Characterisation of the organophosphate hydrolase catalytic activity of SsoPox

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SsoPox is a lactonase endowed with promiscuous phosphotriesterase activity isolated from Sulfolobus solfataricus that belongs to the Phosphotriesterase-Like Lactonase family. Because of its intrinsic thermal stability, SsoPox is seen as an appealing candidate as a bioscavenger for organophosphorus compounds. A comprehensive kinetic characterisation of SsoPox has been performed with various phosphotriesters (insecticides) and phosphodiesters (nerve agent analogues) as substrates. We show that SsoPox is active for a broad range of OPs and remains active under denaturing conditions. In addition, its OP hydrolase activity is highly stimulated by anionic detergent at ambient temperature and exhibits catalytic efficiencies as high as $k_{cat}/K_{M}$ of $10^5$ M$^{-1}$s$^{-1}$ against a nerve agent analogue. The structure of SsoPox bound to the phosphotriester fensulfothion reveals an unexpected and non-productive binding mode. This feature suggests that SsoPox's active site is sub-optimal for phosphotriester binding, which depends not only upon shape but also on localised charge of the ligand.

Organophosphates (OPs) are well-known potently toxic compounds because they irreversibly inhibit acetylcholinesterase, a key enzyme of the central nervous system. They have been extensively used since the end of World War II, primarily as agricultural insecticides. Their toxic properties have also been exploited for the development of chemical warfare agents (such as sarin, soman and VX). Enzymes that are capable of degrading OPs are therefore attractive as potential anti-dotes because of their intrinsic potential in decontamination/detection systems for organophosphates-based pesticides and nerve agents. Enzymatic detoxification of OPs has become the subject of numerous studies because current methods of removing them, such as bleach treatments and incineration, are slow, expensive and cause environmental concerns. For this application, OP hydrolases are appealing due to their broad substrate specificity and their high catalytic rate.

Bacterial phosphotriesterases (PTEs) are members of the amidohydrolase superfamily, enzymes catalysing the hydrolysis of a broad range of compounds with different chemical properties (phosphoesters, esters, amides, etc.). PTEs hydrolyse insecticide-derivatives such as paraoxon with diffusion limit like kinetic parameters. Moreover, PTEs catalyse the hydrolysis of various nerve agents with high efficiency. Because the widespread dissemination of these man-made chemicals began only in the 1950’s, it has been postulated that the PTEs might have evolved specifically to hydrolyse insecticides over a relatively short period of time.

A protein from the hyperthermophilic archaeon Sulfolobus solfataricus, SsoPox, was cloned, characterised and related to the PTE family (and was accordingly named paraoxonase). SsoPox indeed shares approximately 30% sequence identity with mesophilic PTEs, but hydrolyses paraoxon and other pesticides with a lower efficiency. SsoPox is an extremely thermostable enzyme, with an evaluated Tm of 104 °C and a denaturation half-life of 90 minutes at 100 °C. The thermostability of SsoPox was mainly attributed to a large hydrophobic dimer interface and an extensive salt bridge network. Two classical features of hyperthermostable proteins are considered as an excellent starting point for biotechnological applications and directed evolution (which was briefly explored). Isolated by virtue of its phosphotriesterase activity, biochemical and phylogenetic evidence later suggested that SsoPox belongs to another closely related protein family, the Phosphotriesterase-Like Lactonase (PLLs). The structure of SsoPox confirmed that it is a natural lactonase with a promiscuous phosphotriesterase activity. In particular, the activity detected against N-acyl-homoserine lactones (AHLs) may relate SsoPox and the PLLs to the AHL-based quorum sensing system and its inhibition by quorum quenching. The crystal structures of SsoPox free and in complex with an AHL mimic have illustrated the molecular adaptation of a dedicated lactonase to an optimised phosphotriesterase within the last few decades.
is approximately 2.5 times less efficient at 25 µM generated using ChemDraw software. All these compounds are phosphotriesters with the exception of the last three compounds that are phosphodiesters.

Figure 1 | Chemical structure of organophosphorus compounds used in this study. Chemical structure of (ethyl-)paraoxon (I), methyl-paraoxon (II), (ethyl-)parathion (III), methyl-parathion (IV), fensulfothion (V), malathion (VI), CMP-coumarin (VII), IMP-coumarin (VIII) and PinP-coumarin (IX) generated using ChemDraw software. These compounds are phosphotriesters with the exception of the last three compounds that are phosphodiesters.

Results

SsoPox OP hydrolase activity characterisation. Catalytic parameters of SsoPox with (ethyl-)paraoxon (Fig. II) were characterised at 70°C (kcat/KM = (1.22±0.21)×10⁸ M⁻¹ s⁻¹) (Table I). These results are in agreement with previous studies²⁰ (Table SI). Kinetic assays were also performed at 25°C (Table I), and reveal, for the first time, that SsoPox is approximately 2.5 times less efficient at 25°C than at 70°C (kcat/KM = (5.19±1.31)×10⁶ M⁻¹ s⁻¹). SsoPox is thus the most efficient PLL against paraaxon at both 70°C and 25°C (Table I & II). These values, however, are very low compared to the best organophosphate degrading enzyme, PTE from Pseudomonas diminuta, which exhibits second order rate constants for paraaxon near the diffusion limit (kcat/KM ~ 10⁸ M⁻¹ s⁻¹)²⁰. This observation is in agreement with the nature of PLLs which exhibit a promiscuous phosphotriesterase activity, whereas PTEs are natural phosphotriesterases²⁰,²¹.

Others OPs were also tested as substrates at 25°C (Table I), including the phosphotriesters methyl-paraoxon, (ethyl-)parathion, methyl-parathion, malathion and the phosphodiester CMP-coumarin, IMP-coumarin and PinP-coumarin (cyclosarin, sarin and soman derivatives, respectively, in which the fluoro substituent of cyclosarin has been replaced by a cyanocoumarin group²⁰; see Methods for more details) (Fig. I VII, VIII & IX). These assays showed that SsoPox exhibits about 2.5 times higher catalytic efficiency toward methyl-paraoxon than against (ethyl-)paraoxon (kcat/KM = (1.27±0.70)×10⁷ M⁻¹ s⁻¹ and (5.19±1.31)×10⁶ M⁻¹ s⁻¹, respectively). This preference is mainly due to a tenfold lower KM for methyl-paraoxon than for paraaxon. In a similar fashion, SsoPox shows higher catalytic efficiency for methyl-parathion (kcat/KM = 9.09 ± 0.90 M⁻¹ s⁻¹), compared with paraaxon for which no catalysis could be detected. This result suggests that the bulkiness of the substituent groups of certain phosphotriesters prevents a catalytically efficient binding. This feature was also previously observed for SsoPox and SacPox at 70°C,²² and for DrOPH at 35°C²⁶.

Although methyl-paraoxon and methyl-parathion differ by only one atom (the terminal oxygen of the phosphorous moiety is a sulphur atom in parathion), the reaction with methyl-paraoxon approximately 100 times more efficiently (kcat/KM = (1.27±0.70)×10⁷ M⁻¹ s⁻¹ and 9.09 ± 0.90 M⁻¹ s⁻¹, respectively). The observed KM values actually suggest that the Michaelis complex formation is more favourable with methyl-parathion than with methyl-paraoxon (approximately ten fold). However, the kcat decreases approximately 1000 times with methyl-parathion compared to methyl-paraoxon, which may reveal a less productive binding of thiono-phosphotriesters compared to that of oxons. This phenomenon was named the thiono-effect and a similar tendency was previously observed in Agrobacterium radiobacter PTE with chloryprifos and chlorpyrifos oxon²⁷. However, PTEs do not exhibit such a drastic difference regarding paraoxon which is only a slightly better substrate than parathion²⁸,²⁹.

Kinetik parameters were also recorded for the hydrolysis of another sulphur-containing organophosphate, the insecticide malathion (kcat/KM = 5.56 ± 1.26 M⁻¹ s⁻¹). This substrate possesses an equivalent KM value for the enzyme as for methyl-parathion, but the turnover is slower. Finally, SsoPox does not exhibit any detectable activity against PinP-coumarin, but hydrolyses the nerve agent analogs CMP-coumarin and IMP-coumarin with moderate efficiencies.

**Table I**

| Compound     | kcat/KM (M⁻¹ s⁻¹) |
|--------------|-------------------|
| (ethyl-)paraoxon | (1.22±0.21)×10⁸ |
| methyl-paraoxon | (5.19±1.31)×10⁶ |
| (ethyl-)parathion | (1.27±0.70)×10⁷ |
| methyl-parathion | (5.19±1.31)×10⁶ |
| fensulfothion   | (1.27±0.70)×10⁶ |
| malathion       | (5.19±1.31)×10⁶ |
| CMP-coumarin    | (1.27±0.70)×10⁶ |
| IMP-coumarin    | (5.19±1.31)×10⁶ |
| PinP-coumarin   | (5.19±1.31)×10⁶ |

The Pox activity towards various organophosphorus compounds is shown in Table SI. Kinetic assays were also performed at 25°C (Table I), and reveal, for the first time, that SsoPox is approximately 2.5 times less efficient at 25°C than at 70°C (kcat/KM = (5.19±1.31)×10⁶ M⁻¹ s⁻¹). SsoPox is thus the most efficient PLL against paraaxon at both 70°C and 25°C (Table I & II). These values, however, are very low compared to the best organophosphate degrading enzyme, PTE from Pseudomonas diminuta, which exhibits second order rate constants for paraaxon near the diffusion limit (kcat/KM ~ 10⁸ M⁻¹ s⁻¹). This observation is in agreement with the nature of PLLs which exhibit a promiscuous phosphotriesterase activity, whereas PTEs are natural phosphotriesterases. Others OPs were also tested as substrates at 25°C (Table I), including the phosphotriesters methyl-paraoxon, (ethyl-)parathion, methyl-parathion, malathion and the phosphodiester CMP-coumarin, IMP-coumarin and PinP-coumarin (cyclosarin, sarin and soman derivatives, respectively, in which the fluoro substituent of cyclosarin has been replaced by a cyanocoumarin group; see Methods for more details).

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DOC concentrations were used during the kinetic experiments to enhance paraoxon hydrolysis catalysed by SsoPox. Anionic detergents enhance paraoxon hydrolysis catalysed by SsoPox. Previous results have shown that SDS increases paraoxon hydrolysis by wt SsoPox. In this study, we tested various concentrations of SDS (Fig. 2A); the highest effect occurs at 0.01% with a velocity enhancement of approximately 5.5 times. The catalytic parameters of SsoPox at 25°C were thus characterised in the presence of 0.01% of SDS (Table I) and highlight an approximately 12.5 times catalytic efficiency enhancement compared to that without detergent at 25°C (Fig. 2H).

Enhancement of SsoPox paraoxonase activity by DOC. Different DOC concentrations were used during the kinetic experiments (Fig. 2B). The greatest effect was obtained with 0.05% DOC (k_cat/K_M = (1.72 ± 0.21) × 10^4 M⁻¹s⁻¹) (Table I). With approximately 33 fold catalytic efficiency improvement (Fig. 2H), the observed effect with 0.05% DOC is more pronounced than that observed with SDS. These results indicate that despite having two very different chemical structures (Fig. S1), the two anionic detergents SDS and DOC improve the kinetic parameters of SsoPox against paraoxon. Interestingly, this improvement might have different causes, because SDS produces an apparently equivalent effect on k_cat and K_M whereas DOC positively impacts K_M but decreases k_cat (Table I).

Effect of anionic detergents on nerve agent analogues hydrolysis. The ability of DOC and SDS to enhance the CMP-coumarin hydrolysis by SsoPox has been tested using 0.01% of each detergent (Table I). Catalytic parameters show that DOC and SDS allow catalytic efficiency enhancements of approximately 6 and 23 times, respectively, (DOC: k_cat/K_M = (4.63 ± 1.16) × 10^4 M⁻¹s⁻¹; SDS: k_cat/K_M = (1.92 ± 0.11) × 10^3 M⁻¹s⁻¹), which illustrates the potential of SsoPox for nerve agent decontamination.

SsoPox was characterised to be homodimeric in crystals and a rapid equilibrium between monomeric and dimeric forms was observed with size exclusion chromatography experiments. A possible explanation for the observed paraoxon hydrolysis enhancement is a detergent-induced dissociation of the SsoPox homodimer that could influence the kinetic parameters of the enzyme. Another possibility is a conformational change of the enzyme induced by the detergents. These possibilities were investigated by size exclusion chromatography (Fig. S2A), DLS experiments (Fig. S2B), and tryptophan fluorescence (Fig. S2C), but these studies did not reveal any significant differences with or without SDS.

Effect of other denaturing agents on SsoPox paraoxonase activity. Other denaturing compounds (guanidinium chloride, urea, tween 20, DMSO; see methods for more details) were used and their effect on the paraoxonase activity was recorded. All of the tested compounds can improve the hydrolysis of paraoxon catalysed by SsoPox (Fig. 2), albeit with variable amplitudes. Interestingly, unlike the two anionic detergents tested in this study, tween 20 only induces a mild improvement (Fig. 2C). Chaotropic agents such as guanidinium chloride (Fig. 2D) or urea (Fig. 2E), as well as the organic solvent DMSO (Fig. 2F), show very little paraoxon hydrolysis enhancement. The highest enhancements are observed with the anionic detergents SDS and DOC (Fig. 2G).

Fensulfothion is an inhibitor for SsoPox. Fensulfothion is a substrate for the P. diminuta PTE1 but was previously mentioned as a potent inhibitor for SsoPox. It thus constitutes a good candidate for structural studies, given the very high similarity between the chemical structures of fensulfothion and paraoxon (Fig. 1V & I). Preliminary experiments with SsoPox demonstrated that the K_I of fensulfothion, given that this molecule is not water-soluble, is too high to be characterised in classical conditions (data not shown). However, the K_I could be determined in 0.01% SDS (7.78 ± 1.23 mM) (Fig. S3). This value is very similar to the K_M values of the enzyme for paraoxon (24 mM without SDS; 4 mM with 0.01% SDS). Because of technical limitations, such as the very low solubility of fensulfothion in water, we were unable to determine experimentally the nature of the inhibition by kinetic experiments.

Unexpected binding mode of the fensulfothion. SsoPox is an enzyme that catalyses two different types of reaction: the hydrolysis of lactones, its preferred substrate, and the hydrolysis of promiscuous substrates, the OPs. The mechanism by which the two different chemical reactions occur within the same active site is not known yet, but some evidence suggests that the promiscuity originates in lactonases from an overlap between the stabilisation of the transition state species of the native substrate and the binding of the promiscuous one28,29. To understand how phosphotriesters bind to the active site of SsoPox, we performed co-crystallisation experiments.
with various phosphotriesters. Attempts with triethylphosphate and diethyl (4-methylbenzyl) phosphonate failed (Elias et al., unpublished). A complexed SsoPox structure was obtained with the inhibitor fensulfothion at medium resolution (2.68 Å). Interestingly, the binding mode of fensulfothion to the SsoPox’s active site is completely unexpected. Indeed, being (i) a very close mimic of paraoxon, and (ii) a substrate for the P. diminuta PTE, fensulfothion’s binding mode was expected to reveal the phosphotriester-binding mode of SsoPox. However, the structure reveals that the phosphorous moiety of fensulfothion does not bind to the bi-metallic centre, whereas the methyl sulfinyl group occupies the active site (Fig. 3A). This unexpected configuration is unambiguous from the
electronic density map, despite the medium resolution of the structure (Fig. 3B). Indeed, the presence of two heavy groups on both sides of the molecule (sulfinyl group on one side, the phosphothio-moity on the other) enables easy interpretation of the electronic density maps. It is noteworthy that putative nonproductive crystallographic complexes were also previously described with bound phosphotriesters to the \textit{P. diminuta} PTE structure \textsuperscript{30}.

The active site loop (loop 8) adopts a different conformation with fensulfothion binding, altering the dimer orientation. The fensulfothion-bound crystal structure of SoPox reveals conformational changes within the active site, especially related to the loop 8 conformation. This active site loop has been described as a key feature for lactone binding, because it creates a hydrophobic channel that binds the aliphatic acyl chain of lactones and undergoes conformational changes upon the lactone binding\textsuperscript{15}. The structural comparison of the fensulfothion-bound structure with the apo SoPox structure (Fig. 3A) reveals that the whole loop 8 adopts an alternate conformation. Residues W263, T265 and A266 interact with fensulfothion and undergo significant conformational changes (up to 2.5 Å).

The conformational changes that occur in loop 8 upon fensulfothion binding also influence the conformation of residues from the other monomer, because loop 8 is also involved in the dimerization of SoPox (Fig. 3A). Consequently, we observe a relative re-orientation of the second monomer in the other structures. The loop 8 conformation observed in the fensulfothion-bound structure is similar to the one observed in the lactone-bound structure. This is further illustrated by the similar dimer orientations of both bound structures compared to the apo structure (Fig. 3C).

Active site configuration. The sulfinyl group of fensulfothion binds to the bi-metallic active site, and more precisely to the more buried

Figure 3 | Structural studies. (A) Structural superposition between the apo structure of SoPox (PDB ID: 2vc5; monomer A) (in light grey sticks) and the complexed structure of SoPox with fensulfothion (monomer C; in blue sticks). The contacting monomers (forming the SoPox homodimer) are shown in dark grey (apo structure) and cyan (fensulfothion-bound structure). The fensulfothion molecule is shown as green sticks, and the two metal ions in the active site are represented by two spheres. (B) Fourier difference electronic density omit map for the bound fensulfothion. The F\textsubscript{obs} – F\textsubscript{calc} electronic density map was calculated omitting the fensulfothion molecule from the model in all monomers. The electronic density map is shown as a blue mesh (contoured at 2.5σ) and as a black mesh (contoured at 3.5σ). (C) Ribbon representation of SoPox homodimers from the apo structure (PDB ID: 2vc5; in red), the C10-homocysteine thiolactone (C10-HTL) bound structure (PDB ID: 2vc7; in yellow), and the fensulfothion-bound structure (in blue). The structures are superposed onto one monomer of the apo structure of SoPox (right monomer). This enables the observation of the relative re-orientation of the second monomer in the other structures. (D) Close view on the binding of fensulfothion (green sticks; monomer C) in the active site of SoPox (blue sticks). Metal cations and active site water molecules (in red) are shown as spheres. W2 corresponds to W362 (monomer A) in the deposited PDB coordinate file. Distances are indicated in Ångstrom.
metal (2.7 Å) (iron cation, α-metal) (Fig. 3D). The free doublet of electrons of the tetrahedral sulphur atom interacts with a water molecule (W2) bound to the more exposed metal (2.1 Å) (cobalt cation, β-metal). This water molecule also makes a hydrogen bond with the Y97 hydroxyl group (3.1 Å), a key residue for lactone binding[19] that is conserved amongst all PLLs and known lactonases from the metallo-β-lactamase superfamily[16]. The rest of the fensulfothion molecule is bound in the hydrophobic channel of SsoPox, formed by loop 8.

Discussion

SsoPox, an appealing candidate for OPs biodecontamination, is a native lactonase with promiscuous phosphotriesterase activity that belongs to the PLL family[14]. Our kinetic characterisation experiments highlight the fact that SsoPox exhibits by far the highest paraoxonase activity amongst PLLs at both 70°C and 25°C[14,17,21]. Moreover, we provide kinetic characterisations for various organophosphorus compounds, including methyl-paraoxon, methyl-parathion, malathion, IMP-coumarin and CMP-coumarin. Interestingly, SsoPox exhibits very low catalytic efficiency towards P=S containing organophosphates (e.g., the insecticides parathion, methyl-parathion and malathion). Between P=S and corresponding P=O substrates (e.g., methyl-parathion and methyl-paraoxon), the kcal value differs by 3 orders of magnitude. PTEs, albeit preferring paraoxon to paraoxon as a substrate, do not exhibit this marked thiono-effect[2,9], although some thiono-effect has been observed for ArPTE with chlorpyrifos and its oxon derivative[27]. PTEs constitute a protein family that is believed to have evolved in the last few decades to specifically hydrolyse man-made insecticides[8]. They have possibly emerged from native lactonases such as PLLs[24], and thus may have evolved to suppress this thiono-effect for certain insecticides. Recently, a study succeeded in reconstructing a PPL-like lactonase from the pPTE illustrating this potent evolutionary history[26].

The ability of SsoPox to hydrolyse insecticides as well as nerve agent analogues such as CMP-coumarin and IMP-coumarin strengthens the potential of this enzyme in biodecontamination. Moreover, we show an increase of the catalytic efficiency with the strength of the metal bond with the Y97 hydroxyl group (3.1 Å), a key residue for lactone binding[15] that is conserved amongst all PLLs and known lactonases from the metallo-β-lactamase superfamily[16]. The rest of the fensulfothion molecule is bound in the hydrophobic channel of SsoPox, formed by loop 8.

Methods

Production-purification of SsoPox. The gene encoding for SsoPox was optimised for E. coli expression, synthesised by GeneArt (Germany), and subsequently cloned into the pET23b plasmid using Ncol and HindIII as restriction enzymes. Protein production was performed in E. coli BL21(DE3)-pGro7/GroEL strain cells (TaKaRa) in 8 litres of ZYP medium[16] (100 µg/ml ampicillin, 34 µg/ml chloramphenicol) and grown by an overnight pre-culture. Cultures grew at 37°C to reach OD600nm = 1.5. The induction of the protein was made by starting the consumption of the lactose in ZYP medium with an addition of 0.2 mM CoCl2 and a temperature transition to 25°C for 20 hours. Cells were harvested by centrifugation (3000 g, 4°C, 10 min), re-suspended in lysis buffer (50 mM HEPES pH 8, 150 mM NaCl, CoCl2 0.2 mM, 0.25 mM mg/l lysozyme, 0.1 mM PMSF, 10 µg/ml DNase and 20 mM MgSO4) and stored at ~8°C. Suspended frozen cells were thawed and disrupted by three steps of 30 seconds of sonication (Branson Sonifier 450; 80% intensity and microtip limit of 8). Cell debris was removed by centrifugation (12000 g, 4°C, 30 min). As SsoPox is hyperthermostable[14], host proteins were precipitated by incubation for 30 minutes at 70°C and harvested by centrifugation (12000 g, 4°C, 30 min). A second step of heating at 85°C for 15 minutes and centrifugation was performed to precipitate more host proteins. Thermoresistant proteins from E. coli were eliminated by performing ammonium sulphate precipitation (326 g/l). SsoPox was concentrated by ammonium sulphate precipitation (476 g/l) and suspended in buffer 50 mM HEPES pH 8, 150 mM NaCl, 0.2 mM CoCl2. Remaining ammonium sulphate was removed by dialysis against the same buffer and the protein sample was then concentrated for separation on exclusion size chromatography (S75-16-60, GE Healthcare) to obtain pure protein.

Oligomerisation state-interaction analysis. Size exclusion chromatography. Experiments were performed in buffer 50 mM HEPES pH 8, 150 mM NaCl and 0.2 mM CoCl2 at room temperature with approximately 5 mg of purified protein using a GE Healthcare S75-16-60 column connected to an Amino A10M4 column. Experiments were performed to precipitate more host proteins. Thermoresistant proteins from E. coli were eliminated by performing ammonium sulphate precipitation (326 g/l). SsoPox was concentrated by ammonium sulphate precipitation (476 g/l) and suspended in buffer 50 mM HEPES pH 8, 150 mM NaCl, 0.2 mM CoCl2. Remaining ammonium sulphate was removed by dialysis against the same buffer and the protein sample was then concentrated for separation on exclusion size chromatography (S75-16-60, GE Healthcare) to obtain pure protein.

Fluorescence experiments. Experiments were performed in activity buffer with the addition of 0%, 1% and 0.01% (v/v) of SDS. SsoPox was excited at 280 nm (maximum excitation wavelength of tryptophan) and the emitted fluorescence was measured between wavelengths of 300 to 500 nm.

Kinetic assays. The time course of paraoxon hydrolysis by SsoPox at 70°C was monitored by following the production of p-nitrophenolate at 405 nm (ε = 17000 M⁻¹·cm⁻¹) in a 1-cm path length cell with a Cary WinUV spectrophotometer (Varian, Australia) using the Cary WinUV software. Standard assays (500 µL) were
performance in buffer 50 mM CHES pH 9, 150 mM NaCl, 0.2 mM CoCl₂, 6% (v/v) EtOH, with pH adjusted with NaOH at 70°C and using 5 μL of a Pox (1.5 mg/mL) for each experiment. Catalytic parameters were evaluated using a substrate (paraaxon) concentration range from 0 to 6 mM. The initial velocities at each substrate concentration were obtained by fitting the data to the MM equation using Graph-Pad Prism software.

Malathionase activity was performed in activity buffer supplemented with DTNB 2 mM to follow the malathion hydrolysis at 412 nm (ε = 13400 M⁻¹·cm⁻¹) over a range of concentrations between 0 and 2 mM. The substrate solvent final concentration (DMPO) was 1% (v/v). Time course hydrolysis of CMP-coumarin (methylphosphonic acid 3-cyano-4-methyl-2-oxo-2H-coumarin-7-yld ester cyclohexyl ester), IMP-coumarin (methylphosphonic acid 3-cyano-4-methyl-2-oxo-2H-coumarin-7-yld ester isopropyl ester) and PinP-coumarin (methylphosphonic acid 3-cyano-4-methyl-2-oxo-2H-coumarin-7-yld ester pinacolyl ester) were evaluated by following the release of cyanocoumarin at 412 nm (ε = 37000 M⁻¹·cm⁻¹) in the activity buffer. Experiments of CMP-coumarin hydrolysis in the presence of 0.1% SDS and DOC were performed in the same conditions as previously explained.

Catalytic parameters were evaluated over the substrate concentration range 0–750 μM. All catalytic experiments were performed in triplicate.

The initial velocities of these experiments were calculated using Gen5.1 software, the background of substrate hydrolysis subtracted (Table SIII) and catalytic parameters were obtained by fitting the data to the MM equation using Graph-Pad Prism 5 software.

Detergent assays. Simple kinetic experiments were performed in the presence of various detergents: SDS (0.1% (v/v), 0.5%, 1%, 1.5%, 5%, 10% and 20%), sonicated detergent (0.1% (v/v), 0.5%, 1%, 1.5%, 5% and 10%) and deoxycholate (0.1%, 0.5%, 1% and 2% (v/v)). The initial velocities at each substrate concentration were obtained by fitting the data to the MM equation using Graph-Pad Prism 5 software.

Inhibition assay. The inhibition of the enzyme by fensulfothion was evaluated by performing kinetic assays against paraaxon (concentration range 0–300 μM) and by using different fensulfothion concentrations in buffer 50 mM CHES pH 9, 150 mM NaCl, 0.2 mM CoCl₂, 6% (v/v) EtOH with 0.01% (v/v) of SDS. Because of the low solubility of fensulfothion in water, in methanol and ethanol the lack of solubility was overcome by adding 5% (v/v) of NaOH. The substrate and the inhibitor were incubated for 15 min at 25°C before the addition of the enzyme (concentration 0.35 mg/mL). The resulting velocities were evaluated at 0.1%, 0.25% and 0.5% (v/v) of fensulfothion. The inhibition constant (Kᵢ) was determined using the double reciprocal plot (Graph-Pad Prism 5 software).

Data collection and structure determination. Crystals were first transferred to a cryoprotectant solution composed of the reservoir solution and 25% (v/v) glycerol containing a 1:30 (v/v) ratio of a 100 mM fensulfothion solution. Crystals were then flash-cooled in liquid nitrogen. X-ray diffraction data were collected at 100 K using synchrotron radiation at the ID23-1 beam line (ESRF, Grenoble, France) with a wavelength of 0.979 Å using a Pilatus 100K detector. The data set was recorded at 2.68 Å resolution (Table II, Fig. S4). X-ray diffraction data were integrated, scaled and merged with the XDS program and the CCP4 program suite. The phases were obtained using the native structure of SoPox (PDB ID: 2vc5), performing a molecular replacement with MOLREP [29]. The model was built with Coot [30] and refined using REFMAC [31]. The anisotropic displacement parameters were refined via four groups of translation-liberation-screw (TLS) parameterisation identified by REFMAC. The final stereochemistry was checked using the MOL-PROBITY program [32]. Structure illustrations were performed using PyMol [33]. The coordinate file and the structure factors file of the fensulfothion bound SoPox structure have been deposited to the Protein Data Bank under the accession number 3uj9.

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**Author contributions**

J.H, G.G and M.E designed the experiments. J.H, G.G and M.E performed the experiments. J.H, G.G, M.E and EC analysed the results. J.H, ME and EC wrote the paper. All the authors offer a critical review of the paper.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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