

Directed evolution of phosphotriesterase from *Pseudomonas diminuta* for heterologous expression in *Escherichia coli* results in stabilization of the metal-free state

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Phosphotriesterase from *Pseudomonas diminuta* (PTE) is an extremely efficient metalloenzyme that hydrolyses a variety of compounds including organophosphorus nerve agents. Study of PTE has been hampered by difficulties with efficient expression of the recombinant form of this highly interesting and potentially useful enzyme. We identified a low-level esterolytic activity of PTE and then screened PTE gene libraries for improvements in 2-naphthyl acetate hydrolysis. However, the attempt to evolve this promiscuous esterase activity led to a variant (S5) containing three point mutations that resulted in a 20-fold increase in functional expression. Interestingly, the zinc holoenzyme form of S5 appears to be more sensitive than wild-type PTE to both thermal denaturation and addition of metal chelators. Higher functional expression of the S5 variant seems to lie in a higher stability of the metal-free apoenzyme. The results obtained in this work point out another—and often overlooked—possible determinant of protein expression and purification yields, i.e. the stability of intermediates during protein folding and processing.

Keywords: apoenzyme/directed evolution/*Escherichia coli* heterologous expression/organophosphorus hydrolase/phosphotriesterase/promiscuity/recombinant

Introduction

Phosphotriesterase (PTE), originally isolated from the soil bacterium *Pseudomonas diminuta*, catalyses the detoxification of a wide variety of organophosphate triesters, thioesters and fluorophosphate compounds, including parathion, VX, soman and sarin (Caldwell and Raushel, 1991). Even though phosphotriesters are not naturally occurring compounds, the enzymatic hydrolysis of these substrates by PTE is remarkably efficient, with k_{cat} and $k_{\text{cat}}/K_{\text{M}}$ values for paraoxon ($\sim 10^4 \text{ s}^{-1}$ and $10^8 \text{ s}^{-1} \text{ M}^{-1}$, respectively) that approach the limit of diffusion of the reactants in solution (Omburo *et al.*, 1992).

The crystal structure of PTE led to the identification of its major structural and functional components (Omburo *et al.*, 1992; Kuo and Raushel, 1994). PTE is a homodimeric $(\beta/\alpha)_8$ barrel, its catalytic centre being composed of two closely spaced divalent cations (zinc in the native enzyme) ligated to the protein via direct interactions with four histidines and one aspartate. In addition, the two metal ions are bridged by a water molecule and a carbamoylated lysine residue (Vanhook *et al.*, 1996). It is suggested that the reaction mechanism proceeds via the bridging water molecule (or hydroxide) attacking the phosphorus centre of the bound substrate (Benning *et al.*, 1995).

Being the most efficient phosphotriesterase identified to date, PTE seems to have much potential in the decontamination of hazardous organophosphate compounds. These chemicals range in toxicity from agricultural insecticides to nerve agents and their persistence in the environment has become an issue of increasing worldwide concern (Dave *et al.*, 1993; Hoskin *et al.*, 1995; Rastogi *et al.*, 1997). As such, the development of an environmentally friendly and efficient detoxifying tool would be of great value. The utility of PTE may be further enhanced by various methods of immobilization and delivery. These include the production of PTE–silicone biocomposites (Gill and Ballesteros, 2000), PTE foams for surface decontamination (LeJune *et al.*, 1998) and the display of PTE on *Escherichia coli* and *Pseudomonas putida* for *in situ* biodegradation (Richins *et al.*, 1997; Shimazu *et al.*, 2001, 2003). Many efforts have been made to improve PTE's catalytic performance with various organophosphates by both rational design (Chen-Goodspeed *et al.*, 2001a,b) and directed evolution (Cho *et al.*, 2002, 2004; Griffiths and Tawfik, 2003; Yang *et al.*, 2003).

Unfortunately, structural and mechanistic studies, and also practical applications of large-scale enzymatic decontamination, have been limited by the production costs associated with the problems of expressing stable PTE (Shimazu *et al.*, 2001). Some effort has been applied to improving PTE's expression levels, although each system, so far, has been limited in the quantity and stability of enzyme produced (Lai *et al.*, 1994). Heterologous expression using insect tissue cultures, followed by sequential purification by ion-exchange and gel filtration chromatography, has been reported (Dumas *et al.*, 1989). However, a PTE yield of only 0.03% of the total cell mass was achieved. Over-expression in *E.coli* has also been described, but again with low efficiencies: active PTE comprised only $\sim 0.1\%$ of the total cell mass and most of the expressed enzyme was found in an aggregated, inactive form (Omburo *et al.*, 1992; Dave *et al.*, 1993).

Other features that make PTE an attractive object of study are its unknown biological role and its intriguing evolutionary origin. Phosphotriesters—clearly very efficient substrates for this enzyme—have been in existence for only a few decades (Raushel and Holden, 2000) and a naturally occurring substrate has not, as yet, been identified. This enigma has led to the suggestion that PTE's enzymatic activity evolved in the last few decades from a pre-existing hydrolase (Dumas *et al.*, 1989; Raushel and Holden, 2000). In addition to the wide range of phosphotriester substrates that have been reported to be efficiently hydrolysed by PTE, traces of thiophosphonate and phosphodiester hydrolysing activities (more than five and seven orders of magnitude, respectively, lower than phosphotriesterase specific activity) have been detected (Shim *et al.*, 1998; DiSioudi *et al.*, 1999). Promiscuous activities have been proposed to play a key role in the evolution of enzymatic activities, providing a starting point from which new functions

may evolve (O'Brien and Herschlag, 1999; James and Tawfik, 2001; Aharoni *et al.*, 2005). Conversely, these promiscuous activities may comprise vestiges of the function of the protein from which an enzyme originated.

To characterize the promiscuity profile of PTE further, we performed kinetic studies using 2-naphthyl acetate (2-NA) as substrate. Previous studies indicated no esterolytic activity of PTE towards *p*-nitrophenyl acetate (PNPA) (Dumas *et al.*, 1989). It is likely that the high spontaneous hydrolysis rate of PNPA masked the low level of PTE's esterolytic activity. 2-NA does not share this characteristic with PNPA and we could detect its hydrolysis by PTE at a modest but significant rate. In an attempt to increase this activity by directed evolution, we screened PTE gene libraries of random mutations using 2-NA as a substrate. This strategy led to a variant that maintains the specific activities of wild-type (wt) PTE, but expresses functionally at 20 times higher levels in *E.coli*. The results of the evolution for higher esterase activity are discussed elsewhere (Aharoni *et al.*, 2005).

Materials and methods

Expression and purification of PTE variants

LB medium (3 ml) containing ampicillin (100 $\mu\text{g ml}^{-1}$) and ZnCl_2 (0.5 mM) was inoculated with a single colony of *E.coli* DH5 α cells containing either pMAL-c2x/PTE (see below) or pMAL-c2x/S5 plasmids (pMAL-c2x, NEB) and grown overnight at 30°C with shaking at 250 r.p.m. The resulting culture was added to 500 ml of the same medium and grown at 30°C until $\text{OD}_{600} \sim 0.6$. Enzyme over-expression was induced by adding IPTG (0.4 mM final concentration) and shaking (250 r.p.m.) at 20°C for 40 h. Cells were harvested by centrifugation, resuspended in lysis buffer [50 mM Tris pH 8.5, 10 mM NaHCO_3 , 100 μM ZnCl_2 (assembly buffer, AB), plus 0.5 mM DTT and 1 mM PMSF] and disrupted by sonication. After centrifuging, the supernatant was passed through an amylose column (NEB) equilibrated with column buffer (50 mM Tris pH 8.0, 0.25 M NaCl). The fusion protein was eluted with maltose (10 mM)-supplemented column buffer. All purification steps were performed at 4°C. The enzymatic activity of the collected fractions was monitored by kinetic analysis with paraoxon. The pooled aliquots were extensively dialysed against AB at 4°C. The purity of the fusion enzymes and their relative concentrations were established by 12% SDS-PAGE. Quantification of the overexpressed proteins in crude lysates was performed by measuring the SDS-PAGE band density using NIH Image 1.6. Total PTE expression was determined by adding the band density observed in the supernatant to that of the pellet, while ensuring that the pellet has been resuspended in the same buffer volume used for cell lysis. The results were normalized to total bacterial mass according to the band densities of endogenous *E.coli* proteins observed in the same electrophoresis patterns.

Over-expression with BL21(DE3)pLys cells transformed with pET-20b/wt-PTE or pET-20b/S5 plasmid, was induced with IPTG (0.4 mM), as detailed for MBP fusions. The induced cultures were shaken at 20°C for 8–40 h.

For analytical purposes of crude lysates, over-expression of the enzyme variants was carried out with 3 ml cultures and cells were disrupted using 300 μl of BugBuster (Novagen).

Gene cloning, libraries construction and DNA shuffling

The PTE-encoding *opd* gene from *Ps. diminuta* (without the leader peptide-coding fragment) was subcloned into pMAL-c2x (NEB) using *EcoRI* and *PstI* restriction sites. DNA libraries were generated by random mutagenesis either by error-prone (EP) PCR (Vartanian *et al.*, 1996) or by the use of 8-oxo-dGTP and dPTP as base analogues (BA) during the amplification step (Zaccolo *et al.*, 1996). Five randomly chosen clones from each library were used for DNA sequence determination. The average rate of mutation for the EP library was 2.2 mutations per gene, while the BA library contained 1.4 mutations per gene. Both libraries contained 14% transversions and a residual activity with paraoxon of $\sim 1\%$. The resulting gene libraries were subcloned into pMAL-c2x and transformed into *E.coli* DH5 α cells by electroporation for screening and over-expression. The genes corresponding to the selected mutants were transferred to pIVEX (Griffiths and Tawfik, 2003) using *AccI* (internal) and *KpnI* restriction sites, rendering the S5 sequence without the Pro70 and Ala78 silent mutations. The genes were transferred from pIVEX to pET-20b, using the *NdeI/XhoI* restriction sites in the vector and appending them by amplifying the genes with *ad hoc* oligodeoxynucleotides. Electrocompetent *E.coli* BL21(DE3)pLys cells were transformed with the pET-20b constructs for over-expression of the PTE variants with no fusion protein.

For DNA shuffling, the genes corresponding to the selected variants were amplified using mal E and biotinylated S1224 primers (NEB) and pMAL-c2x/PTE variant plasmid as template. The PCR products were then pooled in equimolar amounts and incubated with *DpnI* (NEB) to remove plasmid template DNA. After gel purification (Promega), the DNA was subjected to digestion with DNase I (NEB). Fragments 25–250 bp long were purified by gel electrophoresis (QIAEX II, Qiagen) and assembled by PCR in the absence of primers, based on a previously described methodology (Stemmer, 1994), with some modifications: 1.5 μg of purified DNA fragments were applied to the 25 μl assembly PCR reaction, for which ExTaq DNA polymerase (Takara) was used. A falling gradient of temperatures was used in the annealing step. The biotin-labelled self-assembled PCR product was purified by binding to M-280 streptavidin-coated magnetic beads (Dynabeads, Dynal). Briefly, the beads were resuspended in B&W buffer (10 mM Tris pH 7.5, 1 mM EDTA, 2.0 M NaCl) and added to the PCR product. After a 2 h incubation at ambient temperature, beads were rinsed five times with 2 \times B&W buffer and twice with 1 \times ExTaq polymerase-specific buffer. Beads were finally resuspended in the 1 \times ExTaq polymerase-specific buffer. Specific oligodeoxynucleotides containing the pMAL-c2x cloning restriction sites *EcoRI* and *PstI* were used for a subsequent nested PCR step, which produced DNA that was digested and ligated into pMAL-c2x plasmid and used to transform *E.coli* DH5 α by electroporation.

Screening procedures

E.coli DH5 α cells transformed with the libraries were grown in plates containing LB/amp/ Zn^{2+} medium (LB-agar, 100 $\mu\text{g ml}^{-1}$ ampicillin, 0.5 mM ZnCl_2) and replicated with velvet cloth on IPTG-containing LB/amp/ Zn^{2+} plates. After growing colonies at 30°C overnight, a layer of soft agar (LA, 0.5% in AB) supplemented with 2-NA (0.5 mM) and Fast Red (1.3 mg/ml, Sigma) was added (Khalameyzer *et al.*, 1999;

Aharoni *et al.*, 2004). Replicates of colonies that turned red first were used to inoculate LB/amp/Zn²⁺ (500 μ l) in 96-deep-well plates. These were duplicated and over-expression was induced with IPTG (0.4 mM). Cells were disrupted with BugBuster (Novagen) and cleared lysates assayed for hydrolysis of 2-NA (0.3 mM) and paraoxon (0.25 mM), as described below. To verify the activity and ensure the monoclonality of the selected variants, the hydrolysis rates were re-assayed after growing three subclones from each original colony. Plasmids were extracted and used for mutation analysis and as templates for subsequent rounds of shuffling and screening.

Determination of catalytic parameters

Esterase and phosphotriesterase activities were monitored by spectrophotometry in 96-well plates with 200 μ l reaction volumes, in 100 mM Tris buffer (pH 8.5), at ambient temperature. Products, i.e. 2-naphtholate or *p*-nitrophenolate, were detected at 320 and 405 nm, respectively. Plots of v_0 vs $[S]_0$ were fitted to the Michaelis–Menten equation, $v_0 = k_{cat}[E]_0[S]_0 / ([S]_0 + K_M)$. Background hydrolysis of 2-NA was subtracted from enzyme kinetic parameters. At least two independent measurements were performed for each experiment. All reactions were carried out in the presence of the same amount (0.25 or 0.4%) of organic solvent (DMSO or methanol, respectively).

Cell-free transcription/translation

Wild-type PTE and S5 variant genes were amplified from the pIVEX construct using vector-specific oligodeoxynucleotides. The PCR products were gel purified and used as templates for *in vitro* transcription/translation (IVT, Promega) reactions. Recombinant T7 RNA polymerase (5 μ g/50 μ l IVT mixture) was added to the IVT mixture along with the DNA template at final concentrations of 2 nM. The reaction was allowed to proceed for 3 h at 25°C, whereupon the IVT product was incubated at room temperature for 2 h in AB to allow metal assembly. The enzyme was then assayed for activity as described above.

Stability assays

All stability experiments were performed in duplicate. Heat denaturation of clarified lysates from over-expressed PTE cultures (diluted 1:40 in Tris buffer pH 8.5) was achieved by heating each sample to one temperature within the 40–70°C range for 20 min, then cooling to 25°C. The samples were further diluted to 1:100 in Tris pH 8.5 (100 mM) and residual activity with paraoxon was measured. Controls with non-heated samples were used to determine the 100% activity value. Chemical denaturation was carried out on similarly prepared lysates (diluted to 1:20) by adding guanidinium hydrochloride at seven final concentrations (from 0.2 to 2 mM), for 1 h at room temperature. Samples were further diluted (1:200) in Tris pH 8.5 (100 mM) without Zn²⁺ and incubated for 1 h at room temperature, before measuring residual activity with paraoxon. Diluted clarified lysates were also incubated for 90 min at ambient temperature with different concentrations of the metal chelator phenanthroline (5–300 μ M). All samples, including the untreated sample, contained the same concentration of methanol (0.3% during incubation with phenanthroline). The samples were subsequently diluted 1:50 in Tris buffer pH 8.5 (100 mM), incubated for 30 min at room temperature and assayed with paraoxon (0.25 mM). Two

independent experiments were performed in each case and their results averaged.

The levels of apo-enzyme expression and activity were determined by over-expressing the pET-20b/PTE variants in *E.coli* BL21(DE3) cells, followed by their resuspension and washing in Tris buffer pH 8.5 (100 mM). Cells were then lysed either in Tris buffer (TB) or in AB. The lysates were incubated at room temperature for 1 h, centrifuged and the clarified lysates were assayed for paraoxonase activity.

Results and discussion

Over-expression of the MBP-PTE fusion protein

In an attempt to find an efficient method for PTE over-expression and purification, the *opd* gene was cloned into the pMAL-c2x vector for expression in the bacterial cytoplasm, N-terminally fused to maltose-binding protein (MBP). Following IPTG-induced over-expression, the bacterial culture was lysed and analysed by SDS–PAGE. A band of the expected molecular weight (\sim 82 kDa) was observed (data not shown). Although a fraction of the enzyme was present in the supernatant and proved to be catalytically active, the yield of functional PTE was at best 4 mg/l of *E.coli* culture and >95% of the expressed protein was aggregated in the pellet.

The recombinant MBP-fusion enzyme was purified by affinity chromatography through the MBP tag and was found to display a K_M with paraoxon of $32 \pm 4 \mu$ M—in agreement with previously determined parameters (Caldwell *et al.*, 1991)—and a turnover rate (k_{cat}) that is about one-third that of non-fused wt-PTE (Table I). This lower activity may be due to the MBP fusion, but the unchanged K_M value suggests that is more likely to result from partially inactive enzyme fractions.

Identification of esterase activity in PTE

We tested the 2-NA-hydrolysing activity of purified fusion enzyme. A modest but significant activity was detected, with a k_{cat}/K_M of \sim 250 $M^{-1} s^{-1}$ (Figure 1a). To assign this weak activity to PTE rather than to a contaminating enzyme, the assay was performed after incubation of the sample with 1,10-phenanthroline (a metal chelator) or PMSF (a serine/cysteine hydrolase inhibitor). Almost no inhibition was observed with PMSF, whereas complete inhibition of both

Table I. Kinetic parameters of the different PTE variants

PTE variant	2-Naphthyl acetate k_{cat}/K_M ($s^{-1} M^{-1}$)	Paraoxon		
		k_{cat} (s^{-1})	K_M (μ M)	k_{cat}/K_M ($s^{-1} M^{-1}$)
wt ^a	NA ^d	2280	35	6.2×10^7
wt-MBP ^b	250 ± 60	780 ± 37	32 ± 4	2.5×10^7
1.5E-MBP ^b	240 ± 34	1280 ± 56	37 ± 3	3.5×10^7
1.6F-MBP ^c	NA	1430 ± 35	80 ± 6	1.8×10^7
2.S5-MBP ^b	480 ± 70	2150 ± 217	54 ± 6	4.0×10^7

^aKinetic parameters for wt-PTE were determined by Hong and Raushel (1999) at pH 9.0.

^bResults represent the average of two or three independent measurements. Error ranges were calculated as the highest difference between any value and the average.

^cResult derives from one experiment. Error ranges represent the error in the fitting to the Michaelis–Menten equation.

^dNA: not analyzed.

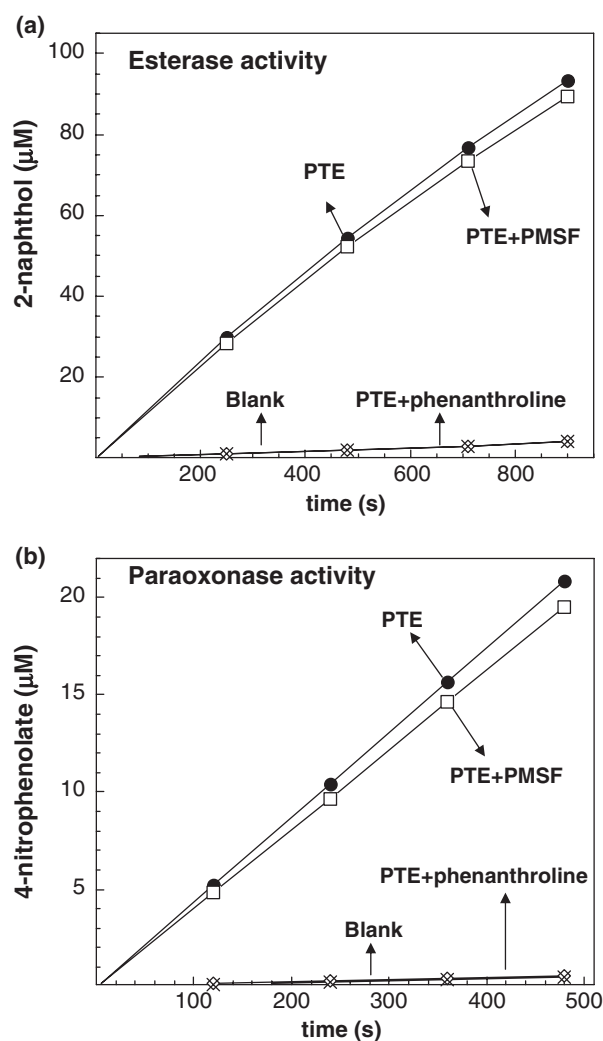


Fig. 1. The esterase and paraoxonase activities of PTE. (a) Esterase activity of PTE (10^{-6} M) with 0.25 mM 2-NA, monitored at 320 nm, measured after 45 min of incubation at room temperature, in the absence or presence of 2 mM 1,10-phenanthroline or 1 mM PMSF. (b) The same samples were assayed for the paraoxonase activity of PTE (7×10^{-11} M) with 0.25 mM paraoxon.

the phosphotriesterase and esterase activities was achieved with 1,10-phenanthroline (Figure 1a and b). This supports the notion that the measured esterolytic activity is mediated by PTE.

Directed evolution of PTE for higher esterase activity

We chose to improve the modest PTE esterase activity by directed evolution, using a colony screen (Khalameyzer *et al.*, 1999). Colonies from PTE gene libraries were grown on agar plates and these were duplicated on to IPTG-containing agar plates using a velvet cloth. After incubation at 30°C overnight, top agar containing 2-NA and Fast Red was poured on the plate. Fast Red reacts with the released 2-naphthol to generate an insoluble red product in the 'positive' colony. Colonies that produced this colour were isolated, grown in liquid medium using 96-deep-well plates, lysed and assayed for 2-NA and paraoxon hydrolysis.

Two libraries were generated by random mutagenesis, either by error-prone PCR (EP) or by the use of a base analogue (BA) (Vartanian *et al.*, 1996; Zaccolo *et al.*, 1996). Adjusting the PCR conditions for each strategy achieved the desired mutation

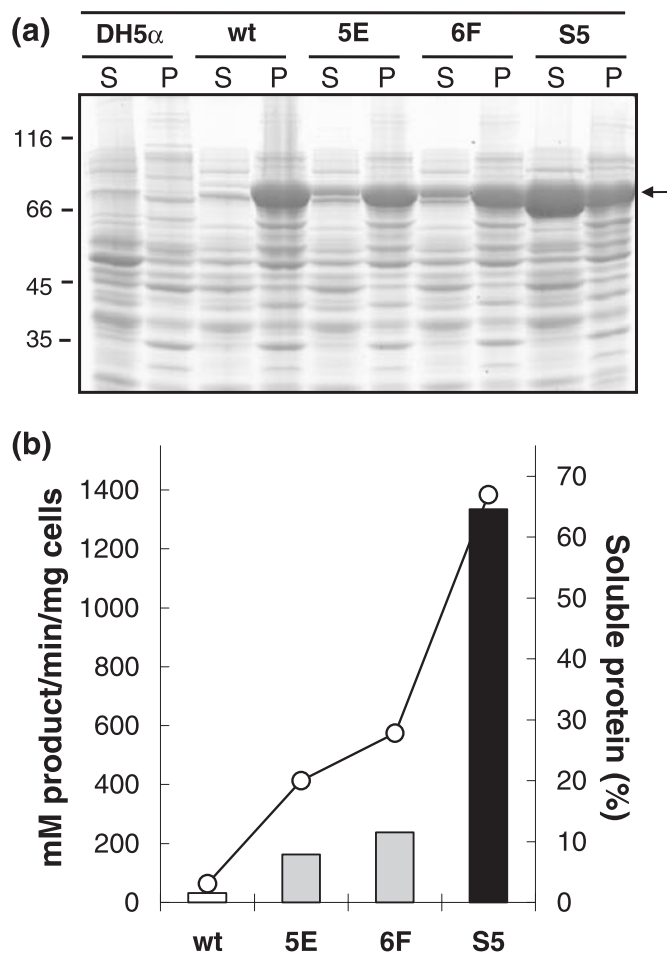


Fig. 2. Evolution of higher functional expression of MBP-PTE fusion. (a) SDS-PAGE (10%) of crude cell lysate supernatants (S) and pellets (P) of first-generation variants 5E and 6F and the second generation variant S5, compared with wt-PTE. (b) The paraoxonase activity per mg of cells measured in crude lysates (bars, left ordinate)—normalized according to total protein expression for each clone—and percentage of protein found in the supernatant, as determined by densitometry from (a) (circles, right ordinate).

rate of about two base substitutions per gene. Screening ~10 000 agar colonies from each library produced 150 positive clones that were transferred to liquid medium in 96-deep-well plates. Lysates from these cultures were prepared and assayed for 2-NAase and paraoxonase activity. Fourteen clones were selected from this screen and re-grown in liquid medium, lysed and re-assayed with 2-NA. The seven clones that exhibited the highest esterase activities were recombined by DNA shuffling and the resulting library was subjected to a second round of screening. Of the four clones isolated, the most active esterase was S5, with an apparent ~50-fold improvement over wt-PTE. However, a parallel increase in the paraoxonase activity was observed.

Having achieved a fair improvement in activity, it was decided to characterize S5 and the best first-generation variants (5E and 6F). SDS-PAGE indicated a 45-fold higher MBP-fused enzyme content in the soluble fraction for the second-round S5 enzyme (~360 mg/l culture) when compared with wt-PTE (~8 mg/l culture, Figure 2a). Normalization of protein content in the supernatant to total recombinant protein expression levels showed a 3% soluble PTE content for wt-PTE and 66% for S5, i.e. a 20-fold increase in the soluble content.

The total expression levels (soluble plus pellet) of the S5 variant was also higher than wt-PTE's (by a factor of ≤ 2). The intermediates 5E and 6F gave increases in soluble expression relative to wt-PTE of 3- and 4-fold, respectively. Correcting wt-PTE and S5 paraoxonase rates for (absolute) soluble protein content indicated that the differences between their activities were due to the increase in soluble expression and it can be deduced that the evolved S5 variant possesses comparable specific activities to wt-PTE (Figure 2b).

Characterization of the evolved PTE variants

Kinetic analysis. Variants 5E, 6F and S5 and wt-PTE were over-expressed as MBP fusion enzymes and purified. Kinetic analyses with 2-NA and paraoxon were then performed. Because of the limited solubility of 2-NA in aqueous solution, independent k_{cat} and K_{M} constants could not be determined for this substrate, so $k_{\text{cat}}/K_{\text{M}}$ (specificity constant) was defined instead (Table I). With paraoxon, MBP-fused wt-PTE gave a lower k_{cat} than the non-fused form [$780 \pm 37 \text{ s}^{-1}$ vs 2280 s^{-1} (Hong and Raushel, 1999)]. This is presumably due to a proportional loss of functional enzyme caused by protein instability or misfolding. In contrast, variant S5 displays a k_{cat} that is essentially identical with previously reported values for non-fused wt-PTE ($2150 \pm 217 \text{ s}^{-1}$). The ~ 2 -fold higher $k_{\text{cat}}/K_{\text{M}}$ parameter with 2-NA (480 ± 70 vs $250 \pm 60 \text{ s}^{-1} \text{ M}^{-1}$) is also in agreement with a higher fraction of active protein in the S5 variant. Despite the somewhat higher K_{M} value obtained for S5 with paraoxon (54 ± 6 vs $32 \pm 4 \mu\text{M}$ for wt-PTE), no major changes in the kinetic parameters of the evolved PTE variants were observed. Although the possibility that the active site of the enzyme has been altered by these three mutations cannot be ruled out completely, it can be concluded that the differences between wt-PTE and S5 are predominantly at the level of functional expression.

Sequence analysis. As seen in Table II, the most improved clone, S5, incorporated four synonymous (silent) mutations and three non-synonymous mutations; Lys185Arg and Asp208Gly emerged in the first round of screening in two different clones, and Arg319Ser may have been introduced during the assembly step of the shuffling process (Table II). It is possible that some of the silent mutations in S5 increased total expression

levels compared with wt-PTE (by ≤ 2 -fold), through a favoured codon usage (Figure 2a). The positions of the non-synonymous mutations do not clearly indicate how they affect the functional expression of the enzyme. All three amino acids are located towards the protein surface, although Lys185 is only partially exposed to the solvent via its side-chain amino group (Table II). In addition, they are found distant from the active site, and also from the monomer–monomer contact area (Benning *et al.*, 1994).

Over-expression of the S5 variant without MBP fusion. To test the expression of S5 on its own (with no MBP fusion), pET-20b was used as the expression vector in *E. coli* BL21(DE3) cells. The recombinant protein proved to have the expected molecular weight (i.e. $\sim 35 \text{ kDa}$), as judged by SDS-PAGE (Figure 3a). Total protein expression levels were

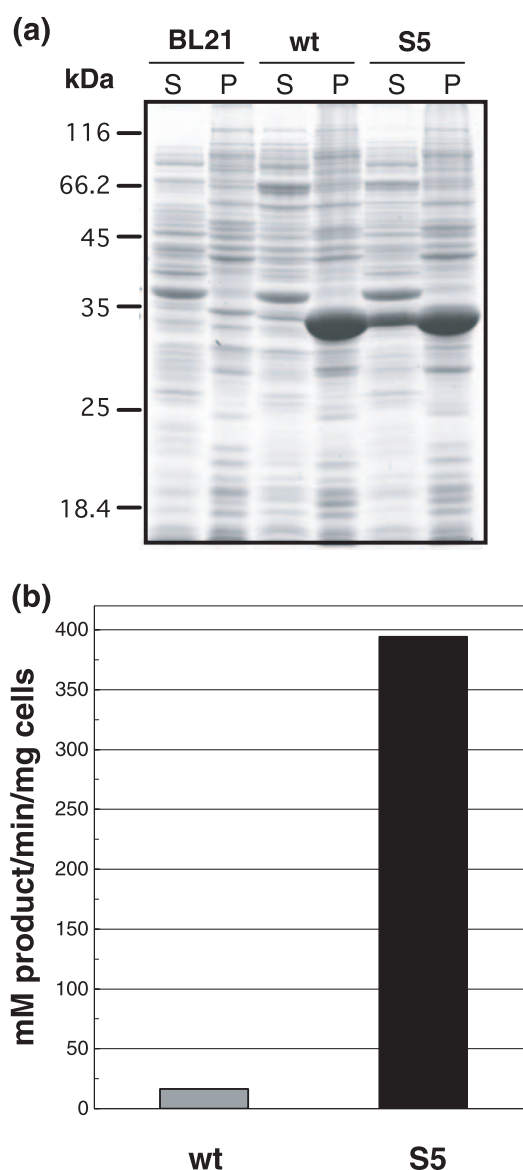


Fig. 3. Overexpression of PTE variant S5. (a) SDS-PAGE (14%) analysis of the supernatant (S) and the pellet (P) of BL21(DE3)pLys cells with no expression vector (BL21) and the same cells overexpressing wt-PTE or the S5 variant, without the MBP fusion. (b) Activity assays of S5 vs wt-PTE lysates [corresponding to supernatants in (a)] normalized according to total protein expression for each clone, with 0.25 mM paraoxon.

Table II. Mutations identified in the different PTE variants

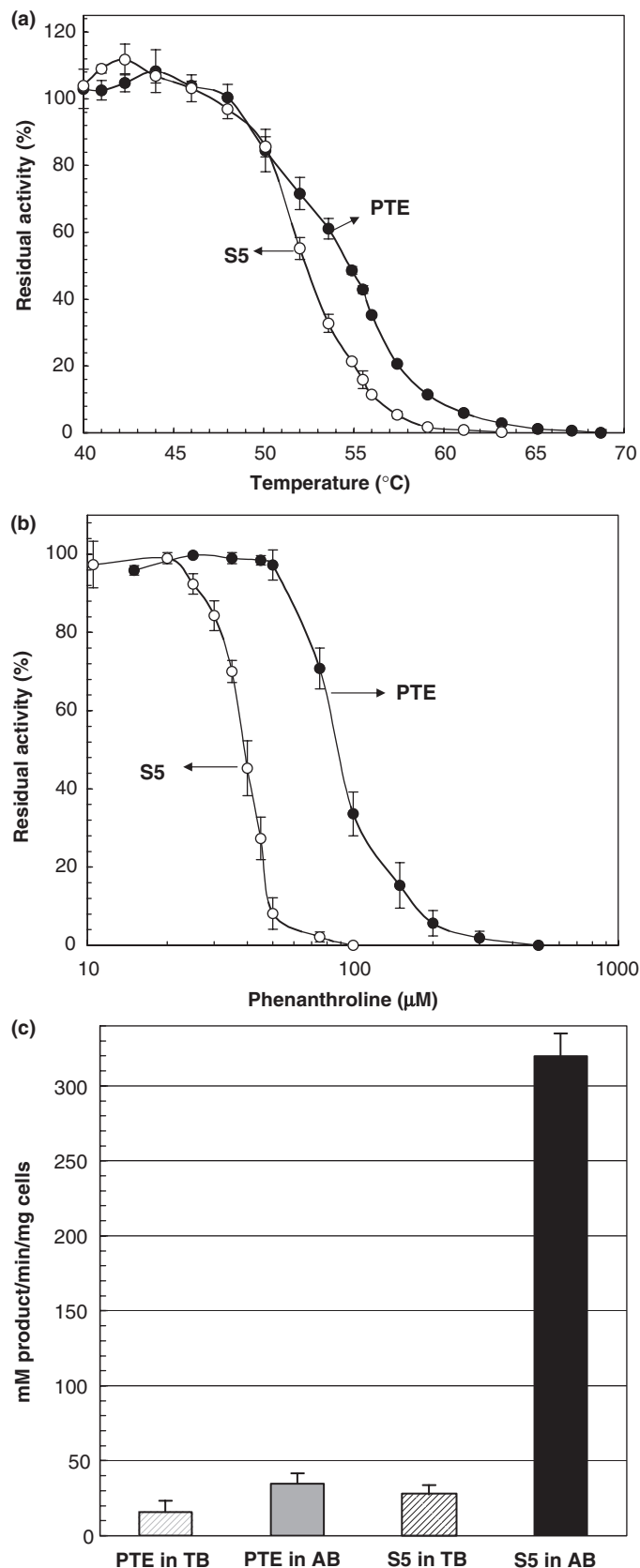
Variant	Round of screening	Library ^a	Non-silent mutations	Silent mutations	Codon	Location
5E	1	EP	Lys185Arg Gln211Leu	T 352	agg ctg act	α -Helix 4 α -Helix 5
6F	1	BA	Asp208Gly		ggt	Loop 5
S5	2	DNA shuffling	Lys185Arg Asp208Gly Arg319Ser	Pro70 ^b Ala78 ^b Asp109 Thr352	agg ggt agc cgc gcc gat act	α -Helix 4 Loop 5 α -Helix ^c

^aEP: library generated by randomization by error-prone PCR. BA: library generated by randomization with a base analogue.

^bThese silent mutations were absent in the pET-20b/S5 construct.

^cLocated out of the (β/α)₈ barrel scaffold, within loop 5.

essentially identical and somewhat lower than those obtained for the MBP fusion, especially for S5 (probably owing to the absence of Pro70 and Ala78 silent mutations in pET-20b-expressed S5). However, as observed with the MBP fusions, the different levels of functional expression were mainly due to



changes in the fraction of soluble protein, rather than total protein expression. The yields of wt-PTE and S5 varied according to growth temperature and time. According to these parameters, the ratio of S5 to wt-PTE ranges from 10-fold (40 h) to 25-fold (8 h) (data not shown). A typical comparison is shown in Figure 3. Even though the percentage of protein in the supernatant is somewhat lower than the corresponding ones as MBP fusions (35 vs 66% for S5 and <2 vs 3% for wt-PTE), the increase in soluble content of S5 relative to wt-PTE (~17-fold) is maintained and in agreement with the paraoxonase activities of the soluble fractions (Figure 3b). The expression levels of S5 under the above conditions were ~50 mg/l culture, whereas those of wt-PTE were ~2.9 mg/l culture.

This difference in soluble enzyme content coincided with that from a cell-free transcription/translation experiment with *E. coli* S30 extracts (Griffiths and Tawfik, 2003). Approximately 50-fold higher paraoxonase activity was observed upon *in vitro* translation of S5—relative to the wt-PTE—genes without MBP fusion (data not shown).

The origins of S5's higher functional expression. To shed light on the possible origins of variant S5's higher functional expression, we performed a series of experiments with crude lysates from over-expressing bacterial cells. Empirical evidence suggests that PTE is a very stable enzyme once assembled with the metal ions, correctly folded and in the dimeric form (Grimsley *et al.*, 1997). Furthermore, its structural stability has been found to be correlated to the nature of the bound metal ion (Rochu *et al.*, 2004). We compared the stability of both wt-PTE and S5 holo-enzymes through chemical and thermal denaturation assays. The stability towards guanidinium hydrochloride denaturation proved similar for both wt-PTE and its S5 variant (data not shown). However, S5 displays a somewhat lower thermal stability, with $T_{50} = 52.5^{\circ}\text{C}$ and $T_m = 61^{\circ}\text{C}$, relative to $T_{50} = 55^{\circ}\text{C}$ and $T_m = 67^{\circ}\text{C}$ for wt-PTE (Figure 4a). We also examined the stability and rate of zinc complexation. The assembly of PTE with metal ions is known to be a requirement for activity and the coordination of at least one of the metals has been suggested to play a role in determining the structural organization of the protein (Lai *et al.*, 1994). In addition, the nature of the bound metal ion has been found to contribute to the enzymes' thermal and pH stability (Rochu *et al.*, 2004). We compared the residual paraoxonase activity of S5 and wt-PTE after incubation with various concentrations of metal chelators (Figure 4b). Interestingly, S5 loses its zinc ions at 10-fold lower phenanthroline concentrations than wt-PTE (a similar ratio was obtained with EDTA;

Fig. 4. Stability of wt-PTE and its S5 variant. (a) Thermal denaturation. BL21(DE3) lysates over-expressing wt-PTE or S5 were incubated at different temperatures for 20 min, cooled to 25°C and the residual activity measured with paraoxon. The results are given as % of the activity of the untreated sample and represent the mean value of two independent experiments. (b) Metal chelation. The residual paraoxonase activity was measured after 90 min of incubation of wt-PTE or S5, lysates with different amounts of phenanthroline. Results are given as % of the activity of the untreated sample and represent the mean value of two independent experiments. (c) Assembly of metal-free apo-enzyme. *E. coli* BL21(DE3) cells were grown in zinc-containing medium while over-expressing either wt-PTE or S5. The paraoxonase activity of the crude lysates was determined after lysis with a buffer without (striped bars, TB) or with zinc (filled bars, AB); similar results were obtained with cobalt chloride (data not shown). Rates were normalized to the total cell mass of each sample. The results represent the mean value of five independent growth and lysis experiments.

data not shown), suggesting that the affinity towards zinc is significantly reduced in the evolved variant. A similar rate of metal assembly was observed with both wt-PTE and S5 (0.026 and 0.04 min⁻¹, respectively) under similar conditions (25–100 µM zinc chloride and 10 mM potassium bicarbonate), suggesting that the main difference between the two is the rate of metal dissociation.

If the S5 variant is less stable than wt-PTE, what then makes it functionally express at much higher levels? The question of availability of metal ions is, of course, central to the formation of correctly stable proteins in cytoplasmic expression systems. *E.coli* possesses an intracellular zinc concentration of around 0.2 mM, yet there is no persistent pool of free zinc ions in the cytoplasm, probably owing to the presence of a myriad of tight metal-ion binding sites which greatly outnumber the total metal ion content of the cell (Outten and O'Halloran, 2001). The tight homeostasis of zinc (Hantke, 2001) suggests that, despite the addition of zinc to growth medium, the intracellular levels are limited and metal assembly of the expressed PTE may occur primarily upon cell lysis. We therefore explored the possibility that the S5 apo-enzyme—and not the metal-assembled holo-enzyme—is more stable than the corresponding wt-PTE intermediate. PTE and S5 were over-expressed in *E.coli* BL21(DE3) cells under conditions optimized for wt-PTE functional expression. The cells were lysed in either Tris buffer or in buffer plus ZnCl₂ and the activities of the crude lysates were measured (Figure 4c). Even under conditions that are optimal for wt-PTE rather than S5, the sample corresponding to the S5 cells lysed in the presence of the zinc ions exhibited ~10-fold higher activity than the corresponding wt-PTE sample. We also observed that the activity level of the S5 sample was about 12-fold higher when the cells were lysed in the presence of the zinc-containing buffer. In contrast, the amounts of active enzyme present in the wt-PTE fractions were similar when the cells were lysed with or without zinc ions.

These results suggest that, even when the bacterial culture was supplemented with zinc ions, the available intracellular zinc concentration was sufficient for assembly of only a small fraction of the PTE molecules that were expressed. The amount of stable and soluble apo-enzyme corresponds to the difference in activity between cell lysates that contain zinc and those that do not. It can be seen that, in the case of S5, most of the enzyme (>96%) is maintained in its apo form until the cell is lysed and exposure to the zinc-supplemented buffer enables formation of the holo-enzyme. In contrast, most of the wild-type apo-enzyme (~99%) was unstable and aggregated and an insignificant amount of stable apo-enzyme was found upon cell lysis (Figure 4c).

It therefore appears that S5 has recruited mutations that cause a stabilization of the apo-enzyme, thereby increasing the ultimate yield of functional protein, possibly at the cost of reducing the holo-enzyme's thermostability and affinity to the zinc ions.

Conclusions

In an attempt to increase PTE's low promiscuous esterase activity, we subjected the enzyme to mutation and selection with an ester substrate. The outcome was not a more active esterase variant, but a higher expressing one. Low levels of expression have inhibited attempts to study interesting and useful proteins such as PTE. As a result, many efforts have been made to increase the functional expression levels and

stabilities of various proteins, including enzymes (Arnold *et al.*, 2001; Turner, 2003; Roodveldt *et al.*, 2005). The work presented here suggests that selection based on a promiscuous activity as a first screen can be of use, particularly when no suitable native substrates are available for facile screening or when the detection of the native activity falls out of the dynamic range of the screen. The snag is that this approach can obviously lead to variants with increased promiscuous activity as well as or instead of, increased expression. For example, serum paraoxonases PON1 and PON3 were both evolved using family shuffling for library making and esterase activity with 2-NA as a screen (Aharoni *et al.*, 2004). Whilst evolution of PON1 led to a mutant with wild-type-like enzymatic properties and much increased expression, evolution of PON3 by the very same method led to a mutant with increased esterase activity and higher expression.

This work also addressed a key and often overlooked aspect that can be exploited in future attempts to improve the functional expression of proteins. It is widely assumed that protein expression levels are determined by the 'solubility' or 'stability' of the expressed protein. This rationale is reflected in strategies that have been successfully applied to improve over-expression yields (Shusta *et al.*, 2000; Morawski *et al.*, 2001; Esteban and Zhao, 2004). However, the main determinant of over-expression for PTE and probably for many other proteins seems to be defined by the energetics of an intermediate (apo-protein, pro-protein or an on- or off-pathway folding intermediate), rather than by the stability or solubility of the fully processed protein (Roodveldt *et al.*, 2005).

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