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Short communication

High resolution crystal structure of a fluoride-inhibited organophosphate-degrading metallohydrolase

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ABSTRACT

Metal ion-dependent, organophosphate-degrading enzymes (OP hydrolases) have received increasing attention due to their ability to degrade and thus detoxify commonly used pesticides and nerve agents such as sarin and VX. These enzymes thus garner strong potential as bioremediators. The OP hydrolase from *Agrobacterium radiobacter* (OpdA) is one of the most efficient members of this group of enzymes. Previous studies have indicated that the choice of the hydrolysis-initiating nucleophile may depend on the pH of the reaction, with a metal ion-bridging hydroxide being preferred at lower pH (*i.e.* pH ≤ 8.5), and a terminally coordinated hydroxide at higher pH (*i.e.* pH > 9.0). Furthermore, fluoride was shown to be a potent inhibitor of the reaction, but only at low pH. Here, the crystal structure (1.3 Å, pH 6) of OpdA in presence of fluoride is described. While the first coordination sphere in the active site displays minimal changes in the presence of fluoride, the hydrogen bonding network that connects the dimetallic metal center to the substrate binding pocket is disrupted. Thus, the structure of fluoride-inhibited OpdA demonstrates the significance of this hydrogen bond network in controlling the mechanism and function of this enzyme.

Organophosphates (OPs) are non-natural compounds that have been synthesized since the late 1800s and have been used as petroleum additives, plasticizers and, in particular since World War II, as pesticides [1]. Since then OPs have played a crucial role in facilitating the global expansion of agriculture, but due to their inherent toxicity they pose a considerable risk to both human and environmental health [2]. While currently no clean and effective ways for their decontamination are available, microorganisms exposed to such compounds have evolved the enzymatic machinery to utilize them as nutrients to obtain phosphorous for metabolic functions [2]. Of particular relevance are OP-degrading phosphotriesterases (PTEs), the first of which was isolated in 1973 from a strain of *Flavobacterium* [3]. PTEs have since been identified in several microorganisms, including *Pseudomonas diminuta* [4] and *Agrobacterium radiobacter* [5]. The PTE expressed by *P. diminuta* (*i.e.* *O*rgano*P*hosphate *H*ydrolase - OPH) is identical to that found in the *Flavobacterium*, whereas the *A. radiobacter* enzyme (*i.e.* *O*rganophosphate-*d*egrading enzyme from *A. radiobacter* - OpdA) shares approximately 90% sequence identity with the other PTEs.

OPH and OpdA are among the best-characterized PTEs to date. Both contain a di-metallic metal center in their active sites with invariant amino acid ligands (Fig. 1). These include H55, H57 and D301 for the metal ion in the more buried α site, and H201 and H230 for the more solvent-exposed metal ion in the β site (unless stated otherwise residue numbers refer to the sequence of OpdA). In both enzymes the metal ions are bridged *via* a carboxylated lysine residue (K169) and a water/hydroxide molecule [6].

In terms of active site geometry OpdA and OPH are very similar to the di-Ni^{2+} urease; the six amino acids that interact with the two metal ions are invariant, including the carboxylated lysine residue [7,8]. However, despite conserved active site structures urease and the PTEs generally differ in their metal ion selectivity. The former has a strict requirement for Ni^{2+}, a fact that is also reflected in the complex mechanism required to transport Ni^{2+} ions into the active site *via* a series of accessory proteins [9–11]. While crystallographic and, more precisely, isothermal titration calorimetry measurements demonstrated that PTEs also bind two metal ions with high affinity in their active sites [12], their precise metal ion compositions are not firmly established; although an analysis of anomalous scattering data indicated that native OpdA may have a preference for the heterodinuclear Fe^{2+}-Zn^{2+} combination [13], PTEs can readily be activated with a range of divalent metal ions including Zn^{2+}, Co^{2+}, Mn^{2+} and Cd^{2+} [14,15]. Furthermore, at pH 8 OpdA also operates in presence of only Ni^{2+} with a k_{cat} of

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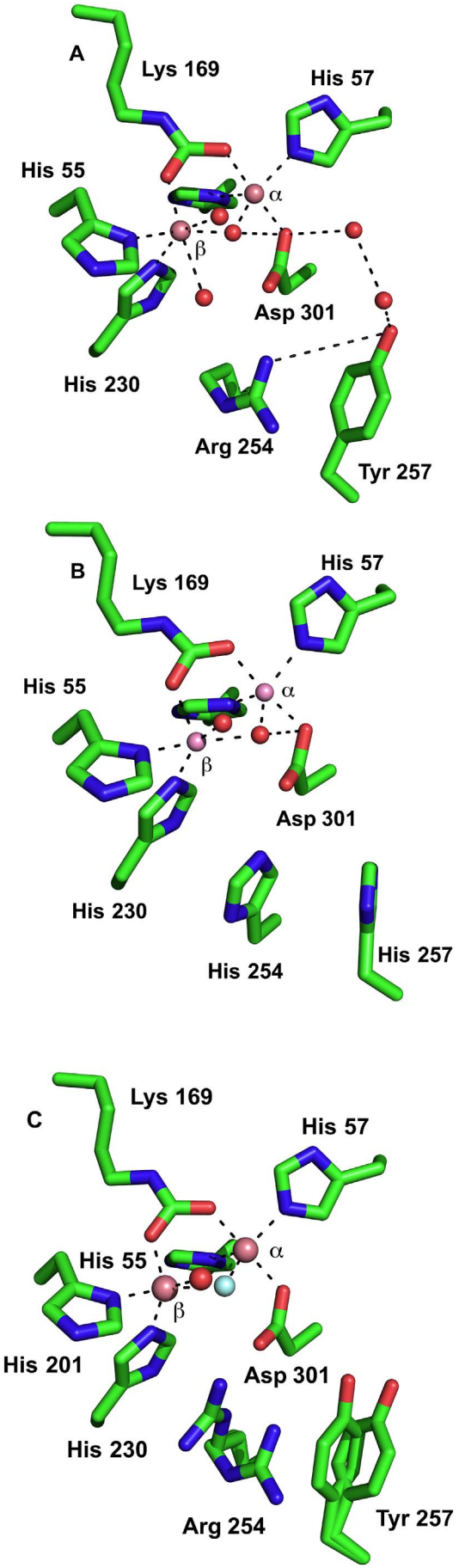

Fig. 1. Structures of OpdA and OPH (2D2J and 1JGM, respectively). (A) Active site of OpdA, illustrating the extensive hydrogen bond network connecting the di-Co^{2+} metal ion center to the substrate binding pocket. (B) Active site of di-Zn^{2+} OPH; no hydrogen bond is present. (C) Active site of di-Co^{2+} OpdA in presence of fluoride (5VEJ). The hydrogen bond network is disrupted and the substrate binding pocket residues R254 and Y257 display conformational flexibility. Water/hydroxide molecules are shown as red spheres, while fluoride is in cyan.

300 s^{-1} and a k_{cat}/K_m of 170 s^{-1} M^{-1}, using paraoxon as substrate (for comparison, under the same conditions the k_{cat} and k_{cat}/K_m of the Co^{2+} derivative of that enzyme are 930 s^{-1} and 1.3 × 10^7 s^{-1} M^{-1}, respectively (12,15); thus, Ni^{2+} strongly destabilizes substrate binding (high K_m)).[1] It is thus likely that PTEs do not have a single preferred metal ion composition, possibly a reflection of their recent evolutionary history that would have benefitted from a degree of flexibility in the selection of metal ions as activity can be reconstituted under diverse environmental conditions with respect to metal ion availability.

Proposed models for the reaction mechanisms employed by the two enzymes are also similar, invoking a role for the metal ion-bridging hydroxide as nucleophile initiating hydrolysis of the OP triester [2]. However, significant variations are observed with respect to their catalytic efficiency, substrate selectivity and metal ion affinities [2,14–17]. Site- directed mutagenesis and *in vitro* evolution studies demonstrated that in particular two residues in the substrate binding pocket may play an important role in mediating these variations: while R254 and Y257 in OpdA are integrated into an extensive hydrogen bonding network that connects the substrate binding pocket to the metal ion center (Fig. 1A), the corresponding residues in OPH are two histidines, and no hydrogen bond network is present (Fig. 1B). In OpdA this network controls the conformation of R254: when present the side chain is kinked, allowing access to the metal center, when absent the side chain is more linear, obstructing access [12]. It is this sequestration of the catalytic site that may underlie (i) the preference of OpdA for smaller substrates, (ii) this enzyme's enhanced catalytic efficiency and (iii) its increased metal ion affinity when compared to OPH [12]. This interpretation may also account for the distinct variation observed between OpdA and OPH with respect to their interaction with fluoride. Fluoride is a potent inhibitor for a number of metal ion-dependent hydrolases, including the di-Ni^{2+} urease [18], the di-Mn^{2+} arginase [19,20] and the Fe^{3+}-M^{2+} purple acid phosphatase (PAP, where M = Fe, Zn or Mn) [21]. OpdA is also inhibited by fluoride (K_i ~ 300 nM at pH 6.5), and the uncompetitive mode of inhibition, together with a strong exchange coupling of the metal ions is consistent with fluoride displacing the hydrolysis-initiating μ-OH [12].

Considering that OPH is expected to employ a mechanism similar to that of OpdA (*vide supra*) it is surprising that this enzyme is not inhibited by fluoride [12]. In order to better understand the interaction between OpdA and fluoride the enzyme was crystallized in presence of this inhibitor. The Co^{2+} derivative of OpdA was selected for this study, not only because it is the best studied derivative and crystallized readily, but also because its catalytic properties at the pH of crystallization (pH 6) are similar to those of other metal ion derivatives of this enzyme (Fig. S1 and [12,15]).

Recombinant di-Co^{2+} OpdA was expressed and purified using a well-established procedure [12,15,22,23]. The isolated enzyme was > 95% pure, judged by SDS PAGE analysis, and concentrated to 20 mg/mL for crystallization in presence of 100 μM fluoride (NaF). The enzyme was crystallized at 20 °C using the hanging-drop vapor-diffusion method. The crystallization solution was made up in 10 mM MES, pH 6.0, with 0.16 M calcium acetate, 80 mM sodium carbonate and 14% v/v PEG 1000. Crystals formed after five days.

[1] The Ni^{2+} derivative of OpdA was prepared using the exact procedure described for the preparation of the Co^{2+}, Zn^{2+} and Cd^{2+} derivatives of that enzyme [15]. The pH dependence of the catalytic parameters of Ni^{2+} OpdA is very similar to that observed for the Co^{2+} derivative (Fig. S1 in the Supplementary Section).