Improving a Natural Enzyme Activity through Incorporation of Unnatural Amino Acids

Isaac N. Ugwumba,‡,* Kiyoshi Ozawa,‡,§ Zhi-Qiang Xu,‡,§ Fernanda Ely,∥
Jee-Loon Foo,‡ Anthony J. Herlt,‡ Chris Coppin,‡ Sue Brown,‡ Matthew C. Taylor,‡
David L. Ollis,‡ Lewis N. Mander,‡ Gerhard Schenk,‡,∥∥∥ Nicholas E. Dixon,§
Gottfried Oting,‡ John G. Oakeshott,‡ and Colin J. Jackson‡,¶,*

Commonwealth Scientific and Industrial Research Organization, Black Mountain, Canberra, Australia,
Research School of Chemistry, Australian National University, Canberra, Australia, School of Chemistry,
University of Wollongong, Australia, School of Chemistry and Molecular Biosciences, University of
Queensland, Australia, Department of Chemistry, National University of Ireland Maynooth, Ireland

Received August 1, 2010; E-mail: cjackson@rsc.anu.edu.au

Abstract: The bacterial phosphotriesterases catalyze hydrolysis of the pesticide paraoxon with very fast turnover rates and are thought to be near to their evolutionary limit for this activity. To test whether the naturally evolved turnover rate could be improved through the incorporation of unnatural amino acids and to probe the role of peripheral active site residues in nonchemical steps of the catalytic cycle (substrate binding and product release), we replaced the naturally occurring tyrosine amino acid at position 309 with unnatural L-(7-hydroxycoumarin-4-yl)ethylglycine (Hco) and L-(7-methylcoumarin-4-yl)ethylglycine amino acids, as well as leucine, phenylalanine, and tryptophan. Kinetic analysis suggests that the 7-hydroxyl group of Hco, particularly in its deprotonated state, contributes to an increase in the rate-limiting product release step of substrate turnover as a result of its electrostatic repulsion of the negatively charged 4-nitrophenolate product of paraoxon hydrolysis. The 8–11-fold improvement of this already highly efficient catalyst through a single rationally designed mutation using an unnatural amino acid stands in contrast to the difficulty in improving this native activity through screening hundreds of thousands of mutants with natural amino acids. These results demonstrate that designer amino acids provide easy access to new and valuable sequence and functional space for the engineering and evolution of existing enzyme functions.

Introduction

The development of experimental techniques that allow incorporation of unnatural amino acids (uAAs) into proteins has opened new avenues in protein engineering and characterization of protein function, permitting the synthesis of a wide range of unnatural enzymes with new or improved activities and novel mechanistic probes. Methods for translational incorporation of uAAs can be broadly separated into global and specific approaches. Global approaches exploit either natural misacylation of tRNA by close structural analogues based on the substrate promiscuity of existing tRNA synthetases, or utilize chemical misacylation, to allow incorporation of uAAs in place of natural amino acids. An alternative, site-specific, approach based on suppressor tRNA allows incorporation of uAAs during suppressor read-through of a stop codon, most often amber (UAG). This approach can be used in both in vitro protein translation systems and in vivo translation systems that have been modified by the addition of an orthogonal tRNA—amino-acyl tRNA synthetase pair. This work describes the incorporation of uAAs into a bacterial phosphotriesterase (arPTE) that has been cloned from an Agrobacterium radiobacter strain and catalyzes the hydrolysis of toxic organophosphate (OP) phosphotriester pesticides such as paraoxon. A 90% identical enzyme (pdpTE) has been isolated from Pseudomonas diminuta and Flavobacterium sp. strains and also exhibits OP degrading activity. These

‡ To whom correspondence should be addressed.
§ Commonwealth Scientific and Industrial Research Organization.
¶ University of Wollongong.
∥ University of Queensland.
∥∥∥ National University of Ireland Maynooth.
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proteins are of interest because of their rapid evolution into efficient catalysts of the hydrolysis of synthetic OPs and their potential use for OP detoxification because these compounds pose a serious health and environmental risk.16

Many studies of the structure and function of the bacterial PTEs have been described, with crystal structures of artPTE and pdPTE revealing very close structural similarity (backbone rmsd of 0.34 Å) and nearly identical active sites with a binuclear metal ion center.17,18 Each metal ion plays a specific role in the reaction (Scheme 1), with the more solvent exposed β-metal ion apparently involved in substrate coordination and the α-metal ion involved in stabilizing a hydroxide ion as the initiating nucleophile.19–23 Paraoxon is known to be hydrolyzed via an S$_2$2-type reaction with net inversion of configuration at the phosphorus center,24 and the rate of product release ($k_3$) has been identified as the slowest and rate-limiting step of the catalytic cycle (Scheme 2).20,25–27

Discussion of the catalytic power of enzymes often focuses on the chemical step,28 but the subtle interactions that facilitate substrate binding and product release are also important; without fast rates for formation of the Michaelis complex and product release, the efficiency of the chemical step can be obscured. Because the chemical step is not rate-limiting for PTE-catalyzed paraoxon hydrolysis, the PTEs are a valuable model system to study these physical steps in the catalytic cycle. Although

\[ E+S \xrightarrow{k_1} ES \xrightarrow{k_2} EPQ \xrightarrow{k_3} E+P+Q \]

substrate binding in the PTEs is driven in part by metal–substrate interactions,29 an important role for aromatic and hydrophobic enzyme–substrate (ES) interactions is implied by crystal structures of Michaelis and product complexes, which show the aromatic ring of the leaving group sandwiched between the aromatic groups of Tyr309, Trp131, and Phe132 (Figure 1).22,27 Moreover, mutation of these residues to alanine results in an increase in the Michaelis constant ($K_M$).29,30 suggesting that these residues are involved in the formation of the Michaelis complex. However, theoretical studies have either indicated that the substrate approaches for nucleophilic attack without significant aromatic interactions with these residues,31,32 or have omitted these residues altogether.33

There have been several reports of protein engineering experiments to improve the catalytic function of the PTEs, involving rational design,29,30 combinatorial libraries,34,35 and laboratory (directed) evolution.18,27,36–38 Most of these studies have focused on improving inefficient, promiscuous, activities of the PTEs, such as the turnover of OPs used as chemical warfare agents or other slowly turned over insecticides. Attempts to improve already efficient activities, such as the turnover of paraaxon, have produced no, or only modest, improvements in artPTE in our laboratories, despite screening hundreds of thousands of variants.27,37 The inability to significantly improve the artPTE-mediated turnover of paraaxon by targeted and random mutagenesis suggests that there is likely to be little easily accessible sequence space available for improvement of this activity using naturally occurring AAs.

In this study, we describe the incorporation of unnatural 7-methyl- and 7-hydroxycoumarinyl amino acids (Mco and Hco;...
The β-ketoester (5.3 g, 12 mmol) was added slowly to a solution of resorcinol (6.6 g, 60 mmol) in methanesulfonic acid (48 mL) at room temperature. After stirring for 1 h, 310 mL of diethyl ether was added and the mixture cooled to −35 °C. The mixture was stirred for 90 min to give a yellow precipitate, which was filtered off. The residue was dissolved in 100 mL of water and lyophilized overnight. It was then dissolved in 100 mL of water at pH 1.3 and 1 M NaOH solution added until the pH of the mixture was 3.5 when a pink solid separates. The mixture was cooled in ice for 1 h, filtered and the residue washed with three portions of ice-water at pH 3 followed by three portions of acetone to give 1.9 g of crude L-(7-hydroxycoumarin-4-yl)ethylglycine as the zwitterions, HPLC purity approximately 90%. The amino acid was further purified by dissolving in 50 mL of water adjusted to pH 1 with methanesulfonic acid. Aliquots (4.2 mL) were injected onto a Waters Symmetry (7 μm, C18 150 mm × 19 mm) semipreparative HPLC column and eluted at 8.5 mL/min with a 1:9 acetonitrile–water containing 0.1% triﬂuoroacetic acid for 15 min followed by a 10 min wash with 100% acetonitrile. Fractions containing the triﬂuoroacetate salt of the amino acid were pooled and rotary evaporated (12 mbar, bath 40 °C). The residue was dissolved in 15 mL 1 M sodium hydroxide at 0 °C and 36% HCl added to adjust the pH to 3.5. The white solid was ﬁltered off, washed three times with ice-water at pH 3.5, and dried under high vacuum for 60 h at room temperature. The yield of the target compound as the zwitterion was 1.2 g (38%). HPLC purity 99%. HPLC analysis was performed with a Waters Symmetry (5 μm C18 150 mm × 4.6 mm) HPLC column, eluted at 1 mL/min with a 20 min linear gradient from 10 to 100% acetonitrile in water containing 0.1% triﬂuoroacetic acid, and monitoring at 323 nm. At 22 °C, the compound eluted at 7.0 min.

Stock solutions (50 mM) of Hco in 100 mM KOH were made and 1 mL aliquots stored at −80 °C until needed.

Preparation of L-(7-methylcoumarin-4-yl)ethylglycine (Mco). Synthesis of L-(7-methylcoumarin-4-yl)ethylglycine hydrochloride (Mco) was performed as follows. The β-ketoester derived from Z-Glu-OBzl (3.25 g, 7.4 mmol), prepared as previously described40 was added in portions to a 4 mL stirred solution of 37 mmol m-cresol in 30 mL of methanesulfonic acid at room temperature. After 3.5 h, the deep yellow reaction mixture was poured into 90 mL of ice water and extracted twice with 100 mL of diethyl ether. The pH was adjusted to 4.6 with sodium hydroxide (18.5 g in 30 mL water) followed by 1 M sodium hydroxide. A small amount of a pink oily solid was ﬁltered off and the volume of the ﬁltrate was reduced to approximately 120 mL using a rotary evaporator (condenser −10 °C, bath 40 °C) and the product was allowed to stand for 3 days. The white solid that was separated was ﬁltered off, washed with ice-water, and dried in air. This material (325 mg) was slurried with water and 1 M HCl added until it fully dissolved (total volume approximately 15 mL). The acidifed solution was extracted with two half volumes of distilled dichloromethane. The aqueous phase was then rotary evaporated (condenser −10 °C, bath 30 °C) to remove traces of organic solvent and lyophilized to give 353 mg of 4-(7-methylcoumarinyl)-2-ethylglycine hydrochloride as a cream colored solid in 16% yield. Stock solutions (50 mM) of Mco in 100 mM KOH were made and 1 mL aliquots stored at −80 °C until needed.

Mutagenesis and Cloning. A Tyr309 amber (TAG) mutant was produced by overlapping PCR with primers PTE_MF, PTE_MR, PTE-Y309TAG_F, and PTE-Y309TAG_R (Table 1 of the Supporting Information) using the opdaA gene (encoding arPTE) as a template. Tyr309Leu, Tyr309Phe, and Tyr309Trp mutants were similarly generated from the opdaA gene using the primers PTE_MF, PTE_MR, PTE-Y309L_F, PTE-Y309L_R, PTE-Y309F_F, PTE-Y309F_R, PTE-Y309W_F, and PTE-Y309W_R (Table 1 of the Supporting Information). The PCR products were digested and ligated into pETMCSI vector41 between the NdeI and EcoRI restriction sites. The resulting construct was verified by DNA sequencing.
Expression of Wild-Type arPTE and Variants. Wild-type arPTE (Tyr309) and Tyr309Leu, Tyr309Phe, Tyr309Trp, Tyr309Hco, and Tyr309Mco variants were expressed in vitro using a cell-free protein synthesis system with natural and unnatural amino acids, as has been described previously. The only difference was that the arPTE variants were expressed overnight at 30 °C and no components for unnatural amino acid incorporation were included for the expression of wild-type or natural arPTE variants. The methods for expression and purification of L-(R)-hydroxyxycamphor-4-ylmethyl ethylglycine (Hco) RNA transcriptase (TollRS) and expression and purification of total RNA including an amber suppressor RNA (Sup-tRNA) have been previously described. The soluble protein fraction was loaded onto a DEAE Fractogel column (Merck Biosciences, Australia), to which arPTE and variants did not bind and was collected in the flow-through. The protein was then dialyzed overnight against 20 mM HEPES, pH 7.0, and loaded onto a 1 mL Resource S column (GE Healthcare, UK). Bound arPTE was eluted at approximately 150 mM NaCl using a linear gradient for Tyr309Hco and Tyr309Mco, 28 130 M−1 cm−1 for Tyr309Leu and Tyr309Phe, and 33 820 M−1 cm−1 for Tyr309Trp. Relative concentrations were confirmed using SDS-PAGE.

Confirmation of Insertion of Hco and Mco by In-Gel Tryptic Digest LC/MS. The incorporation of Hco and Mco into arPTE was verified by LC/MS using previously described methodology. Protein samples were separated by SDS-PAGE and visualized by staining with Coomassie blue. Protein bands that migrated at the correct size (∼28 kDa) were excised from the gel, diced into 1 mm cubes, transferred to a clean 0.5 mL Eppendorf tube and washed with a mixture of 150 µL 25 mM ammonium bicarbonate and 10 µL 45 mM dithiothreitol (DTT) on a heating block set at 60 °C for 30 min. The wash was discarded and gel pieces subsequently washed three times with 50 µL of 1 M acetate (pH 5.5) and 25 mM ammonium bicarbonate for 20 min at room temperature. The pieces were further washed with 25 mM Tris-HCl, 150 µM Hco309 was allowed to stand overnight. The condensed fluid under the lid of the tube was transferred to a 37 °C water bath and left to stand overnight. The condensed fluid under the lid of the microtube was discarded and the gels were rehydrated with 10 µL of 0.1% formic acid and left to stand on the bench for 30 min. The solution containing peptides that diffused from the gel pieces was pipetted into a 96-well microplate and analyzed by LC-ESI-Ion Trap Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS) using an Agilent 1100 capillary liquid chromatography system with an Agilent XCT ion trap mass spectrometer. Mass spectral data sets were analyzed by matching with sequence databases using Agilent’s Spectrum Mill software (Agilent Technologies, USA) to generate a peptide map. False positive matches were avoided by using the software’s stringent autovalidation default settings. Mass searches for Hco- and Mco-containing peptides were performed by matching the calculated +1, +2, +3, and +4 charged peptide masses generated using the Protein Prospector tool (University of California, SF, USA) with those identified from the spectral data.

Detection of Diethyl Phosphate and 4-Nitrophenol by Mass Spectrometry. To establish that diethyl phosphate and 4-nitrophenol were both released from the enzyme as a result of the hydrolysis of paraoxon by Tyr309Hco arPTE (Hco309), a reaction mixture containing 10 mM paraoxon in 50 mM Tris-HCl, pH 8.50, 20% methanol, and 0.2 nM Hco309 was allowed to stand for 5 min. A control sample was similarly set up but without enzyme. The solution that had been inactivated by heating at 70 °C for 30 min on a heating block. Standard solutions containing 2 mM pure samples of diethyl phosphate and 4-nitrophenol (Sigma Aldrich, USA) were also prepared in the same buffer. The samples were analyzed by negative mode LC/MS ESI ToF using an Agilent 1100 capillary liquid chromatography system with an Agilent MSD ToF mass spectrometer (Agilent Technologies, USA). The column used was an Agilent Zorbax Eclipse XDB-C18 (inner dimensions 3.5 µm × 30 mm) rapid resolution column (Agilent Technologies, USA). Ten microliters of each sample was injected and separation was achieved using a gradient (0–90%) of acetonitrile with 0.1% formic acid with a flow rate of 0.8 µL/min for 5 min. Analysis of mass spectral data to identify diethyl phosphate and 4-nitrophenol was performed using Analysts QS software (Agilent Technologies, USA).

Enzyme Kinetics and pH-Rate Profiles. Kinetic measurements of the catalyzed hydrolysis of paraoxon were performed according to published procedures. The catalytic activity of the variants of arPTE was assayed by measuring the decrease in absorbance at 347 nm. The catalyzed hydrolysis of paraoxon were performed according to published procedures. The catalytic activity of the variants of arPTE was assayed by measuring the decrease in absorbance at 347 nm.

limited solubility of the substrate. The kinetic parameters, $k_{cat}$ and $k_{cat}/K_M$, were obtained by fitting the data to the Michaelis–Menten equation using the curve fitting software, GraphPad Prism (GraphPad Software Inc., USA). The values of $k_{cat}$ and $k_{cat}/K_M$ were determined by fitting the initial velocity data to the equation
\[
v = \frac{V_{max}[S]}{(K_M + [S])}
\]
where $v$ is the initial velocity, $V_{max}$ is the maximum velocity, $S$ is the substrate concentration, and $K_M$ is the Michaelis constant. In the case of an enzyme for which product dissociation ($k_5$) is rate-limiting, $K_M$ is defined by eq 2, where $k_1$, $k_2$, $k_3$, and $k_5$ are shown in Scheme 2.
\[
K_M = \frac{(k_5(k_2 + k_3))/(k_1(k_3 + k_5))}{(1 + [H]^+)}
\]
pK_a values were determined from pH-rate profiles by fitting the experimental data to eqs 3 or 4 for single-proton and double-proton profiles, respectively.
\[
\log y = \log \left(\frac{c}{1 + [H]^+} \frac{1}{K_1} \right)
\]
\[
\log y = \log \left(\frac{c(1 + \alpha K_2)}{1 + [H]^+ + K_2} \right)
\]
where $[H]^+$ is the proton concentration, $K_1$ and $K_2$ represent protonation equilibria, $c$ and $\alpha$ are the pH independent values of $y$, and $\alpha$ is the kinetic parameter which can be either $k_{cat}$ or $k_{cat}/K_M$.

**Results and Discussion**

**In Vitro Protein Expression and Incorporation of Unnatural Amino Acids in arPTE.** To probe the role of aromatic interactions in ES and EP complexes of arPTE, two unnatural variants were created: Tyr309Hco and Tyr309Mco (Figure 2). These unnatural coumarinyl amino acids were chosen so that the variants would retain aromatic character in this region of the protein structure, whereas the hydroxyl group of Hco can be titrated through a pH region of high catalytic activity for arPTE (pH 8–9), allowing the effect of its protonation state on activity to be monitored. Mco provided a control, being completely analogous to Hco with the exception that the ionizable hydroxyl group is replaced with a methyl group. The codon for Tyr309 in the wild-type gene encoding arPTE was mutated to the *amber* (TAG) stop codon, to be used in conjunction with mutant suppressor tRNA, MfRNA^{75UUA}, which was enzymatically acylated with either Mco or Hco by purified engineered tRNA synthetase CouRS. The translational incorporation of this residue was carried out using a cell-free coupled transcription/translation system that allowed the various nucleic acid, protein, and chemical components required for synthesis of the gene to be added in defined quantities, allowing optimization of the conditions for maximal protein yield using a minimum quantity of tRNA. The purified engineered tRNA synthetase CouRS was then enzymatically acylated with either Mco or Hco, and the protein was expressed in a cell-free reaction mixture. This was expected considering that wild-type expression used naturally evolved tRNA synthetases, while the incorporation of Hco and Mco relied on a single-proton and double-proton synthetase, CouRS, that was engineered to accommodate Hco.

SDS-PAGE was used to confirm that Hco and Mco were incorporated into arPTE (Figure 3). Before staining with Coomassie blue, the gel was imaged by fluorescence with excitation and detection wavelengths of 360 and 460 nm, which are near the excitation (367 nm) and emission (455 nm) maxima of 7-hydroxycoumarin. The full-length Hco- and Mco-containing proteins, which behaved identically to wild-type arPTE under CouS staining, were clearly fluorescent, consistent with incorporation of the uAAs. The Hco-containing protein band had much higher fluorescence than the Mco protein band, consistent with the 260-fold higher fluorescence of free Hco in solution compared with Mco at these wavelengths (Figure 2 of the Supporting Information). Further confirmation for the site-specific incorporation of Hco and Mco was obtained through tryptic peptide mass fingerprinting (Figure 4), which identified peptides from segments of protein both before and after residue 309, indicating the expression of full-length Tyr309Hco and Tyr309Mco proteins. The coumarinyl-glycine-containing peptides (ILVSHDWFGLFSSHcoVTNIMDVM and ILVSHDWLFGFSSMcoVTNIMDVM) were identified by peptic ion masses. The matched peptides covered 44% of the protein, including the predicted N and C termini.

**Mechanistic Analysis of Unnatural Phosphotriesterases.** The effects of the Tyr309Hco and Tyr309Mco mutations on the catalytic behavior of arPTE were examined using pH-rate profiles. Figure 5 shows pH-rate profiles for the catalyzed hydrolysis of paraoxon by wild-type arPTE and the unnatural variants over a pH range of 4.0–10.0 (Table 1). All variants exhibit acidic dissociation constants with $pK_a$ values between 4.0 and 5.0, consistent with previous results for native wild-type arPTE, which indicates a titration of the rate-limiting step from catalysis ($k_3$) to a physical step (product release, $k_3$), as described previously. However, unlike wild-type and Tyr309Mco arPTE, the Tyr309Hco variant undergoes a second deprotonation event at basic pH. We note that the effect of this is less significant than the acidic protonation event, as seen in the lower slope of this curve (ca. 0.2 vs 0.6). The $pK_a$ value of this deprotonation event (8.2–8.4) corresponds to the $pK_a$ of the hydroxyl group of the 7-hydroxycoumarinyl side chain.

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protein is lacking a signal peptide.

Figure 4. Confirmation of Tyr309Hco and Tyr309Mco arPTEs expression by MS-TRAP tryptic digest. Peptides that were identified by MS are shown in red with the digestion site underlined. The 7-hydroxy- or (7-methylcoumarinyl)ethylglycine containing peptide is shown in pink. Unidentified sequence is shown in black. The position at which the uAA is incorporated (309) is shown in blue (X). The starting Met corresponds to residue 35 because the expressed protein is lacking a signal peptide.

Figure 5. pH-dependence of the catalytic rate constants for wild-type (black), Tyr309Mco (red), and Tyr309Hco (blue) variants of arPTE at this position: Leu309, Phe309, and Trp309. Kinetic parameters for the six variants at pH 8.5 are reported in Table 2. For efficient turnover, an enzyme must balance the relative changes of $k_{cat}$ and $K_M$ in the variants clearly show that different mutations affect the various microscopic constants differently. The comparison between wild-type, Tyr309Mco, and Tyr309Hco is particularly informative: for Tyr309Mco we see $K_M$ increase much more than $k_{cat}$, whereas for Tyr309Hco $k_{cat}$ increase similarly. Thus, for Tyr309Hco the concerted increase in $K_M$ and $k_{cat}$ can be largely attributed to an increase in $k_5$, which is not always an easy feat. The data in Table 2 show that improvement in substrate turnover for enzymes such as arPTE, where the chemical step is not rate limiting, depends upon mutations differentially affecting substrate binding and product release. In the case of the PTEs, it is difficult to assess the effects of mutations by analyzing $k_{cat}$ and $K_M$ because it is a combination of $k_1$, $k_2$, $k_3$, and $k_5$ (eq 2). However, the relative changes of $k_{cat}$ and $K_M$ in the variants clearly show that different mutations affect the various microscopic constants

Table 1. Acid Dissociation Constants for Wild-Type arPTE and the Unnatural Variants Mco309 and Hco309

<table>
<thead>
<tr>
<th>variant</th>
<th>$pK_{a1,2}$</th>
<th>$pK_{a2,2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr309</td>
<td>4.7, -</td>
<td>4.8, -</td>
</tr>
<tr>
<td>Mco309</td>
<td>4.7, -</td>
<td>4.8, -</td>
</tr>
<tr>
<td>Hco309</td>
<td>4.7, 8.4</td>
<td>5.2, 8.2</td>
</tr>
</tbody>
</table>

(8.3), suggesting that the change in the protonation state of this hydroxyl moiety causes the change in activity across pH 8–9. This conclusion is also supported by the observation that the turnover rate of the Mco309 variant does not change over the same pH range and Mco is structurally identical to Hco with the exception that the hydroxyl group is replaced by a methyl group.

In addition to Hco and Mco, we made three other natural variants of arPTE at this position: Leu309, Phe309, and Trp309. Kinetic parameters for the six variants at pH 8.5 are reported in Table 2. For efficient turnover, an enzyme must balance strong interactions with the substrate ($K_M$) with fast dissociation of products ($k_2$), around an efficient chemical step ($k_5$). Because of the similarity between substrate and product in many reactions, this is not always an easy feat. The data in Table 2 show that improvement in substrate turnover for enzymes such as arPTE, where the chemical step is not rate limiting, depends upon mutations differentially affecting substrate binding and product release. In the case of the PTEs, it is difficult to assess the effects of mutations by analyzing $K_M$ because it is a combination of $k_1$, $k_2$, $k_3$, and $k_5$ (eq 2). However, the relative changes of $K_M$ in the variants clearly show that different mutations affect the various microscopic constants differently. The comparison between wild-type, Tyr309Mco, and Tyr309Hco is particularly informative: for Tyr309Mco we see $K_M$ increase much more than $k_{cat}$, whereas for Tyr309Hco $K_M$ and $k_{cat}$ increase similarly. Thus, for Tyr309Hco the concerted increase in $K_M$ and $k_{cat}$ can be largely attributed to an increase in $k_5$. In contrast, for Tyr309Mco, the much larger increase in $K_M$ than in $k_{cat}$ suggests that the affinity between enzyme and substrate ($k_1$ and $k_2$) has been adversely affected, without the same increase in $k_5$ as is seen in Tyr309Hco. In combination with the pH profiles that show that the changing protonation state of Hco309 affects turnover, this analysis establishes that the residue at position 309 is directly involved in both Michaelis complex formation and product release.

The possible rotamers of Hco309 are shown in Figure 6. The effect of this mutation on turnover may be explained by the fact that the phosphotriester substrates of PTEs are uncharged, unlike the 4-nitrophenolate leaving group, which is negatively charged at pH values above 7.1. The negative charge associated with Hco309 will therefore electrostatically repel the negatively charged product more than the neutral substrate, thereby affecting product release more than substrate binding. These data, which could only be obtained using mutagenesis with

Table 2. Kinetic Parameters for the Hydrolysis of Paraaxon at pH 8.5 by arPTE Variants with Natural and Unnatural Mutations at Position 309; the Wild-Type Residue is Tyr

<table>
<thead>
<tr>
<th>variant</th>
<th>$k_{cat}$</th>
<th>$K_M$</th>
<th>$k_{cat}/K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr309</td>
<td>$510 \pm 10$</td>
<td>$20 \pm 1$</td>
<td>$2.5 \times 10^7$</td>
</tr>
<tr>
<td>Leu309</td>
<td>$260 \pm 9$</td>
<td>$51 \pm 4$</td>
<td>$5.1 \times 10^6$</td>
</tr>
<tr>
<td>Phe309</td>
<td>$465 \pm 13$</td>
<td>$73 \pm 6$</td>
<td>$6.4 \times 10^6$</td>
</tr>
<tr>
<td>Trp309</td>
<td>$436 \pm 11$</td>
<td>$80 \pm 7$</td>
<td>$5.5 \times 10^6$</td>
</tr>
<tr>
<td>Mco309</td>
<td>$910 \pm 20$</td>
<td>$96 \pm 7$</td>
<td>$9.4 \times 10^5$</td>
</tr>
<tr>
<td>Hco309</td>
<td>$3990 \pm 110$</td>
<td>$138 \pm 8$</td>
<td>$2.9 \times 10^7$</td>
</tr>
</tbody>
</table>

 active site residues altogether.\textsuperscript{33} The experimentally determined activation energy for the rate limiting step, product release, is of the order of 12 kcal/mol,\textsuperscript{27} meaning the energy barrier for the chemical step must be even lower. The results presented here, as well as previous analysis of alanine and phenylalanine mutants of Tyr309,\textsuperscript{19,29} are more consistent with crystal structure snapshots obtained during the catalytic cycle\textsuperscript{22,27} than recent simulations; because both Tyr309 and the bulkier uAAs analyzed here support fast substrate turnover and directly affect $K_M$ and $k_{cat}$, they are unlikely to impede formation of the transition state, and instead most likely contribute to productive alignment of the substrate for in-line attack from an alternative nucleophilic water/hydroxyl to the $\mu$-hydroxo bridge.

**Engineering Enhanced Activity in arPTE with uAAs.** The $k_{cat}$ values for wild-type, Tyr309Mco, and Tyr309Hco arPTEs for paraoxon hydrolysis at pH 8.5 are 510, 910, and $3990\, s^{-1}$, respectively (Table 2); an 8-fold increase in the turnover rate for the Tyr309Hco variant over that of the wild-type enzyme. The magnitude of this increase is even greater at pH values above 8.5 (11-fold at pH 10). However, although $k_{cat}$ is increased, the ratio of $k_{cat}/K_M$, also known as the specificity constant and often considered to be a measure of catalytic efficiency, is essentially unchanged for the Tyr309Hco variant compared to the wild-type enzyme. As discussed above and shown in Scheme 2 and eq 2, the value of $k_{cat}/K_M$ is informative and valuable because it incorporates most of the different rate constants of the catalytic cycle. However, the present data also show that $k_{cat}/K_M$ is not well suited for evaluating catalytic improvements in enzymes: despite wild-type and Tyr309Hco arPTE having equal $k_{cat}/K_M$ values, the Tyr309Hco variant is a substantially more effective catalyst than the wild-type enzyme at all substrate concentrations above 10 $\mu$M (Figure 7), which is less than one tenth of its $K_M$ and much lower than any substrate concentration that is typically used during laboratory protein engineering/evolution experiments (100–500 $\mu$M). In field applications, concentrations higher than 10 $\mu$M are regularly encountered in some agricultural waste waters (e.g., spent animal and horticultural dip liquors).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Potential rotamers (without steric clashes to other amino acid side chains or substrate) of Hco309 relative to the substrate diethylmethoxyphenylphosphate and Tyr309 in the wild-type protein based on the crystal structure of the enzyme–substrate complex (PDB accession code 2R1N). The topology of Hco, bond restraints, and angle restraints were produced using the PRODRG server.\textsuperscript{47}}
\end{figure}

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Compound} & \textbf{Wild-Type} & \textbf{Tyr309Hco} \\
\hline
paraoxon & 510 & 3990 \\
\hline
\end{tabular}
\caption{Comparison of $k_{cat}$ values for wild-type and Tyr309Hco arPTE for paraoxon hydrolysis at pH 8.5.}
\end{table}


\textsuperscript{uAAs, highlight the importance of noncovalent interactions in ES and EP complexes and the trade-off between efficient binding and fast turnover in enzymes.

In principle, the increased turnover rate observed in the Tyr309Hco variant could also result from an alteration in the catalytic mechanism in which the hydroxyl group of Hco acts as a nucleophile in the hydrolysis of paraoxon because it will be positioned in the vicinity of the hydrolyzable P–O bond. Indeed, lysine and tyrosine residues have been shown to be responsible for the stoichiometric breakdown of organophosphates by albumin.\textsuperscript{51} In this scenario, Hco309 would become phosphorylated by the substrate, followed by slow regeneration of the enzyme through water-mediated dephosphorylation. Because the spectrophotometric assay only detects the release of the enzyme through water-mediated dephosphorylation.}
hundreds of thousands of random mutants or via rational mutation of Tyr309 to other hydrophobic residues (Leu, Phe, Trp; Table 2). Griffiths and Tawfik previously isolated a mutant of pdPTE with a 3.6-fold increase in specific activity at a concentration of 250 µM paraoxon, although this variant was identified through the impressive technical achievement of screening 3.7 × 10^7 mutants by in vitro compartmentalization; here we have increased the specific activity of arPTE at 250 µM 5.5-fold with a single unnatural point mutation. These results highlight the potential that man-made amino acids hold for accessing functional space that is not available during natural evolution and engineering with natural amino acids, showing that the already exciting prospects for de novo design of enzyme catalysts may be greatly enhanced through the use of unnatural amino acids.

Summary

In this work two uAAs, Mco, and Hco, were introduced into arPTE in place of Tyr309. The use of these uAAs allowed the detailed analysis of the interaction between enzyme and substrate during formation of the Michaelis complex and product release, and established that the residue at position 309 is directly involved in both of these processes. The data imply that the complexes seen in recent crystal structures of the enzyme captured during substrate turnover are most likely accurate and highlight the importance of nonchemical steps for the maintenance of fast substrate turnover during the catalytic cycles of enzymes. The potential value of uAAs in protein engineering is emphasized by the observation that the single site Tyr309Hco mutation reported here produced a greater increase in activity than has been obtained from screening of many thousands of variants created using natural amino acids.

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Supporting Information Available: Analytical and spectral characterization data for the synthesis of uAAs (Hco and Mco) and other information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Figure 7. Comparison of reaction velocities of wild-type arPTE and variants for hydrolysis of paraoxon at pH 7.5 and 8.5.