Evolution in action

The α/β barrel enzyme phosphotriesterase from soil bacteria appears to have evolved the ability to hydrolyze the insecticide paraoxon at the diffusion limit in only a few decades. A newly-identified open reading frame from *Escherichia coli* may offer a clue to its origins.

Chemistry & Biology February 1995, **2**:71–75

Generic protein structural motifs that can be adapted to different functions are a common occurrence in biology. For example, immunoglobulins all share a common tertiary structural fold that forms a stable framework for loops that can bind a wide range of different antigen molecules. Thus, the fold that gives the protein stability remains relatively constant, while the residues that give the protein specificity can vary with few restrictions. The α/β barrel family of enzymes provides an example of a similar situation in proteins that have evolved to catalyze biochemical reactions [1–4]. These enzymes share a common tertiary fold consisting of eight parallel β-strands connected by α-helices, as shown in Fig. 1 for the archetypical α/β barrel enzyme triosephosphate isomerase (TIM) [5].

The α/β barrel structure appears to be a generic scaffolding for catalytic activity: ~10% of all structurally-characterized enzymes belong to this family [1]. Like the immunoglobulin molecules, which bind many different antigen structures, the α/β barrel enzymes catalyze a range of very different biochemical reactions using a collection of different catalytic mechanisms and cofactors (Table 1) [1]. In the case of TIM, the enzymatic activity of the α/β barrel structure has been refined over the course of evolution to convert dihydroxyacetone phosphate to D-glyceraldehyde-3-phosphate so rapidly that the rate of diffusion, leading to the encounter of enzyme and substrate, limits the rate of catalysis [6]. Recent studies from the laboratories of Raushel and Holden show that an enzyme from soil bacteria plasmids shares two features with TIM: the α/β barrel structural motif and diffusion-limited enzymatic catalysis. Amazingly, this enzyme appears to have evolved this impressive catalytic efficiency in ~40 years.

Phosphotriesterase chemistry and mechanism

Phosphotriesterase (PTE) is an enzyme from the soil bacterium *Pseudomonas diminuta* that catalyzes the hydrolysis of a number of synthetic phosphotriester compounds used as insecticides and chemical warfare agents (Fig. 2). The gene encoding PTE resides on a plasmid in *P. diminuta* [7]. A gene of identical sequence has been found on a different plasmid in the soil microorganism *Flavobacterium sp* [8]. The PTE gene encodes a mature 336-residue protein of molecular mass 39 kDa. So far, no natural biochemical substrate for PTE has been identified, and hydrolysis of the synthetic organophosphates and phosphonates is the only known activity for the enzyme.

In 1989, Raushel and coworkers [9] reported the purification of PTE and embarked on a series of detailed studies to assess the kinetic properties and elucidate the catalytic mechanism of the enzyme. They found that the isolated enzyme contains two divalent zinc ions per monomer and that both zinc ions are required for full catalytic activity [10]. The PTE apoenzyme can also be reconstituted with two equivalents of other divalent metal ions, such as Co^{2+}, Ni^{2+}, Cd^{2+} and Mn^{2+}, giving full enzymatic activity [10]. Results from ^{113}Cd NMR [11] and Mn^{2+} electron paramagnetic resonance spectroscopy [12] indicate that the two metal ions are bridged by a common ligand and that most of the other ligands to the binuclear metal cluster are imidazole side chains of His residues in the protein. Stereochemical experiments with enantiomerically pure organophosphate substrates show that the hydrolysis reaction occurs with inversion of configuration at phosphorus by direct attack of an activated water molecule [13]. The pH–rate profile for PTE hydrolysis indicates that deprotonation of a functional group with an apparent pK_a of 6.1 is required for full

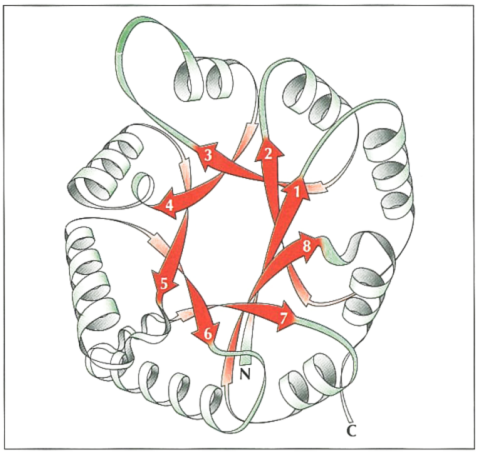

Fig. 1. Schematic structure of triose phosphate isomerase (TIM). The eight β strands which form the β barrel are shown in red, and the connecting α-helices are shown in green.

 ISSN 1074-5521

Table 1. Examples of α/β barrel enzymes.

Enzyme	Cofactor(s)	Substrate	Product
Triose phosphate isomerase	none	dihydroxyacetone phosphate	D-glyceraldehyde 3-phosphate
Tryptophan synthase (α-subunit)	none	indole-3-glycerol phosphate	indole + 3-phosphoglyceraldehyde
Pyruvate kinase	Mg^{2+}, K^+	ATP + pyruvate	ADP + phosphoenolpyruvate
α-Amylase	Ca^{2+}	amylose	glucose + amylose
Mandelate racemase	Mg^{2+}	(S)-mandelate	(R)-mandelate
Glycolate oxidase	FMN	glycolate + O_2	glyoxylate + H_2O_2

FMN, flavin mononucleotide.

activity, and chemical modification experiments provide evidence that a His residue functions at the active site as a general base [14]. Together these data support a catalytic mechanism for PTE that involves zinc-assisted polarization of the substrate P–O bond with His-promoted nucleophilic attack of water (Fig. 3).

PTE is remarkably efficient in hydrolyzing the insecticide paraoxon. The k_{cat} and k_{cat}/K_M values for the PTE-catalyzed hydrolysis of paraoxon are 10^4 s^{-1} and 4 x 10^7 $M^{-1}s^{-1}$ respectively, and the rate enhancement brought about by the enzyme approaches 10^{12} [9, 10]. The high k_{cat}/K_M value for PTE-catalyzed paraoxon hydrolysis is near the bimolecular rate constant for diffusion-controlled encounter of the enzyme and substrate (10^8–10^9 $M^{-1}s^{-1}$) [15]. Moreover, Brønsted correlations and experiments showing rate dependence on solvent viscosity support the conclusion that the physical process of diffusion rather than the chemical process of bond cleavage is rate-limiting in PTE-catalyzed paraoxon hydrolysis [16].

Albery and Knowles [17–19] have proposed, from analysis of TIM, that an enzyme which performs catalysis at the limit of diffusion control has reached its endpoint in evolutionary optimization because mutation and selection can no longer be used to increase the rate of catalysis. Fersht [20] has suggested that both a maximized k_{cat}/K_M and a K_M greater than the physiological substrate concentration should be used as criteria to judge whether an enzyme has evolved to a maximized rate of catalysis. On this basis, Fersht concludes that both TIM and carbonic anhydrase are perfectly evolved for the maximization of rate. It is believed that catalytically ideal enzymes like TIM and carbonic anhydrase achieved this status by operating on a single defined substrate molecule over millions of years of mutation and selection [18]. The PTE enzyme from soil bacteria thus challenges our assumptions about the tempo of molecular evolution. The synthesis of phosphotriester insecticides (including paraoxon) was first reported in 1950 [21] and the gene encoding PTE was first identified in 1989 [7,8]. This

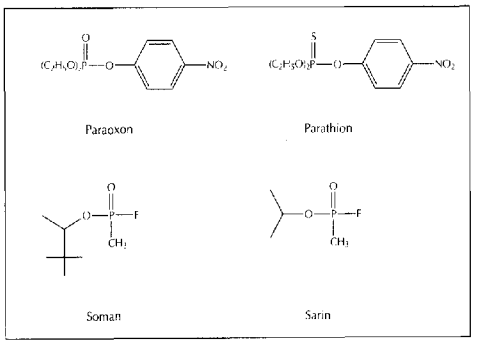

Fig. 2. Substrates for phosphotriesterase. Paraoxon and parathion are used as insecticides, and soman and sarin are chemical warfare agents. These compounds are all extremely toxic to higher organisms, acting primarily through inactivation of acetylcholinesterase.

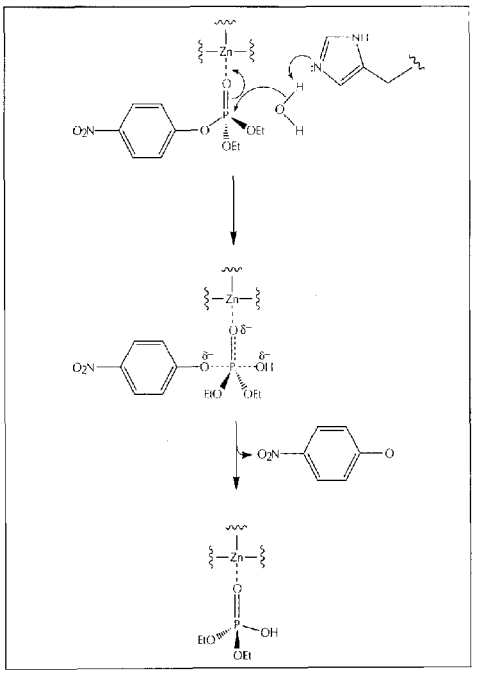

Fig. 3. Proposed catalytic mechanism of phosphotriesterase. The P–O bond of the substrate (shown here as paraoxon, see Fig. 1) is polarized by one of the zinc atoms in the active site of the enzyme, allowing nucleophilic attack by a water molecule, assisted by the proximity of a His residue.

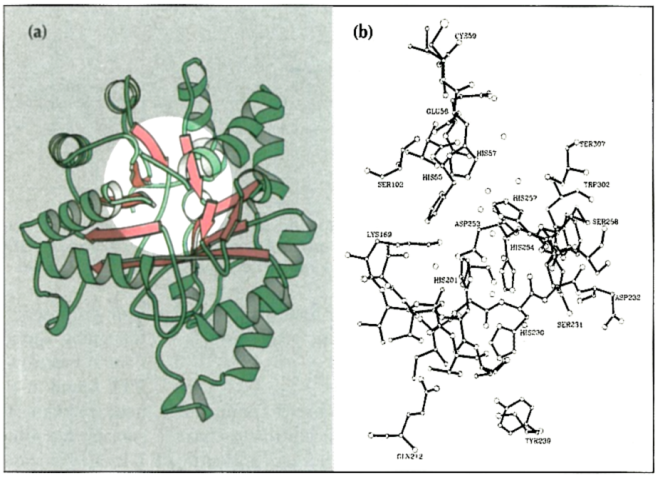

Fig. 4. Structure of phosphotriesterase. **(a)** Ribbon diagram of the overall structure of the apoenzyme with β-strands colored red and the connecting α-helices in green. The white circle shows the region of the protein in which the active site is thought to reside. **(b)** Details of the presumptive active site [23].

places a limit of ~40 years on the time required for PTE to evolve to recognize paraoxon as a substrate and to develop an essentially perfect catalytic mechanism for its hydrolysis. It is conceivable that a form of PTE, performing some other function in soil bacteria, happened to have some level of hydrolytic activity in the initial encounter with paraoxon. It is unlikely, however, that this activity was coincidentally at the diffusion limit. Although a few examples of rapid evolution of enzymatic function have been noted [22], to our knowledge PTE is the only case where diffusion-limited activity has been reached in such a remarkably short period of mutation and selection.

Phosphotriesterase is an α/β barrel enzyme

A recent report [23] on the crystal structure of PTE shows that this enzyme shares a second feature with TIM in that it belongs to the α/β barrel structural family. The 2.1 Å resolution structure is of the apoenzyme form of PTE, which does not contain the binuclear metal cofactor. The PTE structure is a distorted α/β barrel with eight parallel β-strands connected by nine α-helices (Fig. 4). In addition, there are two strands of antiparallel β-sheet at the amino-terminus of the enzyme. The structure also reveals extensive hydrophobic and electrostatic interactions between surface loops of neighboring molecules in the crystalline lattice, suggesting that the enzyme functions in solution as a dimer. Subsequent ultracentrifugation studies have demonstrated that this is the case.

Although this PTE structure is for the apoenzyme, functional studies and structural correlations to other α/β barrel enzymes can be used to define the probable location of the PTE active site. Site-directed mutagenesis has shown that of the seven His residues in PTE, six are either directly involved in the catalytic mechanism or used to ligate the zinc ions [24]. These six His residues (His35, 57, 201, 230, 254, and 257) reside in a cluster at the carboxy-terminal portion of the β-barrel where the active sites of all the structurally-characterized α/β enzymes are located (Fig. 4). These arguments strongly suggest that the cluster of six His residues marks the location of the enzyme active site and the binding site for the two zinc ions. One of the six histidines presumably functions as the general base in the PTE catalytic mechanism. The three-dimensional structure of

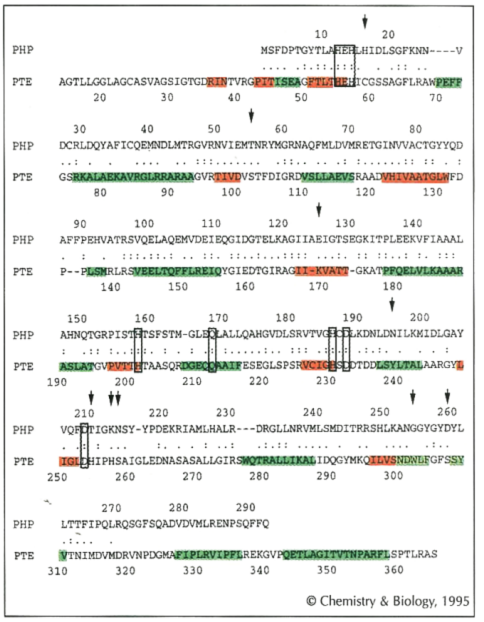

Fig. 5. Alignment of the phosphotriesterase homology protein (PHP) with PTE. Residues that form β-strands in PTE are highlighted in red, and those that form α-helices in PTE are shown in green. Conserved presumptive active site residues are boxed. Non-conserved active site residues are marked with arrows.

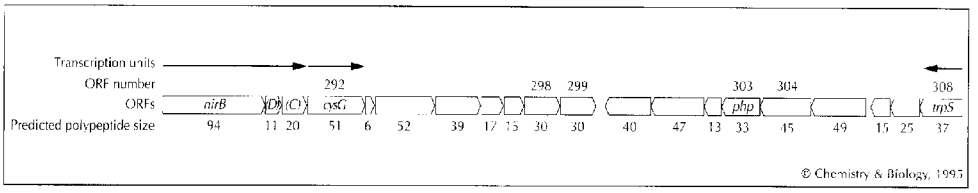

Fig. 6. The region of the *E. coli* genome in which the *php* open reading frame is found. The *php* ORF is the fifth in a series of eight newly-identified ORFs downstream of *trpS*, facing seven other new ORFs downstream of *cysG*. Preliminary homology analysis suggests that the *php* gene may reside in a group of genes involved in organophosphate metabolism.

the holoenzyme will provide a clearer understanding of the PTE active site structure and catalytic mechanism.

The genetic origins of phosphotriesterase

Because PTE is a member of the α/β barrel family, and because of PTE's apparently rapid evolution to near catalytic perfection, it would be interesting to identify the subfamily of bacterial α/β barrel enzymes from which PTE originated. At the time that the crystal structure of PTE was published, no proteins with similar sequences had been identified from homology searches of the sequence data base [23]. In December 1994, however, the sequence of the *E. coli* chromosomal region from 67.4 to 76.0 minutes was entered in the data base [ECOUW67, G. Plunkett, Genbank] as part of the *E. coli* Genome Project (directed by F. Blattner). We have found that the translated sequence of an open reading frame (ORF) in this genomic segment is significantly homologous to the amino acid sequence of PTE, with 28 % identity and 66 % similarity over virtually the entire sequence of the *E. coli* ORF. Structurally-characterized α/β enzymes with this level of sequence identity have been shown to possess highly similar three-dimensional structures [1,2,25]. It therefore seems plausible that this protein, which we call phosphotriesterase homology protein (PHP) is part of the family from which PTE must have evolved.

An alignment of the deduced amino acid sequence of *E. coli* PHP with the PTE amino acid sequence is shown in Fig. 5. Inspection of the alignment reveals that all of the eight β-strands of PTE and the connecting α-helices that make up the α/β barrel core structure are contained within the large region of homology. Analysis of the residues that lie near the putative active site of PTE shows that four of the six potential zinc-ligating histidines, His55, His57, His201, and His230 (PTE numbering), are conserved between the two sequences; the other conserved active site residues are Glu56, Gln212, Asp232, and Asp253.

Based on the homology of the sequence alignment in Fig. 5, the following conclusions and predictions can be made: 1) PHP has an α/β barrel structure; 2) PHP is an enzyme; 3) PHP is a metalloenzyme, probably containing an active site binuclear metal center; and 4) PHP is a member of the subfamily of α/β barrel enzymes from which PTE diverged.

Our proposed relation between PHP and PTE raises intriguing questions for future investigation into the natural function of PHP in bacteria. The gene encoding PTE found in *P. diminuta* is plasmid-borne, whereas the gene encoding PHP in *E. coli* is chromosomal and presumably has some function in its life cycle. The *php* ORF is located at about 74 minutes on the *E. coli* chromosome [26], in a group of 15 of the newly identified ORF's residing between *cysG* and *trpS* (Fig. 6). It is the fifth of a series of eight ORFs downstream of *trpS*, all of which would be transcribed in the same direction as *trpS* itself. Sequence analysis suggests strong similarity between a cluster of ORFs in this region and organophosphate metabolizing operons (Fig. 6). The amino acid sequence deduced from ORF 298 is homologous to a family of proteins that includes ribokinases [27]. The protein encoded by ORF 304 has homology to phosphopentomutase, while that encoded by ORF 299 is homologous to transcriptional regulators and is thus a candidate regulator of some putative operon involving genes in this cluster. Mutational and transcriptional analysis to define the operons of this chromosomal region would be one step toward understanding the physiological relevance of PHP. In addition, biochemical studies aimed at understanding the structure and function of PHP are likely to provide additional insight into PTE's rapid journey to evolutionary perfection.

Acknowledgements: We thank Hazel Holden for kindly providing Fig. 4, and Patsy Babbit for providing a preprint of reference 4. TSS thanks the National Institutes of Health and the American Cancer Society for generous support of his research program. RCR thanks the National Institutes of Health (Program of Excellence grant HL4382) for financial support.

References

1. Farber, G.K. & Petsko, G.A. (1990). The evolution of α/β barrel enzymes. *Trends Biochem. Sci.* **15**, 228–234.
2. Neidhart, D.J., Kenyon, G.L., Gerlt, J.A. & Petsko, G.A. (1990). Mandelate racemase and muconate lactonizing enzyme are mechanistically distinct and structurally homologous. *Nature* **347**, 692–694.
3. Scrutton, N.S. (1994). α/β barrel evolution and the modular assemby of enzymes: emerging trends in the flavin oxidase/dehydrogenase family. *Bioessays* **16**, 115–122.
4. Babbitt, P.C., *et al.*, & Gerlt, J.C. (1995). A functionally diverse enzyme superfamily that abstracts the α-protons of carboxylic acids. *Science*, in press.
5. Banner, D.W., *et al.*, & Waley, S.G. (1975). Structure of chicken muscle triose phosphate isomerase determined crystallographically at 2.5 Å resolution using amino acid sequence data. *Nature* **255**, 609–614.
6. Albery, W.J. & Knowles, J.R. (1976). Free energy profile for the

reaction catalyzed by triosephosphate isomerase. *Biochemistry* **15**, 5627–5631.
7. Serdar, C.M., Murdock, D.C. & Rohde, M.F. (1989). Parathion hydrolase gene from *Pseudomonas diminuta mg*: subcloning, complete nucleotide sequence, and expression of the mature portion of the enzyme in *Escherichia coli*. *Biotechnology* **7**, 1151–1155.
8. Mulbry, W.W. & Karns, J.S. (1989). Parathion hydrolase specified by the *Flavobacterium opd* gene: relationship between gene and protein. *J. Bacteriol.* **171**, 6740–6746.
9. Dumas, D.P., Caldwell, S.R., Wild, J.R. & Raushel, F.M. (1989). Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*. *J. Biol. Chem.* **264**, 19659–19665.
10. Omburo, G.A., Kuo, J.M., Mullins, L.S. & Raushel, F.M. (1992). Characterization of the zinc binding site of bacterial phosphotriesterase. *J. Biol. Chem.* **267**, 13278–13283.
11. Omburo, G.A., Mullins, L.S. & Raushel, F.M. (1993). Structural characterization of the divalent cation sites of bacterial phosphotriesterase by ^{113}Cd NMR spectroscopy. *Biochemistry* **32**, 9148–9155.
12. Chae, M.Y., Omburo, G.A., Lindahl, P.A. & Raushel, F.M. (1993). Antiferromagnetic coupling in the binuclear metal cluster of manganese-substituted phosphotriesterase. *J. Am. Chem. Soc.* **115**, 12173–12174.
13. Lewis, V.E., Donarski, W.J., Wild, J.R. & Raushel, F.M. (1988). Mechanism and stereochemical course at phosphorus of the reaction catalyzed by a bacterial phosphotriesterase. *Biochemistry* **27**, 1591–1597.
14. Dumas, D.P. & Raushel, F.M. (1990). Chemical and kinetic evidence for an essential histidine in the phosphotriesterase from *Pseudomonas diminuta*. *J. Biol. Chem.* **265**, 21498–21503.
15. Fersht, A. (1985). Measurement and magnitude of enzymatic rate constants. In *Enzyme Structure and Mechanism*. (2nd edn), pp. 121–154. W.H. Freeman & Co., New York.
16. Caldwell, S.R., Newcomb, J.R., Schlecht, K.A. & Raushel, F.M. (1991). Limits of diffusion in the hydrolysis of substrates by the phosphotriesterase from *Pseudomonas diminuta*. *Biochemistry* **30**, 7438–7444.
17. Albery, W.J. & Knowles, J.R. (1976). Evolution of enzyme function and the development of catalytic efficiency. *Biochemistry* **15**, 5631–5640.
18. Albery, W.J. & Knowles, J.R. (1977). Efficiency and evolution of enzymatic catalysis. *Angew. Chem. Int. Ed. Engl.* **16**, 285–293.
19. Knowles, J.R. & Albery, W.J. (1977). Perfection in enzyme catalysis: the energetics of triosephosphate isomerase. *Accounts Chem. Res.* **10**, 105–111.
20. Fersht, A. (1985). Enzyme–substrate complementarity and the use of binding energy in catalysis. In *Enzyme Structure and Mechanism*. (2nd edn), pp. 311–346. W.H. Freeman & Co., New York.
21. Schrader, G. (1950). Organische phosphor-verbindungen als neuartige insektizide (auszug). *Angew. Chem.* **62**, 471–473.
22. Benner, S. & Ellington, A.D. (1988). Interpreting the behavior of enzymes: purpose or pedigree? *CRC. Crit. Rev. Biochem.* **23**, 369–426.
23. Benning, M.M., Kuo, J.M., Raushel, F.M. & Holden, H.M. (1994). Three-dimensional structure of phosphotriesterase: an enzyme capable of detoxifying organophosphate nerve agents. *Biochemistry* **33**, 15001–15007.
24. Kuo, J.M. & Raushel, F.M. (1994). Identification of the histidine ligands to the binuclear metal center of phosphotriesterase by site-directed mutagenesis. *Biochemistry* **33**, 4265–4272.
25. Chothia, C. & Lesk, A.M. (1986). The relation between the divergence of sequence and structure in proteins. *EMBO J.* **5**, 823–826.
26. Bachmann, B.J. (1990). Linkage map of *Escherichia coli* K 12, edition 8. *Microbiol. Rev.* **54**, 130–197.
27. Bork, P., Sander, C. & Valencia, A. (1993). Convergent evolution of similar enzymatic function on different protein folds: the hexokinase, ribokinase, and galactokinase families of sugar kinases. *Protein Sci.* **2**, 31–40.

Thomas S Scanlan, Department of Pharmaceutical Chemistry and Ralph C Reid, Biomolecular Resource Center, University of California, San Francisco, CA 94143-0446, USA.