

# Directed evolution of an extremely fast phosphotriesterase by *in vitro* compartmentalization

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**We describe the selection of a phosphotriesterase with a very fast  $k_{\text{cat}}$  (over  $10^5 \text{ s}^{-1}$ ), 63 times higher than the already very efficient wild-type enzyme. The enzyme was selected from a library of  $3.4 \times 10^7$  mutated phosphotriesterase genes using a novel strategy based on linking genotype and phenotype by *in vitro* compartmentalization (IVC) using water-in-oil emulsions. First, microbeads, each displaying a single gene and multiple copies of the encoded protein, are formed by compartmentalized *in vitro* translation. These microbeads can then be selected for catalysis or binding. To select for catalysis the microbeads are re-emulsified in a reaction buffer of choice with a soluble substrate. The product and any unreacted substrate are coupled to the beads when the reaction is finished. Product-coated beads, displaying active enzymes and the genes that encode them, are detected with anti-product antibodies and selected using flow cytometry. This completely *in vitro* process selects for all enzymatic features simultaneously (substrate recognition, product formation, rate acceleration and turnover) and single enzyme molecules can be detected.**

**Keywords:** high-throughput screening/hydrolase/organophosphate/paraoxon/PTE

## Introduction

Mimicking nature by applying directed evolution in the laboratory is a powerful strategy (Georgiou, 2000; Griffiths and Tawfik, 2000; Ness *et al.*, 2000; Petrounia and Arnold, 2000; Pluckthun *et al.*, 2000; Soumillion and Fastrez, 2001; Wahler and Reymond, 2001). Both natural and directed evolution require a link between genotype (a nucleic acid that can be replicated) and phenotype (a functional trait such as binding or catalytic activity) (Griffiths and Tawfik, 2000). *In vitro*, this linkage is usually achieved by physically linking genes to the proteins they encode by a variety of techniques, including display on phage, viruses, bacteria and yeast, plasmid display, ribosome display and mRNA–peptide fusion. These ‘display technologies’ have proved highly successful in the generation of binding proteins (Schatz *et al.*, 1996; Georgiou *et al.*, 1997; Griffiths and Duncan, 1998; Pluckthun *et al.*, 2000; Sidhu, 2000; Amstutz *et al.*, 2001; Keefe and Szostak, 2001; Wittrup, 2001).

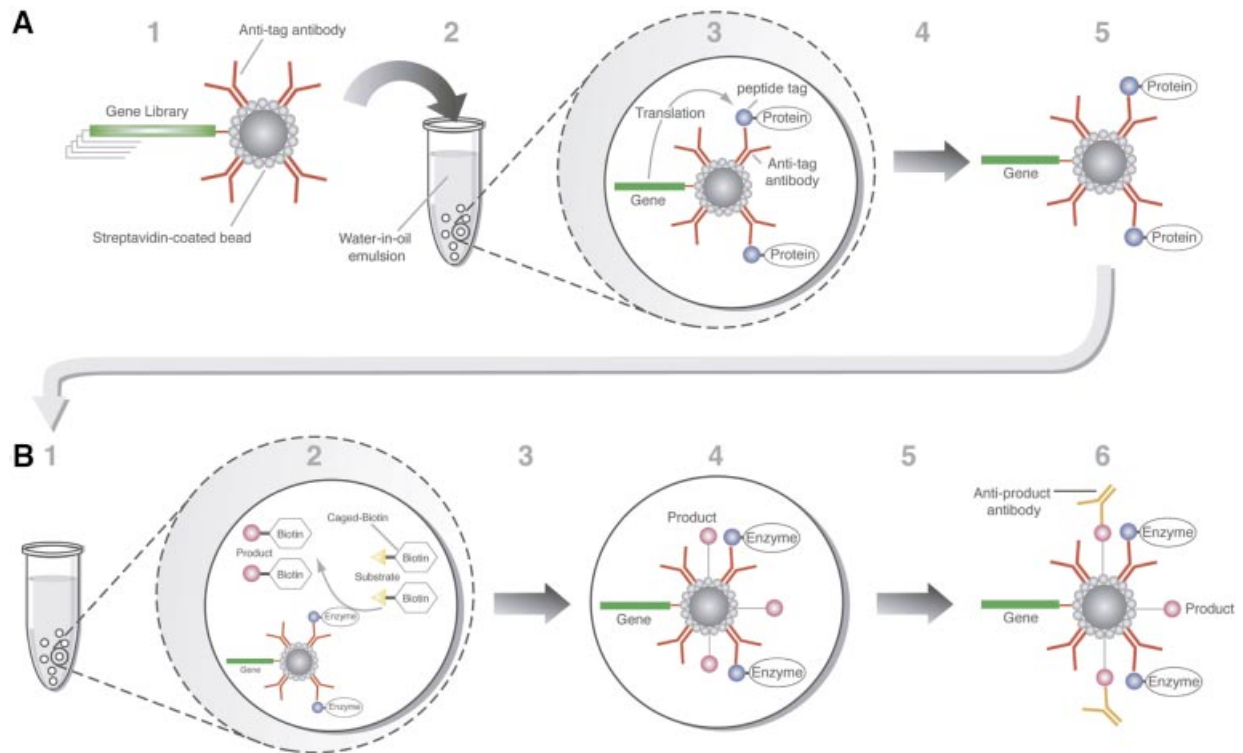
In contrast, selection of enzymes by display approaches has met with little success. Indirect selections, by binding to transition state analogues or enzyme inhibitors have generally failed to produce potent catalysts (Griffiths and Tawfik, 2000). Single-turnover intramolecular selections of enzymes displayed on phage have been demonstrated, but these impose severe limitations (Atwell and Wells, 1999; Griffiths and Tawfik, 2000). To evolve proficient enzymes, the selection (or screen) should be simultaneous and direct for all enzymatic properties: substrate recognition, formation of a specific product, rate acceleration and turnover. The only efficient selection for turnover described to date is for variants of the *Escherichia coli* outer-membrane protein OmpT (Olsen *et al.*, 2000).

Direct selection for all enzymatic properties can be achieved by compartmentalization in cells (as in nature). Unfortunately, *in vivo* selections are usually, but not always (Firestine *et al.*, 2000), restricted to functions that affect the viability of the organism and are often complicated by the complex intracellular environment and the need to transform the gene-library. More commonly,  $10^3$ – $10^5$  clone libraries are screened in a plate assay using a fluorogenic or chromogenic substrate. However, crossing long evolutionary distances, and evolving completely novel proteins and activities, requires much larger libraries (Griffiths and Tawfik, 2000; Keefe and Szostak, 2001). In these cases, selection rather than screening is preferable. Therefore there is little doubt that purely *in vitro* selection systems will eventually prove advantageous (Fastrez, 1997; Minshull and Stemmer, 1999; Pluckthun *et al.*, 2000).

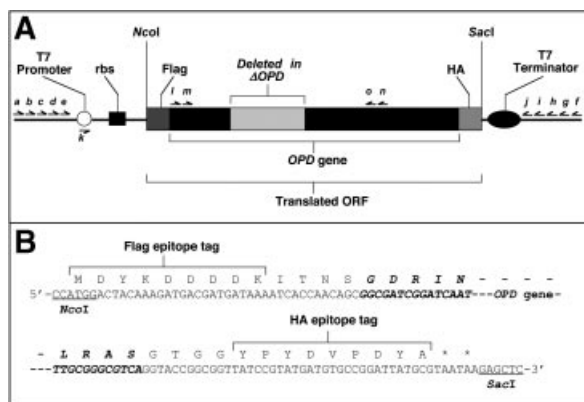
In this context, we have developed a completely *in vitro* system for the selection of enzymes, based on *in vitro* compartmentalization (IVC). Reactions are partitioned into microscopic compartments, each of only ~5 fl, by forming water-in-oil emulsions. A 50  $\mu\text{l}$  reaction can be dispersed in ~ $10^{10}$  aqueous compartments, allowing the selection of many genes and making the system highly sensitive and economical. We have previously used IVC to select DNA-methyltransferases (Tawfik and Griffiths, 1998; Lee *et al.*, 2002). Compartmentalization in emulsions has also been used for the directed evolution of *Taq* DNA polymerase (Ghadessy *et al.*, 2001).

Here, we describe a novel IVC strategy based on creating repertoires of microbeads, each displaying a gene and the protein it encodes (Figure 1A). These ‘microbead-display libraries’ can be selected on the basis of the binding activity of the displayed polypeptide (Sepp *et al.*, 2003) or, as described here, selected for catalysis with a soluble non-DNA substrate under multiple turnover conditions and in a chosen reaction environment (Figure 1B).

We have demonstrated this approach by selecting genes encoding a bacterial phosphotriesterase (PTE) which has attracted considerable interest because of its ability to



**Fig. 1.** Creation of microbead-display libraries and selection for catalysis by compartmentalization. **(A)** Creation of microbead-display libraries. A repertoire of genes encoding protein variants, each with a common epitope tag, is linked to streptavidin-coated beads carrying antibodies that bind the epitope tag at, on average, less than one gene per bead (1). The beads are compartmentalized in a water-in-oil emulsion to give, on average, less than one bead per compartment (2), and transcribed and translated *in vitro* in the compartments. Consequently, in each compartment, the translated protein (10–100 copies) becomes attached to the gene that encodes it via the bead (3). The emulsion is broken (4) and the microbeads carrying the display library are isolated (5). **(B)** Enzyme selection by compartmentalization. Microbead-display libraries are compartmentalized in a water-in-oil emulsion (1) and a soluble substrate attached to caged-biotin is added. The substrate is converted to product only in compartments containing beads displaying active enzymes (2). The emulsion is then irradiated to uncage the biotin (3). In a compartment containing a gene encoding an enzyme, the product becomes attached to the gene via the bead (4). In other compartments, in which the genes do not encode an active enzyme, the intact substrate becomes attached to the gene. The emulsion is broken (5) and the beads are incubated with anti-product antibodies (6). Product-coated beads can then be enriched (together with the genes attached to them) either by affinity purification or, after reacting with a fluorescent labelled antibody, by flow cytometry.



**Fig. 2.** The pIVEX-OPD vector and primer annealing sites. **(A)** The region of the vector pIVEX-OPD around the cloned OPD gene (Mulbry and Karns, 1989). The NcoI and SacI restriction sites used for cloning and the translated open reading frame (Translated ORF), which encodes PTE with N-terminal Flag (Chiang and Roeder, 1993), and C-terminal HA (Field et al., 1988) epitope tags are indicated. The region deleted in pIVEX- $\Delta$ OPD is also shown. The vector contains a T7 promoter, enhancer, terminator and ribosome binding site (rbs). The annealing sites for oligonucleotide primers used for PCR (Table IV) are indicated (a–o). **(B)** Sequence of pIVEX-OPD between the NcoI and SacI sites. The sequence outside this region is as pIVEX2.2b Nde (Roche). The sequences encoding the Flag and HA epitope tags are indicated and the OPD gene sequence is shown in bold italics.

degrade organophosphate pesticides and nerve agents such as soman, sarin and VX (reviewed by Raushel and Holden, 2000). Although PTE is thought to have evolved within ~50 years, its catalytic performance is remarkable. With its best substrate, paraoxon,  $k_{cat}$  is  $2280 \text{ s}^{-1}$  and  $k_{cat}/K_M$  is  $6.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ , close to the diffusion-controlled limit (Hong and Raushel, 1999). Nevertheless, we were able to select a mutant with 63-fold improved turnover number from a library of mutated PTE genes.

## Results

### Microbead display: linking genes to the proteins they encode

First,  $6 \times 10^8$  streptavidin-coated beads of diameter  $1 \mu\text{m}$  coated with biotinylated anti-HA antibodies were incubated with biotinylated OPD genes encoding the enzyme PTE, or with biotinylated  $\Delta$ OPD genes encoding an inactive protein (Figure 2) or with mixtures of these genes at a ratio of 0.3 genes per bead. These microbeads were resuspended in a cell-free translation mixture, compartmentalized in a water-in-oil emulsion, and incubated to allow translation of the genes and capture of the HA-tagged protein by the anti-HA antibody. After the emulsion was broken, the beads, which display the proteins

encoded by the genes attached to them (Figure 1A), were resuspended in a buffer suitable for the enzymatic reaction and which contained zinc and carbonate ions to allow the captured inactive apo-enzyme to assemble into the catalytically active metallo-enzyme (Hong *et al.*, 1995). Each bead captured ~30 active PTE molecules (Table I, row 2, column c), corresponding to more than half of the total *in vitro* expressed protein. Translation and capture of the enzyme onto the beads proceeds in bulk solution and in the emulsion with comparable efficiency (Table I, compare rows 3 and 6, column c). However, compartmentalization in an emulsion ensures that translated proteins become attached to the genes that encode them (via the bead) and not to other genes (attached to other beads).

### The compartmentalized selection of microbeads for enzymatic activity

The microbead-displayed gene-protein complexes created in the first emulsion (Figure 1A) were re-compartmentalized in a second emulsion (Figure 1B). The caged-biotinylated substrate EtNP-cgB (Figure 3B) was then added to the oil phase from where it diffuses into the aqueous droplets. This substrate is a derivative of paraoxon (Figure 3A) where an ethyl group is replaced by a linker connected to caged-biotin (Pirung and Huang, 1996). EtNP-cgB hydrolysis is catalysed by recombinant Zn<sup>2+</sup>-PTE (Dumas *et al.*, 1989) with kinetic parameters comparable to those of paraoxon ( $K_M = 17 \mu\text{M}$ ,  $k_{\text{cat}} = 160 \text{ s}^{-1}$ ) for one of the two enantiomers of the chiral phosphotriester, whilst the other is hydrolysed ~4000 times more slowly, consistent with previous enantioselectivity studies (Hong and Raushel, 1999). The emulsions were incubated for 16 h to allow the hydrolysis of the substrate and then irradiated to yield the biotinylated product or substrate which binds to the streptavidin-coated bead in the compartment.

The emulsions were then broken and the beads fluorescently labelled using antibodies that bind the phosphodiester product (Et-B) but not the unhydrolysed substrate (EtNP-B) (Figure 3B). Flow cytometry can distinguish between beads with different substrate:product ratios (Figure 4). Hence relatively small differences in the

amount of product on beads can be translated into relatively large enrichments by sorting through suitable gates.

Flow cytometry revealed that beads carrying the  $\Delta\text{OPD}$  gene (encoding an inactive protein) carry the substrate and, as expected, exhibit low fluorescence (Figure 5, column 1, row A). In contrast, beads carrying the *OPD* genes (encoding active PTE) carry the product and are highly fluorescent (Figure 5, column 1, row B). Not all the beads are highly fluorescent, as, on average, only one in three had a gene attached. When the *OPD* gene was spiked into an excess of  $\Delta\text{OPD}$  genes, a mixture of 'positive' (high-fluorescence) and 'negative' (low-fluorescence) beads was observed (Figure 5, column 1, row C–E). The percentage of positive beads (Table I, column d) correlates well with the percentage of *OPD* gene (Table I, column a), but as at most one-third of the beads should carry a gene, the number of positive beads is higher than expected. For example, the emulsion prepared with *OPD* genes should have yielded 33% rather than 74.3% of positive beads (Table I, column d, row 2; Figure 5, column 1, row B). This is almost certainly because some compartments contain more than one bead.

To demonstrate the enrichment of genes encoding PTE, 10<sup>5</sup> single unaggregated high-fluorescence beads (gated through R1 and M1) from the experiments with 1:10, 1:100 or 1:1000 starting ratios of *OPD* to  $\Delta\text{OPD}$  genes (Figure 5, columns 1, row C–E) were sorted by flow cytometry. Analysis of the sorted beads showed efficient enrichment (Table I, column e; Figure 5, column 2). The genes attached to sorted and unsorted beads were amplified by PCR (Table I, column f; Figure 5, column 3). The ratio of genes before and after selection indicated up to 200-fold enrichment of the *OPD* gene following selection (Table I, column g). These results demonstrate that compartmentalization in the second emulsion can be used to select for enzymatic activity, and that genes were linked, via microbeads, to the proteins they encode in the first emulsion. Indeed, when translation was performed in bulk solution rather than in an emulsion, the translated enzyme distributed between the beads regardless of whether they carried the *OPD* or  $\Delta\text{OPD}$  genes.

**Table I.** Creation of microbead-display libraries and selections for catalysis

	a	b	c	d	e	f	g
	Starting ratio of <i>OPD</i> : $\Delta\text{OPD}$ genes <sup>a</sup>	Generation of display libraries <sup>b</sup>	Captured PTE molecules per gene <sup>c</sup>	Percentage of positive events in unsorted beads <sup>d</sup>	Percentage of positive events in sorted beads <sup>d</sup>	Final ratio of <i>OPD</i> to $\Delta\text{OPD}$ genes <sup>e</sup>	Enrichment of the <i>OPD</i> gene <sup>f</sup>
1	$\Delta\text{OPD}$ alone	Compartmentalized	0	0	–	–	–
2	<i>OPD</i> alone	Compartmentalized	31.5	74.3	–	–	–
3	1:10	Compartmentalized	3.6	11.4	93.6	1:0.7	14
4	1:100	Compartmentalized	0	1.08	86.6	1:2.1	47
5	1:1000	Compartmentalized	0	0.09	57.5	1:4.6	217
6	1:10	Non-compartmentalized	3.8	61.9	93.2	1:7.6	1.3

<sup>a</sup>Coated at 0.3 genes per bead.

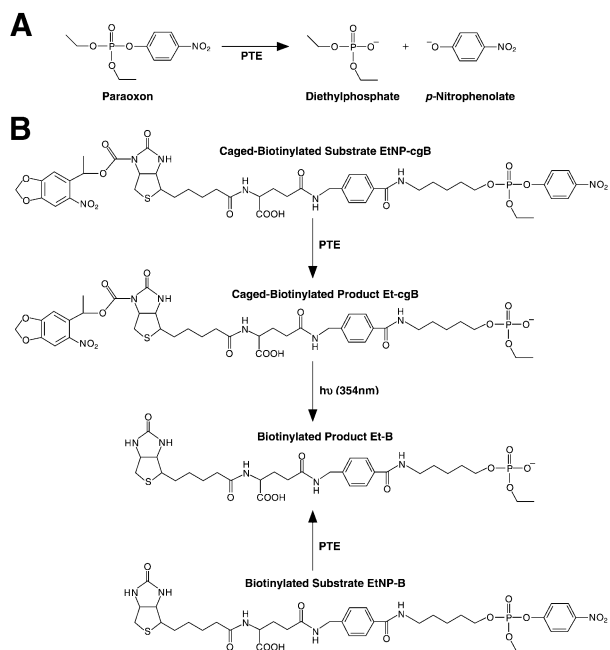
<sup>b</sup>Translation was in bulk (non-compartmentalized) or in an emulsion (compartmentalized).

<sup>c</sup>Calculated by measuring paraoxon hydrolysis using the published kinetic parameters of Zn<sup>2+</sup>-PTE (Hong and Raushel, 1999).

<sup>d</sup>The 'noise', the percentage of events in region M1 with the  $\Delta\text{OPD}$ -coated beads (Figure 5, column 1, row A; 0.21%), was subtracted. See Figure 5, columns 1 and 2.

<sup>e</sup>Determined by densitometry after amplification of DNA from sorted beads. See Figure 5, column 3.

<sup>f</sup>The final ratio of *OPD*/ $\Delta\text{OPD}$  genes amplified from the sorted beads divided by the starting ratio of genes.

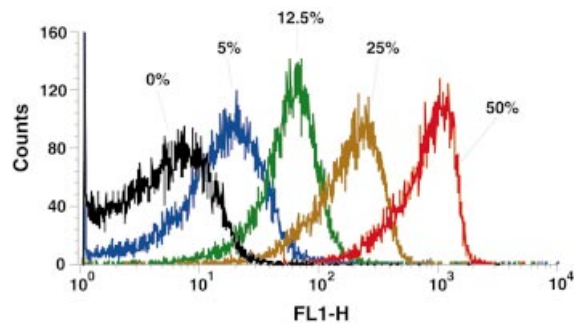


**Fig. 3.** PTE substrates. (A) PTE catalysed hydrolysis of paraoxon. (B) For selection, paraoxon was modified by substituting an ethyl group with a linker connected to caged-biotin (Pirrung and Huang, 1996). PTE-catalysed hydrolysis of the resulting substrate (EtNP-cgB) gives *p*-nitrophenol and the corresponding phosphodiester Et-cgB. Irradiation at 354 nm releases the caging group and carbon dioxide to yield the (uncaged) biotinylated substrate (EtNP-B) or product (Et-B).

Consequently, most of the beads carried at least one enzyme molecule, became labelled with product and were selected as positive (Figure 5, column 1, row F). However, when the DNA on the selected beads (Figure 5, column 2, row F) was amplified, no enrichment for the *OPD* gene was observed (Figure 5, column 3, row F; Table I, row 6).

### Compartmentalization allows single enzyme molecules to be identified

Beads carrying different numbers of PTE molecules were subjected to the enzymatic selection procedure (Figure 1B), either in an emulsion or in bulk solution (Figure 6). When coated with 10.8 PTE molecules per bead, all beads appeared to be highly fluorescent or ‘positive’. When fewer PTE molecules were coated and the reactions were performed in bulk solution, the enzyme concentration was too low to catalyse the complete conversion of substrate into product. The fluorescence was accordingly reduced and, owing to the equal distribution of substrate and product over the entire population of beads, a single population of beads was observed (Figure 6, column 1, row B and C). However, when the same beads were compartmentalized, two populations were observed: ‘positive’ beads, which were as fluorescent as those obtained with 10.8 PTE molecules per bead, and ‘negative’ beads, similar to those observed when no PTE was coated on the beads (Figure 6, column 2, row B and C). When there are far fewer PTE molecules than beads, for example, one PTE molecule per 37 beads (Figure 6, column 2, row C), the vast majority of beads carry no PTE molecules and exhibit low fluorescence, and the rest



**Fig. 4.** Detection of substrate and product on microbeads by flow cytometry. Streptavidin-coated beads were coated with biotinylated anti-HA antibodies and then with mixtures of the biotinylated PTE substrate EtNP-B and product EtNP-B (Figure 3B) at the ratios indicated (% product). After fluorescent labelling using anti-product antibodies, the beads were analysed by flow cytometry. The levels of fluorescence (FL1-H) on single unaggregated beads (gated using forward and side scatter as in Figure 5) are plotted as histograms.

probably carry only one PTE molecule and are highly fluorescent. We believe that this is due to the small volume of the compartments ( $\sim 5$  fl), in which a single PTE molecule is present at a sufficiently high concentration ( $\sim 0.2$  nM) to allow the complete conversion of substrate into product, thereby enabling a bead carrying a single PTE molecule to be identified.

### Construction of PTE libraries

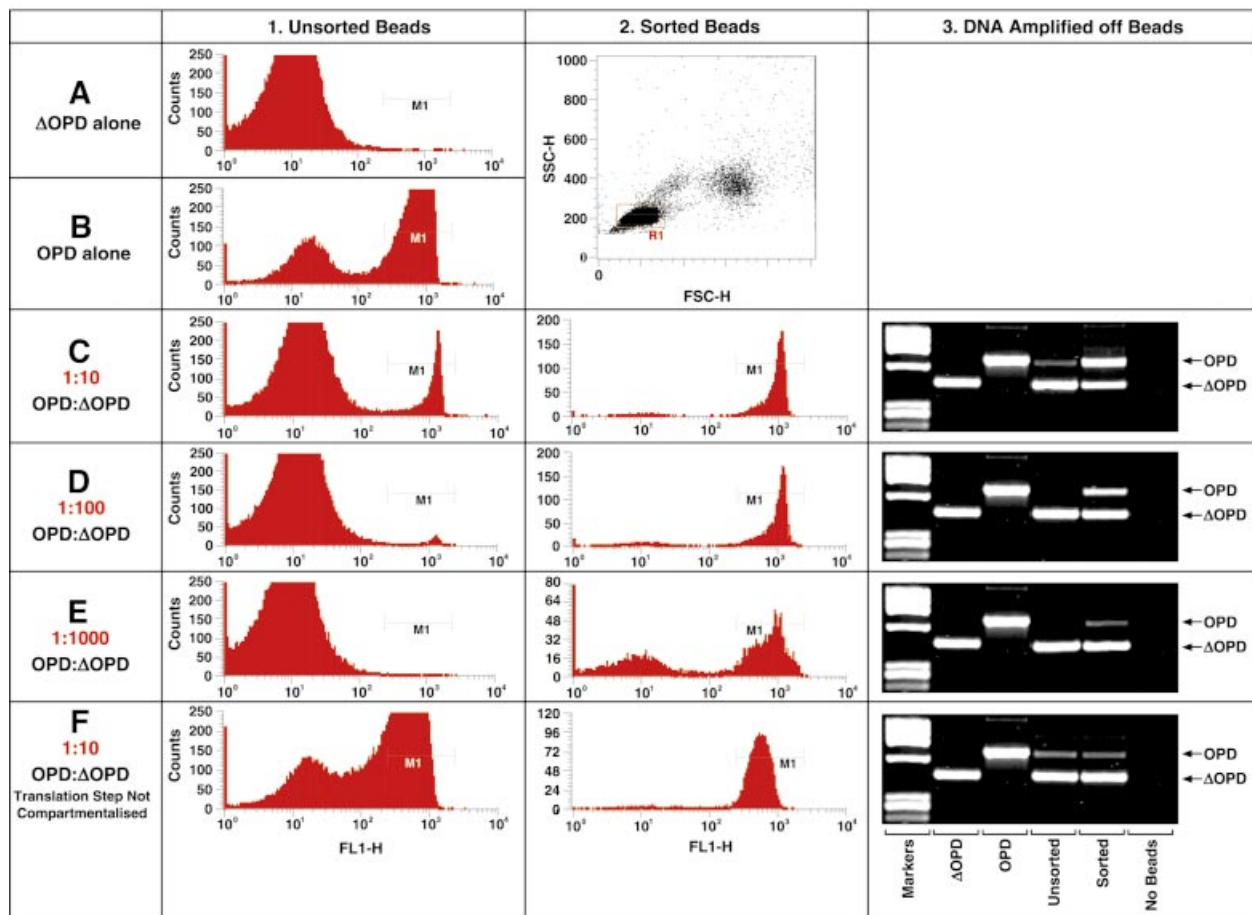
Three substrate binding pockets (the large, small and leaving group pockets) have been assigned within the active site of PTE based on its structure (Vanhook *et al.*, 1996) (Figure 7A). We created a gene library (Library D, diversity  $3.4 \times 10^7$ ) by randomizing the codons for Ile106, Trp131, Phe132, Ser308 and Tyr309 in wild-type PTE (Figure 7B). These residues define the entrance to the active site (the leaving group site) and the small subsite. Phe306 and Leu303 were not randomized as they also help form the large subsite, and Gly60 was left unchanged so as not to reduce further the size of the small subsite. Three sub-libraries were also created, with the following residues randomized: Library A, Ile106 (diversity 32); Library B, Ile106, Ser308 and Tyr309 (diversity  $3.3 \times 10^4$ ); Library C, Ile106, Trp131 and Phe132 (diversity  $3.3 \times 10^4$ ).

### Selection of PTE libraries

For each library,  $2 \times 10^8$  molecules of linear DNA, with a triple biotin at each end, were attached to  $6 \times 10^8$  streptavidin-coated beads and selected for the ability to hydrolyse the phosphotriester substrate EtNP-cgB (Figure 3B) as above (Figure 1).

In the first round of selection, sorting was performed with a gate (M1) set to include no more than 1% of false positives as determined by flow cytometry of beads that were not coated with DNA (Figure 8, row a);  $10^5$  high-fluorescence beads were collected from Libraries A, B and C, and  $5 \times 10^5$  beads from Library D. In subsequent rounds  $10^5$  beads were collected from all libraries.

After each round of selection the DNA was amplified off the sorted beads by nested PCR. To prevent the



**Fig. 5.** Selections for genes encoding PTE. Microbeads displaying the proteins encoded by the genes attached to them (Figure 1A) were created using the *OPD* and  $\Delta$ *OPD* genes and mixtures thereof (Table I). These were then selected for enzymatic activity (Figure 1B) using EtNP-cgB (Figure 3B) as substrate. After fluorescent labelling using anti-product antibodies the beads were analysed by flow cytometry. Forward scatter (FSC-H) and side scatter (SSC-H) indicated that most of the beads were single and unaggregated (95% of total events were in R1 of the dot-plot, column 2). The levels of fluorescence (FL1-H) on single unsorted beads (gated through R1) are plotted as histograms (column 1). The ‘positive’ highly fluorescent beads (in region M1) were sorted from the ‘negative’ low-fluorescence beads and re-analysed (column 2). The genes on the sorted ‘positive’ beads (and on unsorted beads) were PCR amplified and analysed by agarose gel electrophoresis (column 3).

accumulation of PCR artefacts, the *OPD* gene was excised from the amplified DNA using *NcoI* and *SacI* (Figure 2), ligated into the expression vector and re-amplified directly with triply biotinylated primers that anneal in the vector outside the annealing sites of the primers used for nested PCR. The libraries were taken through one to six rounds of selection.

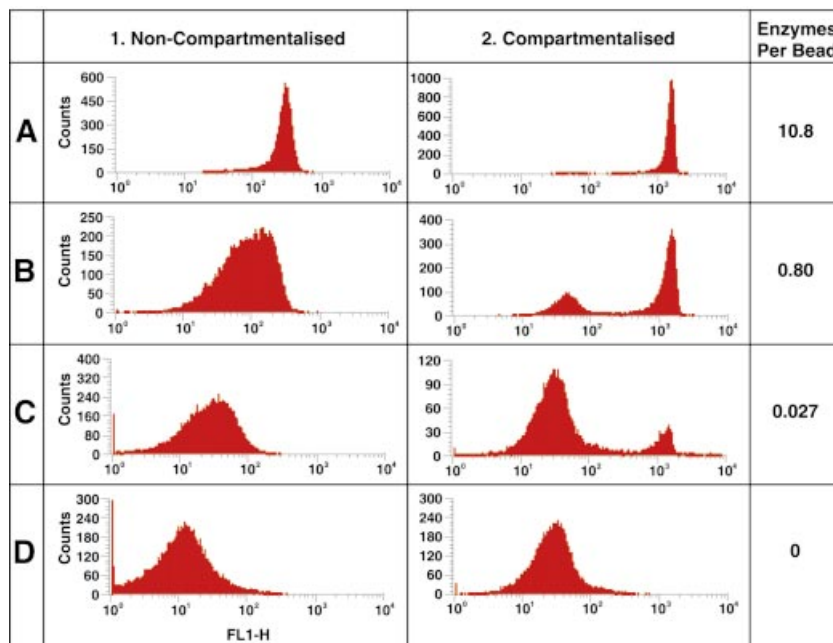
Selection of the largest library (Library D) resulted in an enrichment for PTE activity as seen by the gradual appearance of significant numbers of high-fluorescence (product-coated) beads (Figure 8, column 3), and increased PTE activity following translation of the pool of selected genes (Figure 8, panel 4). There was little detectable PTE activity before selection (0.14% of wild type), but this rose to 44% of wild-type activity after the sixth round.

The selected and unselected libraries were also cloned, and DNA from individual colonies was amplified by PCR and translated *in vitro* and the PTE activity was measured with paraoxon as substrate. Table II shows the activities and sequences of 35 active clones taken at random from the selected libraries. None had the wild-type sequence.

Before selection of Library D, 6% of clones tested had low but detectable [ $\geq 10$  nM/min/ $\mu$ l *in vitro* translation (IVT)] PTE activity (on average 0.18% of wild-type activity). Although the percentage of genes in the library with detectable activity had risen only slightly by the sixth round of selection (to 14%), the mean activity had risen to 31% of wild-type activity. Hence there was a clear enrichment for clones with higher PTE activity.

Similar enrichments were seen with the smaller sublibraries, but fewer rounds of selection were required owing to the higher percentage of genes with significant PTE activity in the unselected libraries (Figure 8).

The PTE activity observed after translation of pools of sorted genes from the final round of selection varied with the gate used to sort the beads (Figure 8, column 1, row C, column 2, row E, column 3, row G), with higher-fluorescence beads yielding higher PTE activity. However, the higher-fluorescence gates did not yield a significantly higher percentage of positive clones, nor was there a significant difference in the activities of the individual clones. This discrepancy is probably due to the relatively small number of clones analysed and the large



**Fig. 6.** Identification of single PTE molecules. Recombinant epitope-tagged PTE (Figure 2) was bound to streptavidin-coated beads via the N-Flag tag at different ratios as indicated. The beads were selected for catalysis (Figure 1B) using EtNP-cgB (Figure 3B) as substrate, either compartmentalized (in an emulsion) or non-compartmentalized. After fluorescent labelling using anti-product antibodies, the beads were analysed by flow cytometry. The levels of fluorescence (FL1-H) on single unaggregated beads (gated using forward and side scatter as in Figure 5) are shown for non-compartmentalized beads (column 1) and compartmentalized beads (column 2).

effect that a few highly active mutants (e.g. h5) can have on the activity of the pool of genes.

#### **Kinetic characterization of selected mutants reveals a phosphotriesterase with a very high $k_{cat}$**

The kinetic parameters for 10 of the PTE mutants were determined using paraoxon as substrate (Table III). The majority had a  $k_{cat}$  higher than wild-type PTE and for one of these (h5)  $k_{cat}$  was  $1.4 \times 10^5 \text{ s}^{-1}$ , which is 63 times higher than the wild type.  $K_M$  was increased for all the mutants (from 5- to 143-fold relative to the wild type) and only the two mutants with the fastest  $k_{cat}$  (h5 and b5) had a  $k_{cat}/K_M$  higher than the wild type. Data obtained with EtNP-cgB (Figure 3B) also indicated that both  $k_{cat}$  and  $K_M$  were increased in clones with activities higher than the wild type (e.g. h5). However, the limited solubility and availability of EtNP-cgB prevented a full kinetic analysis.

## **Discussion**

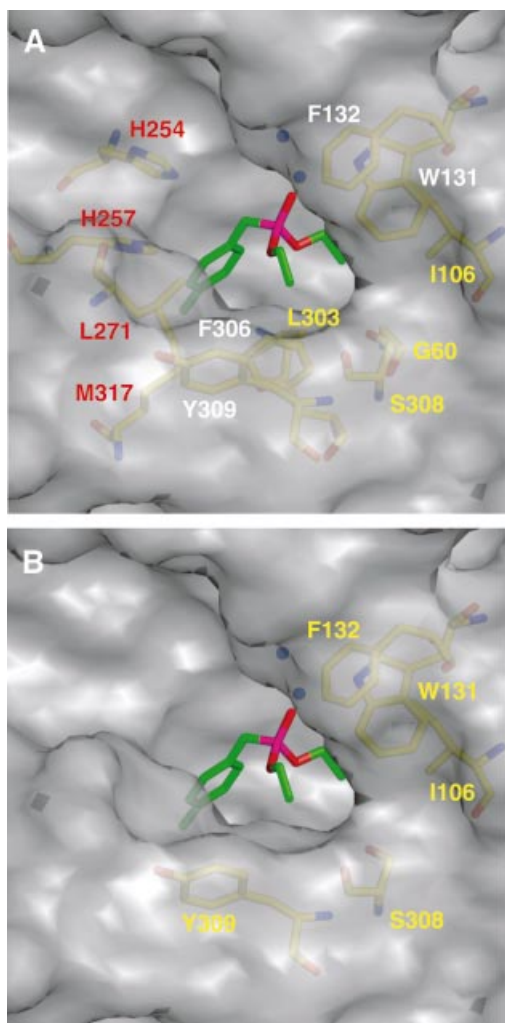
While several completely *in vitro* systems are available for the selection of proteins for binding (Roberts, 1999; Pluckthun *et al.*, 2000), IVC is currently the only way of selecting directly for enzymatic activity. To date, however, IVC has only been used to select enzymes with DNA substrates, where both the enzymatic reaction and translation took place in the same environment, and where selection was not necessarily for multiple turnovers (since the enzyme was in molar excess relative to the substrate) (Tawfik and Griffiths, 1998; Lee *et al.*, 2002).

Here we describe a more general mode of selection with IVC which allows enzymes with soluble non-DNA substrates to be selected under multiple turnover conditions and in a reaction environment of choice. It is based

on creating libraries of proteins displayed on microbeads by translation in an emulsion (Figure 1A) and then re-compartmentalizing the microbead-display libraries in a second emulsion to select for catalysis (Figure 1B). After the second emulsion, product-coated microbeads are sorted by flow cytometry, making this technically a high-throughput screen rather than a selection. However, preliminary results indicate that a genuine selection can be performed by affinity purification of beads using anti-product antibodies, which should also enable selection of larger repertoires.

Selection for enzymatic activity is completely detached from translation, can take place in any buffer or at any temperature and is not complicated by the complex milieu of a cell or an *in vitro* translation system. Even thermophilic enzymes could potentially be evolved since emulsions similar to the ones used here are stable at 99°C (Ghadessy *et al.*, 2001).

Selection is also performed on a soluble substrate (at essentially any given concentration) and is for turnover. A comparison of the fluorescence of beads carrying a single enzyme molecule per bead (Figure 6) with the fluorescence of beads coated with known ratios of substrate and product (Figure 4) indicated that almost all the active enantiomer of the substrate had been converted to product. As  $>10^6$  substrate molecules were added per bead, each enzyme must have catalysed the formation of  $\sim 10^6$  product molecules. At the same time, partial conversion of substrate into product can be detected ( $\geq 5\%$ ; Figure 4) and, typically,  $>30$  enzyme molecules are displayed on each bead. Thus the system has the potential to select enzymes that are at least 300-fold less active than wild-type PTE. Based on PTE having a  $k_{cat}$  for EtNP-cgB of  $160 \text{ s}^{-1}$  and assuming the rate of base-catalysed hydrolysis



**Fig. 7.** The substrate binding pockets of PTE. Based on the coordinates of  $\text{Zn}^{2+}$ -PTE with the bound substrate analogue diethyl 4-methylbenzylphosphate (Vanhook et al., 1996). (A) The amino-acid residues whose side-chains define the substrate binding site. Residues forming the small subsite are annotated in yellow, those forming the large subsite in red and those forming the leaving group subsite in white. (B) The five amino acid residues randomized in the libraries.

of EtNP-cgB to be  $2.4 \times 10^{-7} \text{ s}^{-1}$  at pH 8.5, as for paraoxon (Dumas *et al.*, 1989), this represents a dynamic range (in terms of  $k_{\text{cat}}/k_{\text{uncat}}$ ) from at least  $2 \times 10^6$  up to  $\sim 10^9$ , which is probably sufficient to improve or alter the activity of most existing enzymes (Fastrez, 1997; Griffiths and Tawfik, 2000).

We have demonstrated the utility of this technique by selecting improved enzymes from libraries based on PTE. We created a PTE library with five codons randomized (overall diversity of  $3.4 \times 10^7$ ), which form the entrance to the active site (the leaving group site) and the so-called small subsite (Figure 7). Selection resulted in an enrichment for PTE activity; the activity of the pool of selected genes following translation rose from barely detectable before selection to 44% of wild-type activity after the sixth round (Figure 8). The percentage of clones with detectable activity rose only slightly by the sixth round of selection (from 6% to 14%) but there was a clear enrichment for

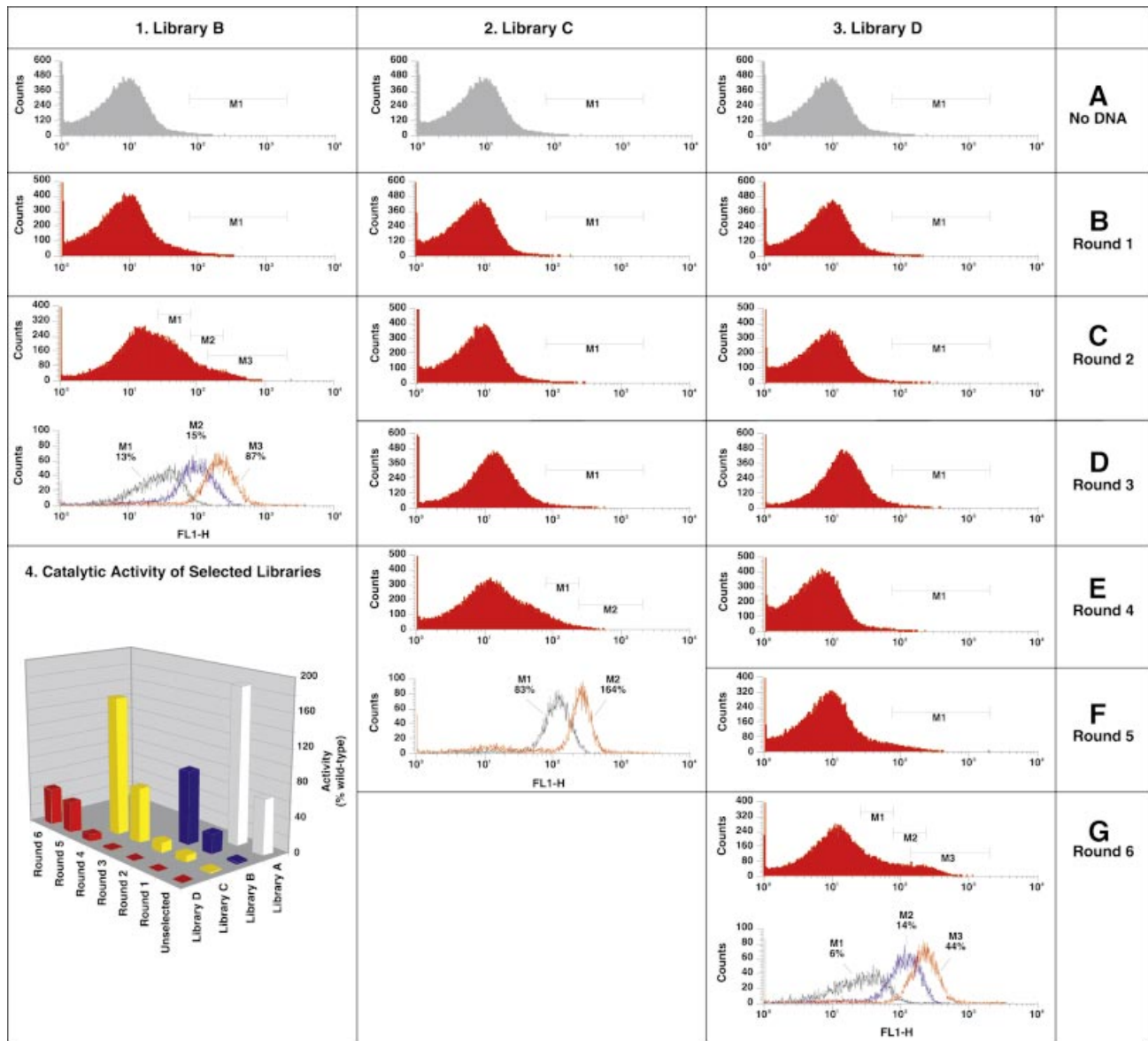
clones with higher PTE activity at the expense of those with lower activity. A similar pattern was seen with smaller sublibraries, but fewer rounds of selection were required owing to the higher frequency of genes with significant activity.

When single clones with PTE activity were analysed after the final round of selection none had the wild-type sequence (Table II). Indeed, at position 106, the wild-type residue isoleucine was present in only two clones with very low activity. Instead, the commonest residues were serine and threonine. At position 132, the wild-type residue phenylalanine was the second most common residue, after leucine. Many different amino acids were seen at the three other positions mutated, but the wild-type residue was the most common. Although the substrate binding site of PTE is predominantly hydrophobic, a potential hydrogen bond between  $N^{\epsilon 1}$  of Trp131 and the phosphoryl oxygen of the substrate has been identified (Vanhook *et al.*, 1996; Benning *et al.*, 2000), and indeed this residue prevailed in the more active clones.

The above sequence preference was reflected in the kinetic properties of the selected PTEs (Table III). The clone (h5) with the fastest turnover number was isolated from Library D in which five codons were randomized. However, this clone had only two mutations relative to wild-type PTE, Ile106 to Thr and Phe132 to Leu. Of the clones analysed kinetically, the majority had a  $k_{\text{cat}}$  higher than wild-type PTE and a lower  $k_{\text{cat}}/K_M$ . Only two clones (h5 and b5) exhibited a  $k_{\text{cat}}/K_M$  higher than the wild type. The increase in  $K_M$  observed in all the selected mutants (5- to 143-fold) suggests that selection occurred under substrate concentrations that are significantly higher than  $K_M$  for the wild type (Fersht, 1999). However, the caged-biotinylated substrate EtNP-cgB was added to a maximum concentration of  $50 \mu\text{M}$  (assuming that all the substrate had partitioned into the aqueous droplets), a concentration that is very similar to the  $K_M$  of both paraoxon and EtNP-cgB. Therefore, it is possible that the effective substrate concentration in the aqueous compartments is higher than expected, perhaps due to surface effects at the water-oil interface of the emulsion.

Wild-type PTE is already a very efficient enzyme;  $k_{\text{cat}}$  for paraoxon hydrolysis (Figure 3A) is  $2280 \text{ s}^{-1}$ , and the  $k_{\text{cat}}/K_M$  of  $6.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  is close to the diffusion-controlled limit (Hong and Raushel, 1999). Despite this, PTE-h5 has a  $k_{\text{cat}}$  of  $1.4 \times 10^5 \text{ s}^{-1}$ , 63 times faster than wild-type PTE, and a  $k_{\text{cat}}/K_M$  slightly higher than that of the wild type ( $1.8 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ ). In the most efficient enzymes,  $k_{\text{cat}}/K_M$  can be as high as  $3 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ , in which case the rate-determining step for  $k_{\text{cat}}/K_M$  is thought to be the diffusion-controlled encounter of the enzyme and the substrate (Fersht, 1999). Thus, PTE-h5 is one of the most efficient enzymes ever described. There are some enzymes with a faster  $k_{\text{cat}}$ , notably catalase with a  $k_{\text{cat}}$  of  $4 \times 10^7 \text{ s}^{-1}$  (Ogura, 1955), but, to the best of our knowledge, the fastest hydrolase described previously is acetylcholinesterase [ $k_{\text{cat}} = 1.4 \times 10^4 \text{ s}^{-1}$  and  $k_{\text{cat}}/K_M = 1.6 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$  (Rosenberry, 1975)] and the  $k_{\text{cat}}$  of PTE-h5 is about 10 times faster than this.

The origins of the remarkable  $k_{\text{cat}}$  of PTE-h5 are currently under investigation. In a previous study, each residue in the substrate binding site of PTE was mutated individually to Ala, and Ile106, Phe132 and Ser308 were



**Fig. 8.** Selection of PTE libraries. PTE libraries were selected for PTE activity as in Figure 1. After fluorescent labelling using anti-product antibodies the beads were analysed and sorted by flow cytometry. The levels of fluorescence (FL1-H) on single unaggregated beads in each round of selection (rows B–G) and on beads not coated with DNA (row A) are plotted as histograms. Results are shown for Library B (column 1), Library C (column 2) and Library D (column 3). Library A was only put through one round of selection as about two-thirds of the beads formed a high-fluorescence population (data not shown) indicating a large percentage of active sequences in the unselected library. Generally, a single gate (M1), set to include ~1% of beads not coated with DNA (row A), was used to sort high-fluorescence beads. However, in the final round of selection multiple gates were used (M1, M2 and M3). The beads sorted through these gates were re-analysed (unfilled histograms). Unselected DNA and DNA on sorted beads from each round of selection was amplified, translated *in vitro* and incubated with  $Zn^{2+}$  to assemble the PTE metallo-enzyme, and PTE activity was measured using paraoxon as substrate. Catalytic activities are expressed as percentage of the activity from *in vitro* translation of an identical number of wild-type *OPD* genes and are indicated as annotations on the histograms of the sorted beads from the final round of selection and plotted for all rounds of selection in panel 4.

also mutated to Gly (Chen-Goodspeed *et al.*, 2001a,b) but no large improvements in the rate of paraoxon hydrolysis were observed with any of these mutations. Simultaneous mutations of Ile106 and Phe132, either both to Ala or both to Gly, gave only a threefold increase in  $V_{max}$ . Hence, the precise nature of the substitutions at each of these positions is of great importance, as is the combination of more than one mutation.

As this work indicates, directed evolution of enzymes poses considerable technical challenges, and most new enzymatic reactions or substrates require the establishment

of a new selection system. However, strategies based on high-throughput screening and selection are extremely valuable as they enable the selection of large repertoires and thereby allow a wide margin of error in our still limited design capabilities.

## Materials and methods

### Synthesis of genes

The *OPD* gene encoding PTE was amplified from *Flavobacterium* sp. (strain ATCC 27551) (Mulbry and Karns, 1989) by PCR using primers

*OPD*-Flag-Bc and *OPD*-HA-Fo and cloned into *NcoI* and *SacI*-cut pIVEX2.2b Nde (Roche) to give pIVEX-*OPD* (Figure 2). pIVEX-*OPD*

**Table II.** Sequences of active PTE mutants

Library	Clone	Amino acid residue <sup>a</sup>					Rate relating to wild-type (%)	
		106	131	132	308	309		
Wild type		I	W	F	S	Y	100.00	
Library A	b5	<b>S</b>	W	F	S	Y	280.00	
	b3	<b>T</b>	W	F	S	Y	74.00	
	a9	<b>R</b>	W	F	S	Y	0.43	
	a10	<b>F</b>	W	F	S	Y	0.11	
	b2	<b>Y</b>	W	F	S	Y	0.02	
Library B	e3	<b>L</b>	W	F	<b>S</b>	<b>Y</b>	47.00	
	f6	<b>T</b>	W	F	<b>T</b>	<b>W</b>	31.00	
	f4	<b>S</b>	W	F	<b>L</b>	<b>L</b>	28.00	
	e2	<b>T</b>	W	F	<b>Q</b>	<b>S</b>	5.00	
	f3	<b>T</b>	W	F	<b>L</b>	<b>V</b>	2.20	
	e6	<b>L</b>	W	F	<b>T</b>	<b>C</b>	0.88	
	f9	<b>S</b>	W	F	<b>F</b>	<b>D</b>	0.29	
	e10	<b>T</b>	W	F	<b>S</b>	<b>Y</b>	0.10	
	e12	<b>I</b>	W	F	<b>T</b>	<b>Y</b>	0.02	
	Library C	d5	<b>S</b>	<b>W</b>	<b>F</b>	S	Y	306.00
c4		<b>V</b>	<b>W</b>	<b>F</b>	S	Y	162.00	
c1		<b>D</b>	<b>Y</b>	<b>S</b>	S	Y	39.00	
c3		<b>S</b>	<b>W</b>	<b>L</b>	S	Y	5.70	
c11		<b>V</b>	<b>W</b>	<b>F</b>	S	Y	1.80	
d2		<b>D</b>	<b>R</b>	<b>R</b>	S	Y	0.52	
d3		<b>I</b>	<b>Y</b>	<b>P</b>	S	Y	0.18	
Library D		h5	<b>T</b>	<b>W</b>	<b>L</b>	<b>S</b>	<b>Y</b>	380.00
		g1	<b>S</b>	<b>W</b>	<b>L</b>	<b>M</b>	<b>N</b>	29.00
		b12	<b>S</b>	<b>W</b>	<b>L</b>	<b>L</b>	<b>R</b>	6.80
		h2	<b>T</b>	<b>W</b>	<b>F</b>	<b>S</b>	<b>Y</b>	5.00
	h7	<b>Q</b>	<b>N</b>	<b>T</b>	<b>K</b>	<b>H</b>	4.50	
	g12	<b>C</b>	<b>S</b>	<b>T</b>	<b>L</b>	<b>N</b>	3.80	
	g3	<b>L</b>	<b>G</b>	<b>V</b>	<b>S</b>	<b>F</b>	3.30	
	h4	<b>C</b>	<b>W</b>	<b>L</b>	<b>E</b>	<b>S</b>	2.60	
	g5	<b>T</b>	<b>H</b>	<b>C</b>	<b>Q</b>	<b>A</b>	0.30	
	h9	<b>T</b>	<b>H</b>	<b>C</b>	<b>Q</b>	<b>A</b>	0.29	
d11	<b>L</b>	<b>G</b>	<b>V</b>	<b>S</b>	<b>A</b>	0.29		
b11	<b>S</b>	<b>G</b>	<b>W</b>	<b>M</b>	<b>T</b>	0.22		
d9	<b>T</b>	<b>H</b>	<b>L</b>	<b>S</b>	<b>A</b>	0.16		
b10	<b>H</b>	<b>G</b>	<b>W</b>	<b>L</b>	<b>T</b>	0.04		

<sup>a</sup>Residues diversified in the libraries are indicated in bold and undiversified residues in normal type.

**Table III.** Kinetics of PTE mutants

Library	Clone	Amino acid residue <sup>a</sup>					$k_{cat}$ (s <sup>-1</sup> ) <sup>b</sup>	$K_M$ (mM)	$k_{cat}/K_M$ (M <sup>-1</sup> s <sup>-1</sup> × 10 <sup>5</sup> )	$k_{cat}$ relative (mut/wt)	$K_M$ relative (mut/wt)	$k_{cat}/K_M$ relative (mut/wt)
		106	131	132	308	309						
Wild type		I	W	F	S	Y	2280	0.023 ± 0.003	990	1.0	1.0	1.0
Library D	h5	<b>T</b>	<b>W</b>	<b>L</b>	<b>S</b>	<b>Y</b>	144 300	0.82 ± 0.07	1760	63.3	36	1.8
	b12	<b>S</b>	<b>W</b>	<b>L</b>	<b>L</b>	<b>R</b>	7410	2.2 ± 0.04	34.0	3.3	96	0.034
	h4	<b>C</b>	<b>W</b>	<b>L</b>	<b>E</b>	<b>S</b>	1710	3.3 ± 0.1	5.2	0.8	143	0.0052
	g1	<b>S</b>	<b>W</b>	<b>L</b>	<b>M</b>	<b>N</b>	570	1.27 ± 0.13	4.5	0.3	55	0.0045
	g3	<b>L</b>	<b>G</b>	<b>V</b>	<b>S</b>	<b>F</b>	513	1.59 ± 0.02	3.2	0.2	69	0.0033
	h2	<b>T</b>	<b>W</b>	<b>F</b>	<b>S</b>	<b>Y</b>	342	0.41 ± 0.02	8.3	0.2	18	0.0084
Library A	b5	<b>S</b>	W	F	S	Y	27 930	0.21 ± 0.02	1330	12.0	9	1.3
Library C	c1	<b>D</b>	<b>Y</b>	<b>S</b>	S	Y	22 800	1.2 ± 0.05	190	10.0	52	0.19
	d5	<b>S</b>	<b>W</b>	<b>F</b>	S	Y	13 680	0.17 ± 0.02	810	6.0	7	0.81
Library B	e3	<b>L</b>	W	F	S	Y	7980	0.12 ± 0.014	670	3.5	5	0.67

<sup>a</sup>Residues diversified in the libraries are indicated in bold and undiversified residues in normal type.

<sup>b</sup>The  $k_{cat}$  of clone h5 was calculated by quantifying the enzyme using [<sup>35</sup>S]methionine incorporation. For the remaining clones,  $k_{cat}$  was calculated by quantifying the enzyme using a sandwich ELISA. With this technique the  $k_{cat}$  of clone h5 was 56 800 s<sup>-1</sup>. Error ranges in the Kinetic determination of  $V_{max}$  by fit to the Michaelis-Menten model were all under 5%. In deriving  $k_{cat}$ , errors in determining the concentration of the *in vitro* translated enzymes by ELISA are 20%.

was digested with *HincII* and *NofI*, treated with Klenow polymerase and re-ligated, creating pIVEX- $\Delta$ *OPD* in which the *OPD* gene has a 258 bp in-frame deletion.

DNA for selection (Figure 2A) was prepared by PCR (using Super Taq; HT Biotechnology) using primers pIV-B1 and LMB2-1-tribiotin (Figure 2A; Table IV) and either pIVEX-*OPD* or pIVEX- $\Delta$ *OPD* as the template. The reactions were cycled 30 times (94°C, 0.5 min; 50°C, 0.5 min; 72°C, 2.0 min) with a final step at 72°C for 7 min. DNA was gel purified using Wizard PCR Preps (Promega).

### Synthesis of PTE libraries

Library A was created by PCR amplification of pIVEX-*OPD* with primers LMB2-1-biotin and LibA-Fo, which encodes the diversified codon (NNS) replacing Ile106 and appends a *BsmBI* site to give an 'N-terminal' fragment. A 'C-terminal' fragment was prepared by PCR amplification of pIVEX-*OPD* with primers pIV-B1 and LibA-Bc, which also appends a *BsmBI* site. The two fragments were digested with *BsmBI* and gel-purified. Then, 10<sup>12</sup> molecules of each of the fragments were mixed, ligated overnight and captured on 2 mg Streptavidin M-280 Dynabeads (Dyna). Ligation efficiency, determined using a <sup>32</sup>P-labelled 'C-terminal' fragment, was 10–20%.

Library B was created as above by ligating an 'N-terminal' fragment (created by amplifying Library A with primers LMB2-9-biotin and LibB-Fo, which encodes two NNS codons replacing Ser308 and Tyr309, and appends a *BsmBI* site) and a 'C-terminal' fragment (generated by PCR amplification of pIVEX-*OPD* with primers pIVB-1 and LibB-Bc, which appends a *BsmBI* site).

Library C was created by ligating an 'N-terminal' fragment (created by amplifying Library A with primers LMB2-8-biotin and LibC-Fo, which encodes two NNS codons replacing Trp131 and Phe132, and appends a *BsmBI* site) and a 'C-terminal' fragment (generated by PCR amplification of pIVEX-*OPD* with primers pIVB-1 and LibC-Bc, which appends a *BsmBI* site).

To create Library D, the ligation reactions of Library B and Library C were PCR amplified with primers pIV-B5 and LMB2-5-biotin. The resulting DNA was digested with *BclI* (which cuts between Phe132 and Ser308), and the 'N-terminal' fragment of Library C and the 'C-terminal' fragment of Library B were gel purified and ligated.

The ligated *OPD* genes from all four libraries were PCR amplified with primers LMB2-9 and pIV-B9, digested with *NcoI* and *SacI*, and 10<sup>11</sup> molecules were ligated into 5 × 10<sup>10</sup> molecules pIVEX2.2b Nde (Roche). DNA for selection was prepared by PCR of >10<sup>9</sup> molecules of ligated vector using primers pIV-B1-tribiotin and LMB2-1-tribiotin, and was gel purified. The triple biotins provide a stable link to the beads and resistance against exonucleases. Approximately 12% of the beads were recovered after the two emulsifications, but 57% of the DNA on the beads survived based on quantitative PCR. Library diversity was confirmed by sequencing.

Table IV. Oligonucleotide primers

Name	Sequence	Annealing site <sup>a</sup>
OPD-Flag-Bc	5'-CATTGCCAAGCCATGGACTACAAAGATGACGATGATAAAAATCACCAACAGCGGCGATCGG-ATCAATACCG-3'	
OPD-HA-Fo	5'-CGTCCCGGGAGCTCTTATTACGCATAATCCGGCACATCATACGGATAACCGCCGGTACCT-GACGCCCGCAAGGTCGGTGACAAGAACCG-3'	
LMB2-1 <sup>b</sup>	5'-CAGGCGCCATTTCGCCATT-3'	a
LMB2-5-biotin <sup>c</sup>	5'-CCAGCTGGCGAAAGGGGG-3'	b
LMB2-6	5'-ATGTGCTGCAAGGCGATT-3'	c
LMB2-8	5'-GTTTTCCAGTCACGACG-3'	d
LMB2-9	5'-GTAAAACGACGGCCAGT-3'	e
pIV-B1 <sup>b</sup>	5'-GCGTTGATGCAATTTCT-3'	f
pIV-B5	5'-CCTGCTCGCTTCGCTAC-3'	g
pIV-B6	5'-TTGGAGCCACTATCGAC-3'	h
pIV-B8	5'-CACACCGTCCTGTGGA-3'	i
pIV-B9	5'-TATCCGGATATAGTTC-3'	j
T7	5'-TAATACGACTCACTATAGGG-3'	k
OPDPCR-B5	5'-ACCAACAGCGGCGATCGGATC-3'	l
OPDPCR-B6	5'-AATACCGTGC GCGGTCCTATC-3'	m
OPDPCR-F5	5'-GGATGCCAGGAGGGCTGATG-3'	n
OPDPCR-F6	5'-CACTCGCATTATCTTCTAGAC-3'	o
LibA-Fo <sup>d</sup>	5'-AAGGTTCCAACGTCTCGCGACCSNNATCGAAAGTCGACACATC-3'	
LibA-Ba	5'-AACCTTGGAAACGTCTCGGTCGCGACGTCAGTTTATTGGCC-3'	
LibB-Fo <sup>d</sup>	5'-AAGGTTCCAACGTCTCGGTCGACSNNSNNCGAAAACCCGAAACGCCA-3'	
LibB-Ba	5'-AACCTTGGAAACGTCTCGTACCAACATCATGGAC-3'	
LibC-Fo <sup>d</sup>	5'-AAGGTTCCAACGTCTCCGGGTCSSNNCAAGCCGGTCGCCGCCAC-3'	
LibC-Ba	5'-AACCTTGGAAACGTCTCGACCCGCCACTTTCGATG-3'	

<sup>a</sup>For annealing sites, see Figure 2.

<sup>b</sup>These primers were also synthesized with a triple biotin at the 5'-end (Oswel, UK) and designated LMB2-1-tribiotin etc.

<sup>c</sup>Contains a single biotin at the 5'-end.

<sup>d</sup>N = A, G, C or T; S = G or C.

### Synthesis of PTE substrates

Caged-biotin was synthesized essentially as described (Sundberg *et al.*, 1995; Pirrung and Huang, 1996). Caged-biotin and biotin were coupled via a linker to the *p*-nitrophenyl-ethyl phosphotriester substrate to give the caged-biotinylated substrate EtNP-cgB and biotinylated substrate EtNP-B respectively (Figure 3B). Detailed procedures will be published elsewhere.

### Generation of anti-product antibodies

A *p*-nitrophenyl-ethyl phosphotriester substrate with a glutaryl linker was coupled to keyhole limpet hemocyanin (KLH) and hydrolysed to give the phosphodiester product as described previously (Tawfik *et al.*, 1993a). Antibodies were elicited in rabbits by immunization with EtBG-KLH (*Hd* = 14) (Tawfik *et al.*, 1993b, 1997).

### Selections for PTE

**Coating of beads with anti-HA antibodies and DNA.** One hundred and ninety-five microlitres of 0.95  $\mu$ m streptavidin-coated polystyrene beads (Bangs, CP01N,  $\sim 2 \times 10^7$  beads/ $\mu$ l; Lot 4771; binding capacity 0.545  $\mu$ g biotin-FITC/mg) were spun (3 min, 6500 g) in 1.7 ml MaxyClear tubes (Axygen) (used for all bead manipulations), rinsed twice (by resuspension and centrifugation) in 200  $\mu$ l PBS/T (50 mM sodium phosphate pH 7.5, 100 mM NaCl, 0.1% Tween 20) and 8 mg/ml heparin (sodium salt) and resuspended in the same. After sonication for 1 min (in a Branson 200 Ultrasonic Cleaner), 2500 biotinylated anti-HA antibody molecules (Roche, 3F10) were added per bead and incubated for 2 h at 20°C, mixing at 1400 r.p.m. for 10 s every minute. Biotinylated *OPD* and  $\Delta$ *OPD* genes, and mixtures thereof (see Table I), were added to  $\sim 6 \times 10^8$  beads at 0.33 genes/bead. The beads were incubated for 16 h at 7°C, mixing as above, and then rinsed in 100  $\mu$ l PBS/T, 8 mg/ml heparin, 100  $\mu$ l 5 mM Tris-acetate pH 8.0, 1 mg/ml heparin, resuspended in 18  $\mu$ l 5 mM Tris-acetate pH 8.0 and sonicated for 1 min.

**In vitro translation and emulsification.** Eighteen microlitres of coated beads ( $\sim 6 \times 10^8$  beads) were mixed with 2  $\mu$ l 5 mM methionine and 35  $\mu$ l EcoPro T7 *in vitro* translation reaction mix (Novagen) on ice. Samples were added to 0.5 ml of ice-cold oil mix [0.5% w/w Triton X-100 (Fischer) and 4.5% w/w Span 80 (Fluka) in light mineral oil (Sigma)] and stirred at 1600 r.p.m. for 5 min on ice as described previously (Tawfik and

Griffiths, 1998). After 4 h at 23°C, the emulsions were spun for 7 min at 20 800 g. The oil phase was removed and the concentrated emulsion broken by extracting three times with 1 ml of mineral oil and, after adding 200  $\mu$ l PBS/T, three times with 1 ml hexane. Residual hexane was removed by spinning for 5 min at room temperature in a Speedvac. After spinning for 3 min at 9000 g, the supernatant was removed and the beads were rinsed three times with 100  $\mu$ l PBS/T, 5 mM EDTA and 5 mM EGTA (the second rinse was incubated for 10 min), once with 100  $\mu$ l PBS/T and 8 mg/ml heparin, and once with 100  $\mu$ l Tris-CO<sub>2</sub>-Zn buffer (50 mM Tris-HCl, 10 mM potassium carbonate, 25  $\mu$ M ZnCl<sub>2</sub>, pH 8.5) plus 0.1% Triton X-100. The beads were resuspended in 60  $\mu$ l of the same buffer, sonicated for 1 min and put on ice.

**Assaying PTE activity on the beads.** After incubating  $4 \times 10^7$  beads prepared as described above in Tris/CO<sub>2</sub>/Zn buffer for 16 h at 4°C, activity was measured with 0.25 mM paraoxon in 50 mM Tris-HCl pH 8.5 by monitoring the release of the *p*-nitrophenolate product at 405 nm (Dumas *et al.*, 1989).

**Re-emulsification and uncaging.** The bead display libraries were added to 0.5 ml of ice-cold oil mix, stirred at 1150 r.p.m. for 3 min on ice and then homogenized for 3 min at 11 000 r.p.m. using an Ultra-Turrax T8 Homogenizer (IKA) with a 5 mm diameter dispersing tool. The resulting emulsion was incubated at 25°C for 10 min. Caged-biotinylated substrate EtNP-cgB (1.75 mM in methanol) was added to give a concentration of 5  $\mu$ M in the oil and the emulsions were mixed briefly. After incubating at 25°C for 16 h, 0.5 ml of 7.5 mM acetic acid in oil mix was added and mixed. The emulsions were transferred to a 24-well plate (Nunc) and irradiated for 4 min on ice using a B100 AP 354 nm UV lamp (UVP) from a distance of  $\sim 5$  cm while stirring at 200 r.p.m., and were then incubated for 30 min at 25°C and broken as above. Beads were rinsed three times with PBS/T by resuspension and centrifugation, resuspended in 100  $\mu$ l PBS/T and sonicated for 1 min.

**Labelling beads with anti-product antibodies and flow cytometry.** One hundred microlitres of anti-product rabbit serum (see above), diluted 1:30 in COVAP buffer (2 M NaCl, 0.04% Tween-20, 10 mM phosphate, 0.1 mM *p*-nitrophenol pH  $\sim 6.5$ ) plus 1.5 mg/ml BSA, were added to each bead suspension ( $\sim 2 \times 10^8$  beads) and incubated for 1.5 h. The beads were rinsed twice with PBS/T and resuspended in 100  $\mu$ l of PBS/T. Then,

100  $\mu$ l of 50 ng/ $\mu$ l FITC-labelled goat anti-rabbit Fab (Min.X; Jackson) in COVAp buffer plus 1.5 mg/ml BSA were added and incubated for 1 h. The beads were rinsed twice with PBS/T, sonicated for 2 min, diluted to  $\sim 10^8$  beads/ml in PBS/T and run on a MoFlo flow cytometer (Cytomation) at  $\sim 20$  000 events per second, with a 100  $\mu$ m nozzle, exciting with a 488 nm argon ion laser (Coherent Innova 70; 10 W max. CW output) at full power and measuring emission passing a  $530 \pm 20$  nm bandpass filter. Single unaggregated beads were gated using forward and side scatter and  $10^5$  high-fluorescence beads were collected. Analysis of  $10^5$  events from the unselected samples and  $10^4$  events from the selected samples was performed using a FACScan cytometer (BD) to check enrichment.

**PCR amplification of the selected genes.** First,  $10^5$  sorted and unsorted beads were spun for 5 min at 20 800 g, rinsed twice with 200  $\mu$ l PCR buffer and resuspended in 25  $\mu$ l of the same. Then, 50  $\mu$ l PCR reactions were performed using Super Taq, primers OPDPCRB5 and OPDPCRF5 (Figure 2A; Table IV) and 25  $\mu$ l bead suspensions from above. The reactions were cycled 22 times (94°C, 0.5 min; 50°C, 0.5 min; 72°C, 2.0 min) with a final step at 72°C for 7 min. The amplification was repeated using nesting primers OPDPCRB6 and OPDPCRF6 (Figure 2A; Table II) and 1  $\mu$ l of the first PCR reaction as template, cycling 33 times as above.

### Selection of PTE libraries

$2 \times 10^8$  biotinylated genes from each library were coated onto  $\sim 6 \times 10^8$  streptavidin-coated polystyrene beads and selected as above. Genes were amplified off sorted beads by PCR as above but using PfuTurbo enzyme (Stratagene) with primers pIVB-8 and LMB-2-8 for the first PCR, and pIVB-9 and LMB-2-9 for the subsequent nested PCR.

The PCRs were purified and digested with NcoI and SacI, and  $10^{11}$  molecules were ligated into  $10^{10}$  molecules pIVEX2.2b Nde. Then,  $10^8$  ligated vector molecules (determined by competitive PCR) were PCR amplified (using PfuTurbo polymerase) with primers pIV-B1-tribiotin and LMB2-1-tribiotin in a 200  $\mu$ l PCR reaction cycled 30 times as above. The full-length genes were gel purified as above. Up to six rounds of selection were performed by repeating this procedure.

### Assaying PTE activity in the selected libraries

The wild-type OPD gene and DNA amplified from the ligations of the unselected and selected libraries were translated at 1 nM for 4 h at 23°C in a 10  $\mu$ l *in vitro* translation reaction (EcoPro T7). Then, 30  $\mu$ l Tris/CO<sub>2</sub>/Zn buffer was added and incubated for 1.5 h at room temperature. Activity was measured with 0.25 mM paraoxon as above.

### Characterization of selected clones

Two microlitres of the ligations of the unselected and selected libraries were transformed into XL10-Gold Ultracompetent cells (Stratagene). Colonies were picked into 384-well large-volume plates (Genetix) containing 2xTY, 100  $\mu$ g/ml ampicillin, 8% glycerol and 1% glucose (75  $\mu$ l per well), incubated overnight at 37°C and stored at -70°C.

Bacteria from these plates were transferred using 96-pin replicators (Genetix) into 50  $\mu$ l PCR reactions in 96-well plates using Super Taq and primers pIV-B1 and LMB-2-1, and cycled 30 times as above. Each DNA was translated at  $\sim 2$  nM for 6 h at 30°C in a 2.5  $\mu$ l *in vitro* translation reaction (Rapid Translation System RTS 100, *E.coli* HY Kit; Roche). Then, 15  $\mu$ l Tris/CO<sub>2</sub>/Zn buffer was added and incubated for 1.5 h at room temperature. Activity was measured with 0.25 mM paraoxon as above.

Clones showing detectable activity ( $\geq 10$  nM/min/ $\mu$ l IVT) were re-amplified by PCR from the bacterial stocks as above. The DNA was purified using a QIAquick 96 PCR Purification Kit (Qiagen), sequenced using primers T7 and pIV-B9, translated *in vitro* and assayed for paraoxon hydrolysis as above.

### Kinetic analysis of selected clones

PCR-amplified DNA was translated at 1 nM using the EcoPro T7 *in vitro* translation system and assembled as described above. Rates were measured in 50 mM Tris-HCl pH 8.5 with 0.02–4  $\mu$ l of IVT and 0.014–3.6 mM paraoxon.  $K_M$  and  $v_{max}$  were determined by fitting the data to the Michaelis–Menten model

$$v_0 = v_{max}[S]_0 / ([S]_0 + K_M)$$

using KaleidaGraph. If a  $k_{cat}$  of 2280 s<sup>-1</sup> (Hong and Raushel, 1999) is assumed, the  $v_{max}$  found for wild-type PTE (0.4  $\mu$ M/s/ $\mu$ l IVT) corresponds to an enzyme concentration of 35 nM in the *in vitro* translation mix. The relative concentrations of the wild-type and mutant

PTEs were determined by a sandwich ELISA based on the PTE possessing an N-terminal Flag tag and a C-terminal HA tag (Figure 2) and used to convert  $v_{max}$  to  $k_{cat}$  (Table III). Microtitre plates (Nunc, Maxisorb) were coated with the anti-FLAG M5 antibody (Sigma; 3.5  $\mu$ g/ml; overnight at 4°C) and blocked with BSA. The IVT reactions were serially diluted (25- to 225-fold) in PBS/T and incubated in the coated plates for 1 h. The plates were rinsed and biotinylated anti-HA antibody (3F10; Roche; 0.5  $\mu$ g/ml in PBS) was added, followed (after rinsing) by streptavidin–peroxidase (Sigma; diluted 4000-fold in PBS). The assay was developed using TNB (Nolge). A calibration curve made with *in vitro* translated wild-type PTE was used to determine the concentration of the mutants. Expression levels varied from 10% (h11) to 480% (b5) relative to the wild type. The expression level of PTE-h5 was also determined more precisely by measuring incorporation of [<sup>35</sup>S]methionine and found to be 118% relative to wild-type PTE. *In vitro* translations were analysed by SDS–PAGE and full-length protein quantified using a PhosphorImager (Typhoon 8600; Molecular Dynamics).

## Acknowledgements

We thank Zelig Esshar for rabbit antibodies, Jeremy Agresti for quantitative PCR, Leonid Gaydokov for kinetic assays and Leo James for preparing Figure 7. We acknowledge financial support (to D.S.T.) by the Israeli Ministry of Science.

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Received August 26, 2002; revised November 5, 2002;  
accepted November 7, 2002