coli* DH5α using electroporation.

Structural superimposition studies

The superimposition of MPH with MBL superfamily enzymes was done in two stages in PyMOL. PyMOL's Cealign command was initially used for the superimpositions. Minor adjustments were then added using PyMOL's pair fitting function, by using the two active site metals, the bridging water and metal coordination residues of MPH and a target protein as points of alignment. The PDB IDs used in the superimposition study were 1P9E [10], 4AWY [15], 1BVT [16], 1X8G [17], 1SML [18], 1QH5 [19], 2QED [20], 2BR6 [21] and 2CBN [22].

Library generation

The SSM libraries were constructed using a megaprimed, ligase-free site specific mutagenesis method reported by Tseng et al. [23] and Sanchis et al. [24]. The PCR reactions were performed using 30 ng of template DNA (WTMPH-pJWL1030), 1× HF buffer, 1.5 mM $MgCl_2$, 0.1 μM of each mutagenic and flanking primer, 1 U of Phusion polymerase (Thermo Scientific, Waltham, MA, USA) and 0.2 mM of each dNTP. The amplification program for mutagenesis was as follows: initial denaturation for 3 min at 98 °C, followed by 18 cycles of 1 min at 98 °C, annealing for 1 min at 55 °C and extension at 72 °C. The second stage consisted of 25 cycles of 1 min at 98 °C and extension for 5 min at 68 °C and a final extension for 10 min at 68 °C. Up to four reactions were pooled and treated with 20 U of *Dpn*I twice at 37 °C for 1 h each. The PCR product was subsequently purified and 2 μL of the product was transformed into *E. coli* DH5α using electroporation. Primers P2 and P3 were used to generate the L67P68R72-NDT library; P2 and P4 to generate the R72-NNK library; P2 and P5 to generate the F119-NDT library; P2 and P6 to generate the P150-NDT library; P2 and P7 to generate the W179-NNK library; P1 and P8 to generate the F196-NDT library; P1 and P9 to generate the L258-NDT library and P1 and P10 to generate the L273-NDT library. The sequences of the primers are shown in Table S1. NDT (N = A, C, G or T; D = A, G or T) randomisation was used in all libraries except R72-NNK and W179-NNK.

Shuffling of mutations isolated from the SSM library was performed using the staggered extension process (StEP) [25]. The StEP reaction (25 μL) was performed in a solution containing 30 ng of template DNA, 1 μM of the P1 and P2 primers, 0.2 mM of each dNTP, 1× HF buffer and 1 U of Phusion polymerase. The amplification program for StEP was as follows: initial denaturation for 5 min at 98 °C, followed by 120 cycles for 10 s at 98 °C, annealing for 10 s at 55 °C, extension for 2 s at 72 °C, final annealing for 2 min at 55 °C and final extension for 5 min at 72 °C. Up to four reactions were pooled, purified and digested with *Ase*I and *Pst*I. The digested PCR product was cloned into pJWL1030 as described for the construction of MPH-pJWL1030.

Library screening

The library screening procedures consisted of a primary and secondary screen. The primary screen master plate was prepared either by picking single colonies that were then inoculated in 96-well plates or by culture dilution methods developed by Stevenson et al. [26]. The former was used for most of the generated SSM and StEP shuffling libraries, while the latter was used for the L67P68R72-NDT library due to its much larger size. Mutants were subsequently transformed into *E. coli* DH5α; single colonies were picked and inoculated in 96-well round-bottomed plates (Sarstedt, Nümbrecht, Germany) containing 100 μL LB broth supplemented with 0.1 mM $ZnCl_2$ and 50 μg/mL kanamycin. For the culture dilution method, the transformants were diluted with LB-kanamycin supplemented with 0.1 mM $ZnCl_2$ and dispensed into 96-well round-bottom plates at as density of 3–4 cells per 100 μL. The plates were incubated for 24 h at 37 °C. Prior to activity assays, the cultures were resuspended by gentle pipetting and 10 μL of the 100 μL culture from each well was aliquoted into 96-well flat-bottom plates (Sarstedt, Nümbrecht, Germany) and lyzed with 10 μL of 0.5× BugBuster (Novagen, Darmstadt, Germany) for 15 min. The lysate was assayed with 119 μM ethyl paraoxon (EPO) in 50 mM HEPES, 100 mM NaCl, 0.1 mM $ZnCl_2$, pH 7.6, in a final volume of 200 μL. The activity was monitored by the release of *p*-nitrophenolate at 405 nm using a Spectramax M2e Microplate reader (Molecular devices, Sunnyvale, CA, USA) at 30 °C. The assay procedure was adapted and modified from reference [27]. Mutants that showed improved EPO activity were streaked on fresh LB-kanamycin plates and single colonies were picked for a secondary screen. The secondary screen assay was performed exactly as described for the primary screen. For the culture