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A thermostable phosphotriesterase from the archaeon *Sulfolobus solfataricus*: cloning, overexpression and properties

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Abstract A new gene from the hyperthermophilic archaeon *Sulfolobus solfataricus* MT4, coding for a putative protein reported to show sequence identity with the phosphotriesterase-related protein family (PHP), was cloned by means of the polymerase chain reaction from the *S. solfataricus* genomic DNA. In order to analyse the biochemical properties of the protein an overexpression system in *Escherichia coli* was established. The recombinant protein, expressed in soluble form at 5 mg/l of *E. coli* culture, was purified to homogeneity and characterized. In contrast with its mesophilic *E. coli* counterpart that was devoid of any tested activity, the *S. solfataricus* enzyme was demonstrated to have a low paraoxonase activity. This activity was dependent from metal cations with Co^{2+}, Mg^{2+} and Ni^{2+} being the most effective and was thermophilic and thermostable. The enzyme was inactivated with EDTA and *o*-phenantroline. A reported inhibitor for *Pseudomonas putida* phosphotriesterase (PTE) had no effect on the *S. solfataricus* paraoxonase. The importance of a stable paraoxonase for detoxification of chemical warfare agents and agricultural pesticides will be discussed.

Keywords Phosphotriesterase · Organophosphates · Pesticides · *Sulfolobus solfataricus* · Thermostability

Abbreviations PTE: Phosphotriesterase · OPH: Organophosphate hydrolase · OPD: Organo-phosphate-degrading enzyme · OPs: Organophosphates · *Sso*Pox: *Sulfolobus solfataricus* paraoxonase · ePHP: *E. coli* phosphotriesterase-related protein · *p*NP: *P*-nitrophenyl · LB: Luria-Bertani · IPTG: Isopropyl-β-D-thiogalactopyranoside · PVDF: Polyvinylidene fluoride · EST2: Esterase 2

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Introduction

Organophosphates (OPs) belong to a class of highly toxic compounds that are commonly used in the control of major insect pests and as chemical warfare agents such as sarin, soman and VX (Raushel 2002). These compounds are toxics for all vertebrates since they irreversibly inhibit acetylcholinesterase, a key enzyme of the nervous system. In these molecules phosphorus is linked via a double bond to either an oxygen or a sulfur atom, in oxon or in thion OPs, respectively, and by an ester linkage to phenoxy or others groups.

Enzymatic detoxification of OPs has become the subject of many studies because alternative methods of removing them such as bleach treatment and incineration are impractical due to high costs or environmental concerns (LeJeune et al. 1998 and references therein). For this purpose bacterial OP hydrolases are more appealing owing to their broader substrate specificity and higher catalytic rate.

Enzymes that catalyse the hydrolysis of the phosphoester bonds in OPs are known from several different bacterial species. These enzymes are termed phosphotriesterases (PTEs; EC 3.1.8.1), organophosphorous hydrolases (OPHs), organo-phosphate-degrading enzymes (OPDs) or parathion hydrolases (Raushel 2002). A different class of enzymes, capable of more specifically degrading nervine gasses, is represented by prolidases identified in *Alteromonas* sp.s (Cheng et al. 1993).

In general, bacteria are not affected by the presence of OPs since these compounds do not enter as such into the cell (Richins et al. 1997). However, pesticides can be a good carbon source for some bacteria (Cheng

et al. 1998; Raushel, 2002). Therefore, several bacterial isolates have been identified that are able to hydrolyse a large range of OPs including chemical warfare agents. The genes for OP hydrolases, *opd* (*o*rgano *p*hosphate *d*egradation) genes, were found in *Pseudomonas diminuta* (Munnecke 1976), *Flavobacterium* sp.s (Sethunathan and Yoshida 1973), *Agrobacterium radiobacter* (Horne et al. 2002). The enzymes from *P. diminuta* and *Flavobacterium* are identical at the protein sequence level. Here we will refer to them as PTE/OPD.

In each of these microrganisms the *opd* genes were located on a large plasmid, and seem to be part of transposable elements (Siddavattam et al. 2003; Horne et al. 2003). These enzymes have been thoroughly studied at the structure-function level. *Pseudomonas* OPH has a 'TIM' barrel $(\alpha\beta)_8$ structure with a binuclear metal binding site located at the C-terminal end of the barrel (Dumas et al. 1989; Benning et al. 1994, 1995, 2001). The enzyme activity was metal-ions dependent with higher activity observed with Co^{2+}; substrate specificity was very broad towards organophosphate tri-esters and a high catalytic rate, near the diffusion limit, was observed with certain substrates (Caldwell et al. 1991). In contrast, no activity was detectable with phosphate monoesters or di-esters, nor with common esterase or protease substrates (Donarski et al. 1989; Dumas et al. 1989, 1990). The reaction mechanism has been elucidated through structural and kinetic studies; the reaction proceeds via an SN2-like (Lewis et al. 1988) mechanism in which a metal-bound hydroxide ion attacks the electrophilic phosphorous centre of the substrate (Benning et al. 2000; Aubert et al. 2004).

In *E. coli* and some other organisms phosphotriesterase-related proteins (PHPs) have been identified and grouped into a single family (Hou et al. 1996; Buchbinder et al. 1998). Members of this family have been suggested to represent archetypal type of enzymes from which PTE/OPDs evolved (Hou et al. 1996). The sequence identity of *E. coli* PHP (ePHP) with the *Pseudomonas* enzyme was low but significant (about 30%) as confirmed by the observation that the 3D structure of this protein, solved at 1.7 Å resolution, was very similar to the PTE structure. However, no activity was detected with any tested substrate (Buchbinder et al. 1998). Two Zn^{2+} ions were observed in its structure and residues involved in their coordination were quite well conserved in PTE, except for PTE Lys170 that in ePHP was substituted with Glu125. No data are available on other members of the PHP family.

Here we report the cloning of a gene from the archaeon *Sulfolobus solfataricus* MT4 encoding a protein showing low sequence identity with both PTE and members of PHP family. The gene was expressed in *E. coli* and the recombinant protein purified and characterized biochemically. This is the first paraoxonase to be reported from a hyperthermophilic archaeon.

Materials and methods

Chemicals

p-nitrophenyl (*p*NP) butanoate, β-naphtylacetate, paraoxon (di-ethyl-*p*-NP-phosphate), dursban (*O,O*-diethyl(*p*-(methylsulfinyl)phenyl) phosphorothioate), parathion (*O,O*-diethyl *O*-(*p*-nitrophenyl) phosphorothioate), methyl-parathion (*O,O*-dimethyl *O*-(*p*-nitrophenyl) phosphorothioate), coumaphos (*O,O*-diethyl *O*-(3-chloro-4-methyl-2-oxo-2H-1benzopyran-7-yl) phosphorothioate), diazinon (*O,O*-diethyl *O*-2-isopropyl-4-methyl-6-pyrimidyl phosphorothioate), fensulfothion (*O*-diethyl-*O*-4-methylsulfinylphenylphosphoro-thioate) and bis-*p*NP-phosphate were purchased from Sigma Chemical Co. (St Louis, MO, USA). Molecular mass markers for SDS-PAGE and for gel filtration were obtained from Bio Rad (Hercules, CA, USA) and Amersham Biosciences (Uppsala, Sweden), respectively.

Strains and plasmids

Escherichia coli HB101 (Invitrogene, CA, USA) was used as host for cloning, whereas *E. coli* BL21 *(DE3)* harboured the recombinant plasmid for gene expression. Vector utilized was a derivative (pT7-SCII) of pT7 vector (Studier et al. 1990; Brown and Campbell 1993).

Cloning and expression

Standard molecular cloning techniques were employed throughout. *Escherichia coli* strains, HB101 or BL21 *(DE3)*, were grown at 37°C in Luria-Bertani (LB) medium containing ampicillin (100μg/ml). Restriction enzymes used in this work were from New England BioLabs (Beverly, MA, USA).

A 945-bp fragment containing the entire ORF *Sso*Pox, was amplified using the *S. solfataricus* genomic MT4 DNA, as template, recombinant Taq DNA polymerase, and *S. solfataricus* P2-based oligonucleotides 5′ *SSo*pox (5′-GATATA CATATGAGAATAC-CATTAGTT-3′) and 3′ *SSo*pox (5′-CTTGGG GTCGACTTAGCTGAAGAACTTTTTCGGA-3′) as forward and reverse primers, respectively, in a 30-cycle PCR (1 min 92°C, 1 min 50°C, 1 min 72°C). The amplification primer 5′ *SSo*pox was designed to introduce an *Nde*I restriction site (underlined) upstream from the initiation site, whereas 3′ *SSo*pox was designed to introduce a *Sal*I restriction site (underlined) downstream from the stop codon of *Ssopox*. The PCR product, eluted from agarose gel and digested with *Nde*I and *Sal*I, was ligated into the *Nde*I-*Sal*I-linearized expression vector pT7-SCII to create the pT7-SCII-*Sso*pox construct. The cloned fragment was completely sequenced to verify that only desired mutations were introduced during amplification. In this way, the *Ssopox* gene was

expressed under the direct control of the IPTG-inducible promoter T7 RNA polymerase through the T7 promoter ϕ 10 (Brown and Campbell 1993). The ligation mixture was used to transform *E. coli* HB101.

For protein production 8 l of LB medium containing 100 μg/ml ampicillin were inoculated at an optical density $O.D._{600}$ of 0.005 and grown overnight at 37°C with vigorous bubbling of sterile air. The next day, after addition of $CoCl_2$ (0.2 mM) and isopropyl-β-D-thiogalactopyranoside (IPTG;1 mM) 3 h of induction followed; cells were harvested by centrifugation (3000 *g*, 4°C, 10 min), washed with 25 mM Tris–HCl buffer pH 8.5 and stored at −80°C.

Purification of the recombinant enzyme

All procedures were carried out at room temperature, unless otherwise indicated. Wet frozen cells (45 g) were thawed and re-suspended in 120 ml of buffer A (20 mM Hepes pH 8.0, 0.2 mM $CoCl_2$, triton-X100 0.05% v/v). Cells were broken by French pressure cell disruption (Aminco Co., Silver Spring, MD, USA). A pressure setting of 2,000 lb/in^2 (1.38 MPa) was used. Cell debris was removed by centrifugation (80,000× g, 20 min, 4°C). After 1 : 1 dilution with buffer A, *E. coli* proteins were partially removed by incubating the crude extract for 15 min, at 50, 60 and 70°C, under gentle stirring and with clarification, between each incubation, by centrifugation (80,000× g, 20 min, 4°C). Pellets were discarded. The enzyme solution obtained at 70°C was directly loaded onto Q Sepharose Fast Flow FPLC column (Pharmacia) equilibrated with buffer A. The flow rate was 2 ml/min. After washing, a linear gradient of NaCl (0–0.5 M) was applied. The fractions with paraoxonase activity were pooled, brought to 10% (w/v) $(NH_4)_2SO_4$ and loaded onto an FPLC phenyl-Superose column (Pharmacia) equilibrated in buffer A, containing 10% (w/v) $(NH_4)_2SO_4$. The flow rate was 1 ml/min. After washing, a decreasing gradient of $(NH_4)_2SO_4$ was applied. Fractions showing paraoxonase activity were pooled and stored at 4°C.

Automated Edman degradation

N-terminus amino acid sequence analysis was performed by running the protein onto SDS-PAGE and electrotransfer to polyvinylidene fluoride (PVDF) membrane (Applied Biosystems). The band of interest was cut out and subjected to automate Edman degradation with ABI Procise protein sequencer (Matsudaira 1987), in accordance with the manufacturer's instructions.

Determination of *Sso*Pox molecular mass by gel filtration

The molecular mass of *Sso*Pox was determined by size-exclusion chromatography, using a High Load 16/60 Superdex 75 column run over an FPLC apparatus (Pharmacia, Sweden). The column was equilibrated and eluted with 20 mM Hepes buffer (pH 8.0) containing 0.2 mM $CoCl_2$ and 0.05% (v/v) triton X-100. The flow rate was 0.5 ml/min. The column was calibrated in the condition outlined above, using the following molecular mass markers: ribonuclease A (12,700 Da), ovoalbumin (43,000 Da), bovin serum albumin (67,000 Da), and blue dextran (2,000 kDa) from Amersham Biosciences and esterase 2 (EST2) from *Alicyclobacillus acidocaldarius* (34,000 Da; Manco et al. 1998). The loaded sample had a concentration of 0.1 mg/ml.

Electrophoreses

Electrophoretic runs were performed with a Bio-Rad Mini-Protean II cell unit, at room temperature. 12.5% SDS-PAGE was performed essentially as described by Laemmli (1970). Gels were stained with Coomassie Brillant Blue G-250. As molecular weight standard the 'Prestained SDS-PAGE broad range' (Bio Rad), was used containing: myosin (206 kDa), β-galactosidase (116 kDa), BSA (98 kDa), ovalbumin (55 kDa), carbonic anhydrase (37.4 kDa), soybean trypsin inhibitor (29.6 kDa), lysozyme (20.4 kDa), aprotinin (7.0 kDa). Non-denaturing PAGE was performed at pH 8.5 in 7.5% (w/v) polyacrilamide gel.

Activity staining

Non-denaturing gels were stained for esterase activity as described by Higerd and Spizizen (Higerd and Spizizen 1973). Briefly, after run gels were incubated at room temperature in a solution of 20 mM Tris–HCl (pH 8.0) containing 5 mg of β-naphtylacetate (dissolved in 0.5 ml methanol) and 25 mg of Fast Blue RR. Reactions were stopped after 20 min by rinsing with tap water and placing gels in 7.5 % (v/v) acetic acid. The esterase EST2 from *A. acidocaldarius* was used as control (Manco et al. 1998).

Enzyme assays

The time course of the paraoxonase-catalysed hydrolysis of paraoxon was followed by monitoring of *p*-nitrophenoxide production at 405 nm, in 1-cm path-length cells with a Cary 100 spectrophotometer (Varian, Australia). Initial rates were calculated by linear least-squares analysis of time courses comprising less than 10% of the total substrate turnover. Standard assays were performed at 70°C in a mixture of 20 mM Tris–HCl (pH 8.0), containing paraoxon 0.5 mM. For the other substrates a mixture of 20 mM Tris–HCl (pH 8.0), containing 10 % acetonitrile and substrates at a final concentration of 0.5 mM was used. Stock solutions of the substrates were obtained by dissolving them in pure acetonitrile.

Kinetic measurements were calculated by using paraoxon concentrations over the range 0.005–1 mM. Initial velocity substrate concentration data were fitted to the Lineaweaver-Burk transformation of the Michaelis-Menten equation, by weighted linear least-squares analysis with a personal computer and the GRAFIT program (Leatherbarrow 1992). Assays were done in duplicate or triplicate, and results were means of two independent experiments. Absorption coefficients used for each substrate were calculated after overnight hydrolysis with NaOH in the above reported assay conditions.

The inhibition by fensulfothion was evaluated by assaying *Sso*Pox activity at 30°C in 20 mM Tris/HCl buffer (pH 8) containing 4% acetonitrile, in the presence of three different inhibitor's concentrations (40, 80 and 160 μM) and different concentrations of paraoxon (range 0.015–1 mM). Data were reported as Lineweaver-Burk plots and then the slopes of the three lines were replotted against the inhibitor concentrations in order to calculate Ki.

Determination of pH optimum

The dependence of initial velocity on pH was monitored at 348 nm (the pH-independent isosbestic point of *p*-nitrophenol and *p*-nitropenoxide ion), by using paraoxon (0.5 mM) as substrate. A molar absorption coefficient of 5,300 M^{-1} cm^{-1}, at 70°C, was used in this case. The buffer used was 20 mM Na_2HPO_4/NaH_2PO_4 over the range of 5.0–7.5, 20 mM Tris–HCl over the range of 7.5–8.5, and 20 mM Gly/NaOH over the range of 8.5–9.5.

Thermostability and thermophilicity

The dependence of enzymatic activity on temperature was studied over the range of 30–95°C, with paraoxon (0.5 mM) as substrate, in a buffered mixture of 20 mM Tris–HCl (pH 8.0); pH values were adjusted at each temperature.

Enzyme stability was studied over the range of 70–100°C. Pure enzyme (0.2 mg/ml in 20 mM Tris–HCl buffer pH 8.0) was incubated in sealed glass tubes. Aliquots were withdrawn at times and assayed at 70°C by the standard assay.

Preparation of apo-enzyme

Apo-enzyme of *Sso*Pox was obtained by dialysing the enzyme (0.5 mg/ml) in 20 mM Hepes buffer (pH 8.0), containing 50 mM EDTA and 1 mM β-mercaptoethanol or 1 mM *o*-phenantroline, at 4°C. The dialysis was followed for 10 days; every 24 h aliquots of enzyme were withdrawn and assayed at 70°C by the standard assay. The apo-enzyme was dialysed 24 h at 4°C in 20 mM Hepes buffer (pH 8.0) to remove residual *o*-phenantroline before further use.

Results and discussion

Cloning and overexpression of *Sso*Pox in *E. coli*

By searching the *S. solfataricus P2* genome (She et al. 2001) for ORFs encoding putative PTEs we identified the ORF SSO2522 showing sequence similarity to the PTE from *P. diminuta* (Dumas et al. 1989) and OPD from *Flavobacterium sp.* (Brown 1980; 100% identical to PTE), as well as to the *E. coli* PHP (ePHP). *Sso*Pox residues His22, His24, Lys137, His170, His199 and Asp256 were strictly conserved and correspond to PTE/OPD residues reported to be involved in binding of divalent cations (Fig. 1). *Sso*Pox Lys137 (corresponding to Lys170 of PTE/OPD), which in its carbamylated form is involved in coordination of two metal ions, in ePHP is substituted by a glutamic acid (Glu125). Therefore in this respect the *Sulfolobus* sequence appears more similar to the PTE/OPD sequence. Aiming at verifying whether the product of ORF SSO2522 was devoid of any activity, as reported for ePHP (Buchbinder et al. 1998), we cloned the gene from *S. solfataricus* MT4 genomic DNA, by PCR amplification and inserted the gene into the expression vector pT7-SCII. A clone was sequenced and used for overexpression in *E. coli* BL21(*DE3*). The DNA sequence was identical to the *S. solfataricus* P2 gene, excluding the regions corresponding to the primers used for cloning (see Materials and methods) that were not sequenced.

It was previously reported (Omburo et al. 1992) that the level of PTE activity was dependent on the presence of divalent cations in the growth medium. In order to verify if the same correlation holds for *Sso*Pox activity, 0.2 mM of each: $ZnCl_2$, $CoCl_2$, $MnCl_2$ and $NiSO_4$, were separately incorporated into the growth medium (50 ml).

Activity assays carried out with semi-purified *Sso*Pox, obtained by thermal precipitation of host proteins, showed that the maximum increase of activity with respect to the un-supplemented medium (more than 30-folds) was obtained when Co^{2+} ions were included in the culture medium, suggesting that traces of metal ions contained in the LB medium were not sufficient to sustain activity. Henceforth, *Sso*Pox was prepared at large scale, by using $CoCl_2$ to increase the expression of activity.

Purification and physical properties of *Sso*Pox

Forty milligrams of pure enzyme were obtained starting from an 8-l *E. coli* culture. Identification and purity were evaluated by N-terminal sequencing, SDS-PAGE and HPLC analysis. The recombinant enzyme started with a methionine and the detected sequence was:

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PTE/OPD     1  MQTRRVVLKSAAAAGTLLGGLAGCASVAGSIGTGDRINTVRG-PITISEAGFTLTHEHICGSS
SsoPox      1  ----------------------------------MRIPLVGKDSIESKDIGFTLIHEHLRVFS
ePHP        1  ------------------------------------------MSFDPTGYTLAHEHLHIDL
                                                         :     *.** ***::  .

PTE/OPD    64  AGFLRAWPEFFG-SRKALAEK-AVRGLRR-ARAAGVRTIVDVSTFDIGRDVSLLAEVSRAADV
SsoPox     30  EAVRQQWPHLYN-EDEEFRNA---VNEVKRAMQFGVKTIVDPTVMGLGRDIRFMEKVVKATGI
ePHP       20  SG----FKNNVDCRLDQYAFICQEMNDLM-TR--GVRNVIEMTNRYMGRNAQFMLDVMRETGI
                .    ..          .      .    .. **:.::::.    **:  :.  * :...:

PTE/OPD   124  HIVAATGLWF--DPPLSMR-LRSVEELTQFFLREIQYGIEDTGIRAGII-KVATTG-KATPFQ
SsoPox     89  NLVAGTGIYIYIDLPFYFLN-RSIDEIADLFIHDIKEGIQGTLNKAGFV-KIAADEPGITKDV
ePHP       76  NVVACTGYYQDAFFPEHVA-TRSVQELAQEMVDEIEQGIDGTELKAGIIAEIGTSEGKITPLE
               .:**:** .   .:*  :   **::*: : :: :*  **:.*  :**:: .:.: : ::*.

PTE/OPD   182  ELVLKAAARASLATGVPVTTHTAASQRDGEQQAAIFESEGLSPSRVCIGHSDDTDDLSYLTAL
SsoPox    150  EKVIRAAAIANKETKVPIITHSNAHNNTGLEQQRILTEEGVDPGKILIGHLGDTDNIDYIKKI
ePHP      138  EKVFIAAALAHNQTGRPISTHTSFSTM-GLEQLALLQAHGVDLSRVTVGHCDLKDNLDNILKM
               *.*: *** *   *::*: **. :.   *..* .::  .*:...:: :**. ..*::..: .:

PTE/OPD   245  AARGYLIGLDHIPHSAIGLEDNASASALLGIRSWQTRALLIKALIDQGYMKQILVSNDWLFGF
SsoPox    213  ADKGSFIGLDRY-----GL-D--------LFLPVDKRNETTLRLIKDGYSDKIMISHDYCCTI
ePHP      200  IDLGAYVQFDTI-----GK-N--------SYYPDEKRIAMLHALRDRGLLNRVMLSMD--ITR
               .: *  :.. *.     *. .           .  .*     .*.. *. .::::* *  :.

PTE/OPD   308  SSYVTNIMDVMDRVNPDG-MAFIPLRVIPFLREKGVPQETLAGITVTNPARFLSPTLRAS
SsoPox    262  DWGTAK-PEYKPKLAPRWSITLIFEDTIPFLKRNGVNEEVIATIFKENPKKFFS------
ePHP      247  RSHL------K--ANGGYGYDYLLTTFIPQLRQSGFSQADVDVMLRENPSQFFQ------
                .       .  ..      :    **.*:  *: .. :. :  .**  *..
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Fig. 1 Multisequence alignment among PTE/OPD, *Sso*Pox and ePHP. Several sequences showing significant identity with *Sso*Pox were aligned with the program Clustal W, but only two sequences are shown here, namely PTE/OPD from a *P. putida*//*Flavobacterium* sp. and PTE homology protein from *Escherichia coli* (ePHP). The sequence identity of *Sso*Pox with both proteins was 31% on a global sequence alignment. Identical residues are marked with an asterisk and residues forming the cluster of the binuclear metal centre (His22, His24, Lys137, His170, His199 and Asp256 in *Sso*Pox) were shadowed

MRIPLVGKDS, which was identical to that deduced from the DNA sequence; the apparent molecular mass of the unique band observed on 12.5 % SDS-PAGE was about 34,000 Da (Fig. 2, panel A, lanes 1 and 2), in agreement with a molecular mass predicted from the sequence of 35,565. By HPLC analysis on reverse-phase C18 column a main peak eluting at about 50% of acetonitrile in 0.1% TFA was observed (95% purity; data not shown).

It has been reported that PTE from *P. diminuta* does not show esterase activity (Dumas et al. 1989). *Sso*Pox was tested for this activity after non-denaturing PAGE by in situ activity staining with β-naftyl-acetate as substrate. As shown in Fig. 2, panel B (lane 2) a single band, coincident with the Coomassie-stained one (lane 1), was observed.

The apparent molecular mass of the enzyme in non-denaturing conditions was measured by gel filtration using a High Load 16/60 Superdex 75 column, developed on a FPLC system. A value of 48 ± 2 kDa was estimated by interpolation on a calibration curve (data not shown). This value remains exactly in between the masses expected for a monomer and a dimer (35 kDa and 70 kDa, respectively). Several hypotheses could be formulated to explain this behaviour: (1) a dynamic equilibrium between a monomer and a dimer; (2) a monomeric enzyme with an asymmetrical shape; (3) a more slowly migrating dimer due to non-specific interactions with the matrix. The latter hypothesis was discarded because we observed the same behaviour with different chromatographic supports and in the presence or not of 0.5 M NaCl. In order to address the monomer-dimer equilibrium hypothesis, assuming that this was the case, *Sso*Pox samples of different protein

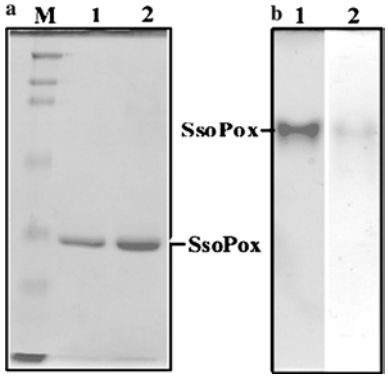

Fig. 2 Electrophoretic characterization of recombinant *Sso*Pox. **a** SDS-PAGE of molecular weight markers (lane M) and 1 and 3μg of recombinant *Sso*Pox (lanes 1 and 2, respectively). As molecular weight standard the 'Prestained SDS-PAGE broad range' (Bio Rad), was used containing; myosin (206 kDa), β-galactosidase (116 kDa), BSA (98 kDa), ovalbumin (55 kDa), carbonic anhydrase (37.4 kDa), soybean trypsin inhibitor (29.6 kDa), lysozyme (20.4 kDa), aprotinin (7.0 kDa). **b** Non-denaturing electrophoresis of Coomassie-stained (*lane 1*) or in situ activity stained (*lane 2*) *Sso*Pox (see text for details)

concentrations were prepared and samples of equal volumes (200μl) were analysed by running onto a Superose 12 analytical gel-filtration column. Again, a single peak of about 50 kDa was obtained and its migration was independent from protein concentration over the range of 0.5–5 mg/ml (data not shown). Therefore, the hypothesized dimer–monomer equilibrium was not influenced from protein concentration, at least under the explored conditions. Higher concentrations could not be analysed due to protein aggregation. Considereing the above, the hypothesis of a

monomer with an anomalous migration seems to be the more appropriate to explain the above reported behaviour. It is worth noting that ePHP has been reported to be monomeric both in solution and in crystals (Buchbinder et al. 1998), whereas PTE has been crystallised as a dimer and reported to be monomeric (Dumas et al. 1989) or dimeric in solution (Benning, et al. 1994).

pH optimum, thermophilicity and thermostability

We first examined the dependence of paraoxonase activity from pH at 70°C (see below). Reading was performed at 348 nm, the pH-independent isosbestic point between the *p*-nitrophenol and *p*-nitrophenolate ion. As reported in Fig. 3a, by using three different buffers over the pH range of 5.0–9.5, the maximum of activity was broad in the range of pH 7.0–9.0. This value is somewhat lower than values reported for PTE (pH 9–10), but values could not be easily compared because in others systems published data were not acquired at 348 nm.

The relationship between activity and temperature over the range of 30–95°C was obtained using paraoxon as substrate (Fig. 3b). It is relevant that in the range of temperature tested we did not find a maximum, but until 95°C activity still keeps growing. For technical reasons it was unfeasible to reach temperatures over 95°C and, for our convenience, all characterizations were performed at 70°C. From the *Arrhenius* plot reported in the inset of Fig. 3b, the calculated value for the activation energy was 20 kcal/mol.

The *Sso*Pox thermal stability was evaluated by measuring the residual activity after incubation of enzyme samples, for different lengths of time (until 4 h), at temperatures spanning the range of 70–100°C. From 70°C to 85°C no activity changes were observed after 4 h incubation (data not shown). A weak decrease was observed at 90°C, whereas at 95°C and 100°C $t_{1/2}$ values of 4 h and 90 min were observed, respectively (Fig. 4a). The time-course of inactivation followed a first-order kinetic for each temperature suggesting that we were looking at a single phenomenon. The exceptional intrinsic thermal stability of this enzyme is a property of valuable interest for biotechnological applications. In fact, usually high thermal stability is also linked to high resistance to other harsh conditions such as the presence of solvents, detergents, proteases and so forth. The reported data of thermal stability on PTEs indicate loss of activity in the range of 35–60°C and complete inactivation at 60°C (Rochu et al. 2004). Although for PTE it has been reported that immobilization dramatically improves enzyme stability (LeJeune et al. 1997), it should be considered that immobilization costs are usually high and the kinetic properties of the enzyme could change following the immobilization procedure. Furthermore, an intrinsically thermostable paraoxonase could be a convenient alternative in other systems such as display on the cell surface; complex stabilization systems have been proposed in order to achieve this objective for the mesophilic PTE (Richins et al. 2000). Obviously, given the very low activity of *Sso*Pox at low temperatures, a mutagenic approach aiming at increasing its activity without loosing stability should be devised before such applications might be implemented.

Preparation of the apo-enzyme

The apo-enzyme form of *Sso*Pox was obtained by dialysing the enzyme in 20 mM Hepes buffer (pH 8.0) containing either 50 mM EDTA and 1 mM β-mercaptoethanol or 1 mM *o*-phenantroline at 4°C, for several days. Fig. 4b shows the time-course inactivation of *Sso*Pox during a 6-day period; after this, time-residual activities of about 30% and 10% were recovered with EDTA and *o*-phenantroline, respectively. The inactivation experimental points could be computer-fitted to curves described by a theoretical rate equation for a single-exponential decay, which gave linear fits on a semi-logarithmic plot. Complete enzyme inactivation was only obtained after 10 days dialysis in *o*-phenantroline; the apo-enzyme was dialysed 24 h at 4°C in 20 mM Hepes buffer (pH 8.0) to remove residual

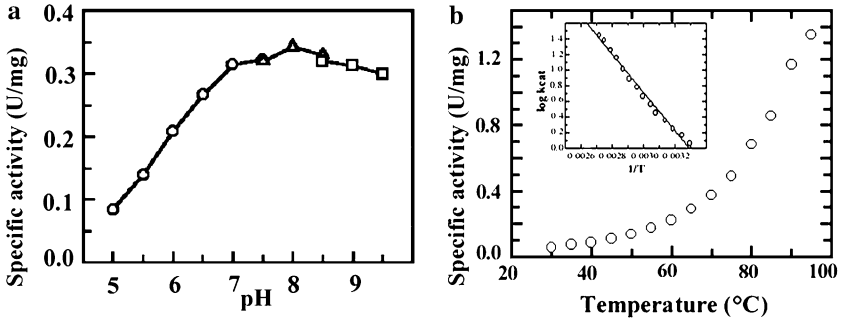

Fig. 3 **a** pH dependence of *Sso*Pox paraoxonase activity. Paraoxon was used as substrate. Buffers used were: 20 mM Na_2HPO_4/NaH_2PO_4 over the range of pH 5.0–7.5 (*circles*); 20 mM Tris–HCl over the range of 7.5–8.5 (*triangles*); and Gly/NaOH over the range of pH 8.5–9.5 (*squares*). Assays were done at 70°C. **b** Thermophilicity of *Sso*Pox. Activity was measured at different temperatures using the standard assay. In the inset data are shown as *Arrhenius* plot

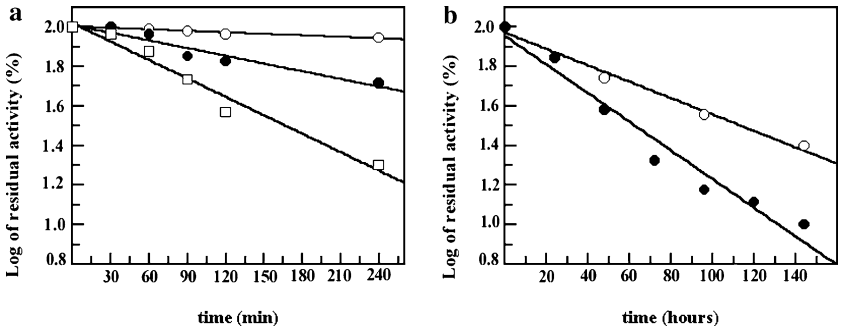

Fig. 4 **a** *Sso*Pox thermal stability. The enzyme at 0.2 mg/ml in 20 mM Tris/HCl buffer pH 8.0 was incubated in sealed glass vials at 90°C (*empty circles*), 95°C (*full circles*) and 100°C (*empty squares*). Samples were withdrawn at indicated times and assayed by the standard assay at 70°C. Data were reported as logarithm of residual activity (%) against a non-incubated sample. **b** *Sso*Pox inactivation with chelating agents. The time courses of *Sso*Pox inactivation in the presence of either 50 mM EDTA and 1 mM β-mercaptoethanol (*open circles*) or 1 mM *o*-phenantroline (*closed circles*) are shown. Aliquots were withdrawn at indicated times and assayed by the standard assay at 70°C. Data were reported as logarithm of residual activity (%) with respect to a non-incubated sample

chelating agent before assays. Further studies are ongoing to analyse in more detail the role of metal ions on *Sso*Pox activity and stability.

Substrate specificity and inhibitors

The Michaelis constant with freshly prepared enzyme was 0.060 ± 0.009 mM. From the calculated specific activity of 0.42 ± 0.02 U/mg and the molecular weight of 35,500, the values of k_{cat} and $s = k_{cat}/K_M$ were 0.24 ± 0.01 s^{-1} and 4.0×10^3 ± 50 M^{-1} s^{-1}, respectively (Table 1). Considering that the second order rate constant for the chemical hydrolysis of paraoxon by KOH is 7.5×10^{-2} M^{-1} s^{-1} (at pH 7.0 and 25°C; Raushel 2002), the catalytic rate enhancement was 5.3×10^4. Quite interestingly, with substrate methyl-paraoxon we observed a 5- and 3.4-fold increase of activity and K_M, respectively. This behaviour is similar to PTE except that in the latter enzyme the K_M value increased more dramatically (Hong and Raushel 1999).

Experimentally determined values for k_{cat}, K_M and s with paraoxon as substrate, at 70°C (Table 1), were compared with the results obtained for *P. diminuta* PTE (Dumas et al. 1989). *Sso*Pox shows a very low activity with a k_{cat} about 10,000-folds lower, but with substantially the same value of K_M. This finding opens the question, yet to be solved, concerning the identity of natural substrates for PTE/OPDs and PHPs. A current hypothesis is that high-activity PTE/OPDs evolved very recently from an enzyme with different substrate specificity, perhaps belonging to the PHP family. The *Sso*Pox paraoxonase activity could be an ancillary reaction of another more physiologically important activity, especially considering that the natural habitat of *S. solfataricus* (about 80°C and low pH) is not favourable for the stability of these xenobiotics, which appeared in the environment only in the last 50 years (Schrader 1963).

The *S. solfataricus* ORF has low sequence identity with both the PTE/OPD and ePHP (Fig. 1). As said before ePHP is devoid of esterase, aminopeptidase, sulfatase, phosphatase, carbonic anhydrase, phosphodiesterase and phosphotriesterase activities. Instead, *Sso*Pox was able to degrade several pesticides, as reported in Table 2. Therefore the enzyme seems more similar to PTE than ePHP. It would be interesting to test the activities of other members classified in the PHP family

Table 2 *Sso*Pox specific activities for the hydrolysis of organophosphorus insecticides

Substrates	λ (nm)	ε_{mM} (OD/mM)	Specific activity ($\times10^{-3}$)(U/mg)
*p*NP-butanoate	405	25	43 ± 4.00
Bis-*p*NP-phosphate	405	25	6.5 ± 0.2
Paraoxon	405	25	420 ± 20
Methyl-paraoxon	405	25	1125 ± 240
Parathion	405	25	19 ± 1.00
Methyl-parathion	405	25	6 ± 0.31
Dursban	276	9	160 ± 13
Coumaphos	348	15.2	92 ± 4.6
Diazinon	228	11.4	11 ± 0.4

In table are reported the wavelengths used (λ), and the absorption coefficients (ε_{mM}) calculated for each substrate at pH 8.0 and 70°C. All substrates were dissolved in pure acetonitrile and used at final concentration of 0.5 mM. Errors are mean values ± SD (n = 5)

Table 1 Kinetic parameters of *Sso*Pox. Parameters were calculated at pH 8.0 and 70°C using paraoxon or methyl-paraoxon as substrates

Substrate	U/mg ($\times10^{-3}$)	k_{cat}(sec^{-1})	K_M(mM)	k_{cat}/K_M(mM^{-1} s^{-1})
Paraoxon	420 ± 20	0.24 ± 0.01	0.060 ± 0.009	4.00 ± 0.75
Methyl-paraoxon	2,200 ± 90	1.30 ± 0.05	0.205 ± 0.023	6.34 ± 0.58

Errors are mean values ± SD (n = 5)

(Hou et al. 1996) in order to ascertain if *Sso*Pox belongs to this family or could be considered an intermediate step towards the evolution of PTE/OPDs. It is also worth noting that the *Ssopox* gene is chromosomally located, as also reported for ePHP (Hou et al. 1996), while PTE/OPDs are located on plasmids (Horne et al. 2003; Dumas et al. 1989). This could strengthen the suggestion of a member of the PHP family as precursor for PTE/OPDs.

The purified *Sso*Pox was also able to catalyse the hydrolysis of *p*NP-butanoate and β-naphtylacetate (Table 2 and Fig. 2b), two typical substrates for carboxylesterases. The pH/activity profile as well as thermofilicity and thermal stability of the carboxylesterase activity were qualitatively very close to the paraoxonase activity profiles (unpublished results), suggesting that the two activities take place at the same site. Furthermore, the *p*NP-butanoate esterase activity was not inhibited by paraoxon (data not shown), which is a powerful inhibitor for carboxylesterases (Oosterban and Janz 1965), allowing to rule out the presence of a contaminating serine-type esterase activity and reinforcing the hypothesis of a unique active site. The ability of these kinds of enzymes to catalyse hydrolysis of C–O and P–O bonds has been recently reported for a human carboxylesterase (Bencharit et al. 2003), a human butyrylcholinesterase (Millard et al. 1998) and a human paraoxonase not related to bacterial PTE enzymes (Harel et al. 2004).

From the analysis shown in Table 1 and 2 it is evident that *Sso*Pox has substrate specificity similar to *P. diminuta* PTE (Dumas et al. 1989; Hong and Raushel 1999). Unlike PTE, *Sso*Pox was unable to degrade the substrate fensulfothion (Dumas et al. 1989). Therefore we asked if this compound binds anyhow at the active site by measuring its effectiveness in a competition assay against paraoxon. Data (not shown) indicate that fensulfothion behaves as a competitive inhibitor with respect to paraoxon with a Ki of 120 μM. The effect of a PTE substrate analogue, namely tri-ethylphosphate (Benning et al. 1994), was also analysed. This compound when assayed with different saturing or sub-saturing paraoxon concentrations and for different incubation times was proven not to be an *Sso*Pox inhibitor (data not shown).

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