Characterization of a Phosphodiesterase Capable of Hydrolyzing EA 2192, the Most Toxic Degradation Product of the Nerve Agent VX†

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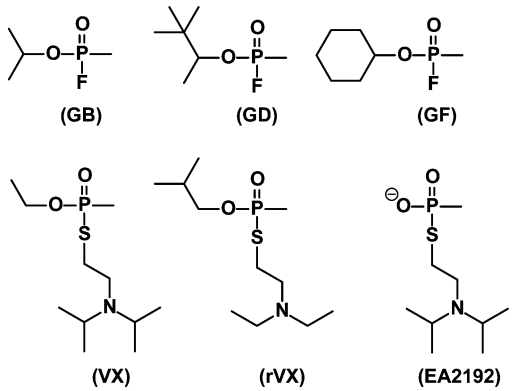
ABSTRACT: Glycerophosphodiesterase (GpdQ) from *Enterobacter aerogenes* is a nonspecific diesterase that enables *Escherichia coli* to utilize alkyl phosphodiesters, such as diethyl phosphate, as the sole phosphorus source. The catalytic properties of GpdQ were determined, and the best substrate found was bis(*p*-nitrophenyl) phosphate with a k_{cat}/K_m value of 6.7×10^3 M^{-1} s^{-1}. In addition, the *E. aerogenes* diesterase was tested as a catalyst for the hydrolysis of a series of phosphonate monoesters which are the hydrolysis products of the highly toxic organophosphonate nerve agents sarin, soman, GF, VX, and rVX. Among the phosphonate monoesters tested, the hydrolysis product of rVX, isobutyl methyl phosphonate, was the best substrate with a k_{cat}/K_m value of 33 M^{-1} s^{-1}. The ability of GpdQ to hydrolyze the phosphonate monoesters provides an alternative selection strategy in the search of enhanced variants of the bacterial phosphotriesterase (PTE) for the hydrolysis of organophosphonate nerve agents. This investigation demonstrated that the previously reported activity of GpdQ toward the hydrolysis of methyl demeton-S is due to the presence of a diester contaminant in the commercial material. Furthermore, it was shown that GpdQ is capable of hydrolyzing a close analogue of EA 2192, the most toxic and persistent degradation product of the nerve agent VX.

Organophosphate triesters have been widely employed as agricultural pesticides and insecticides. The most toxic of the reported organophosphonate esters are the military-based nerve agents sarin (GB), cyclosarin (GF), soman (GD), and two forms of VX (Scheme 1). All of these compounds exert their toxicity by irreversibly inactivating the enzyme acetylcholine esterase (AchE)[1] (*1*). AchE is responsible for hydrolysis of the neurotransmitter acetylcholine, and the toxicity of these nerve agents is dependent on the duration and route of administration (*2*). Due to the environmental and health risks associated with these phosphate and phosphonate esters, serious efforts have been devoted to developing improved enzymes for bioremediation applications (*3*, *4*). Among these enzymes, the bacterial phosphotriesterase (PTE) is the most promising candidate for in vivo and in vitro detoxification and detection of organophosphate nerve agents. The wild-type PTE possesses a broad substrate specificity, and improvements in the catalytic activity for marginal substrates have been obtained by modest changes in the identity of a limited set of key residues within the active site (*5*). However, further enhancements in the catalytic properties of PTE are limited by the efficiency and sensitivity of the screening and/or selection approaches that have been employed for the isolation of improved variants from modified gene libraries (*6*).

Scheme 1



One enzyme that may function as an integral component of an efficient selection system for the identification of PTE mutants with enhanced catalytic properties for selected organophosphate substrates is the glycerophosphodiesterase (GpdQ) from *Enterobacter aerogenes*. This enzyme is part of an operon homologous to the glycerol 3-phosphate (G3P) uptake operon in *Escherichia coli* (*ugp*) and is induced under phosphate-limiting conditions (*7*). The *Enterobacter* phosphodiesterase was initially isolated from cultures of sewage growing on minimal medium enriched with 0.2 mM dimethyl phosphate (*8*). GpdQ was first purified by Gerlt and co-workers, and it is believed to be the first phosphohydrolase demonstrated to hydrolyze stable alkyl diesters such as dimethyl phosphate (DMP) and diethyl phosphate (DEP) (*9*, *10*). The *E. aerogenes* phosphodiesterase has also been

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[1] Abbreviations: AchE, acetylcholine esterase; PTE, phosphotriesterase; GpdQ, glycerophosphodiesterase; DMP, dimethyl phosphate; DEP, diethyl phosphate; GPE, glycerophosphoethanolamine; OpdA, phosphotriesterase from *Agrobacterium radiobacter*; IPTG, isopropyl *β*-thiogalactoside; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CHES, 2-(cyclohexylamino)ethanesulfonic acid; ADH, alcohol dehydrogenase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MOPS, morpholinopropanesulfonate.

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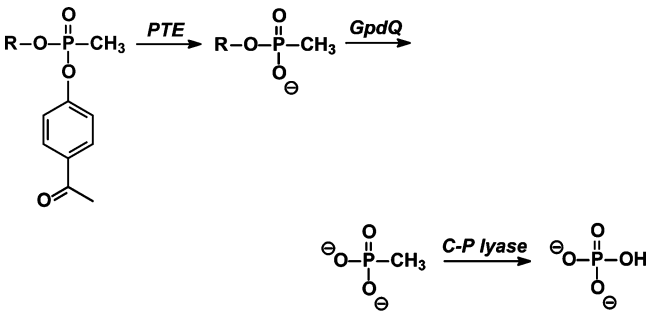
Scheme 2



shown to catalyze the hydrolysis of a broad range of diesters including bis(*p*-nitrophenyl) phosphate, glycerophosphoethanolamine (GPE), ethylene phosphate, and cAMP (*9*, *11*).

Ollis and co-workers demonstrated that expression of GpdQ from *E. aerogenes* enables *E. coli* to utilize alkyl phosphodiesters (e.g., DMP and DEP) as the sole phosphorus source (*11*). An in vivo growth assay where GpdQ was coexpressed with a phosphotriesterase from *Agrobacterium radiobacter* (OpdA) was employed to examine the ability of *E. coli* to transform dimethyl *p*-nitrophenyl phosphate (methyl paraoxon) to inorganic phosphate (*11*, *12*). Their findings showed that under phosphate-limiting conditions, cells expressing only OpdA were unable to utilize the phosphotriester as a source of phosphate. However, cells coexpressing GpdQ and OpdA allowed *E. coli* to grow on methyl paraoxon as the sole phosphorus source (*11*).

In this investigation, a more detailed characterization of the substrate specificity of GpdQ is presented. A series of organophosphate diesters and methyl phosphonate monoesters was synthesized, and the kinetic constants were determined for product formation. The objective of this analysis was to extend the application of the in vivo selection assay to cover a wider range of organophosphate nerve agents including GB, GD, GF, VX, and rVX. The methyl phosphonate hydrolysis product is anticipated to be subsequently degraded by the bacterial C-P lyase complex to ultimately produce methane and inorganic phosphate (*13*). The C-P lyase complex is part of the *phn* operon in *E. coli*, which is induced under phosphate starvation conditions and is known to enable *E. coli* to utilize methyl phosphonate as a sole phosphorus source (*14*). The ability of GpdQ to hydrolyze methyl phosphonate esters would provide an alternative means to conventional screening assays in the search for improved variants of PTE for the detoxification of organophosphonate esters. The proposed transformation of a generic methyl phosphonate diester by this series of enzymes is presented in Scheme 2.

During the course of these studies we discovered the first enzymatic hydrolysis of a close analogue of EA 2192 (**23**), the most toxic degradation product of the nerve agent VX. The United States stockpile of VX is estimated as 1269 tons, which is currently stored at the Newport Chemical Agent Disposal Facility in Indiana (*15*). According to the Chemical Weapons Convention, all stockpiles of VX were slated to be destroyed by the year 2007. However, the Government Accountability Office (GAO) has announced that the projected date for complete destruction of chemical weapons in the United States is 2014 (*16*). Currently, large-scale destruction of the nerve agent VX is achieved by alkaline hydrolysis. At pH 8, EA 2192 is the major product of VX hydrolysis (*17*). EA 2192 is very persistent in the environment, and it is known to retain significant anti-cholinesterase activity (*18*). Although the oral toxicity of EA 2192 is approximately 10−20% that of VX, the intravenous toxicity in mice is very similar to VX (LD_{50} = 18 μg/kg) (*18*). For complete degradation of EA 2192 to occur, VX must be kept at pH 12 for approximately 90 days (*19*).

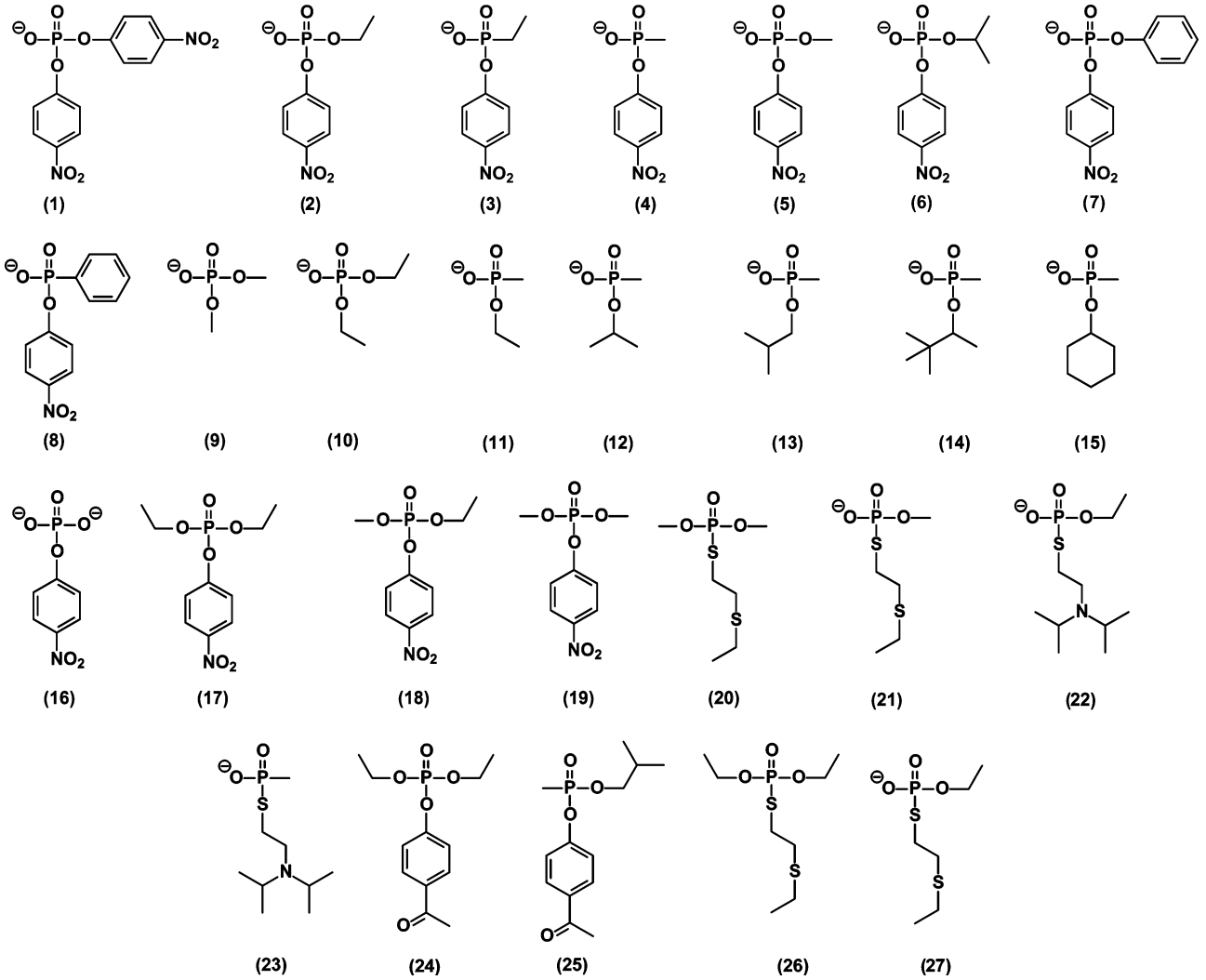
MATERIALS AND METHODS

Materials. E. aerogenes was purchased from the ATCC (ATCC number 13048). *E. coli* BL21(DE3) and XL1-blue cells were obtained from Stratagene. The expression plasmid pET30(a) and *Platinium pfx* DNA polymerase were purchased from Invitrogen. The genomic DNA purification kit was obtained from Promega. Alcohol dehydrogenase and NAD^+ were purchased from Sigma. Organophosphate diesters and methyl phosphonates were synthesized by standard methods and the structures of these compounds (**1**−**27**) are presented in Scheme 3. The details of these syntheses and the physical constants are provided in the Supporting Information. The toxicities for some of these compounds have not been determined, and thus they should be used with caution. Methyl demeton-S (**20**) was purchased from Chem Services and also synthesized by us because the commercial material was shown to be contaminated with a significant amount of a diester hydrolysis product. Compounds **1** and **16** were obtained from Aldrich. DNA sequencing was performed by the Gene Technologies Laboratory, Texas A&M University.

Cloning of GpdQ. Genomic DNA from *E. aerogenes* was isolated and used as the template for PCR amplification. The GpdQ gene was amplified using the primers 5′-GGC GGC CAA GCT TGC AGG TTT AAA ATC ATA TGC TGT TAG CGC ACA TTT CCG ATA C and 3′-CTA CGA ATT CTC ATC ATC ATT AGC GCT CTT CCG TTG GAC. The *Nde*I and *Eco*RI restriction sites are underlined. PCR conditions were 95 °C for 30 s, followed by 30 cycles at 95 °C for 1 min, 55 °C for 1 min, 68 °C for 4 min, and final extension at 68 °C for 10 min. The 824 bp PCR product was purified, digested with *Nde*I and *Eco*RI, and ligated to similarly digested pET30(a). The ligation product was transformed in *E. coli* XL1-blue electrocompetent cells for DNA sequencing.

Purification of GpdQ. The plasmid pET30 encoding the GpdQ gene was transformed in BL21(DE3) electrocompetent cells. Cells were grown in Luria−Bertani medium supplemented with 0.05 mg/mL kanamycin and 1.0 mM $MnCl_2$ at 30 °C until the OD_{600} reached 0.6. Protein expression was induced by the addition of 1.0 mM isopropyl β-thiogalactoside (IPTG), and the culture was grown for an additional 12 h at 30 °C. Cells were harvested by centrifugation and resuspended in 20 mM Tris-HCl buffer, pH 7.6, containing 0.1 mg/mL phenylmethanesulfonyl fluoride (PMSF). All purification steps were carried out at 4 °C. Cells were disrupted by sonication, and the soluble fraction was isolated by centrifugation. The supernatant was treated with 1.5% protamine sulfate to precipitate nucleic acids which were removed by centrifugation. The supernatant was treated to 60% saturation with ammonium sulfate. The pellet from the ammonium sulfate fractionation step was resuspended in 20

Scheme 3



mM Tris-HCl, pH 7.6, and loaded on a Superdex 200 gel filtration column (Amersham Biosciences). Fractions that eluted from the Superdex column were assayed for the hydrolysis of 1.0 mM bis(*p*-nitrophenyl) phosphate (**1**) at pH 9.0. Fractions with the highest activity were pooled and loaded on a Resource Q anion-exchange column (Amersham Biosciences) and subsequently eluted with a 0−1 M sodium chloride gradient in 20 mM Tris-HCl buffer, pH 7.6. SDS−PAGE indicated that the protein was at least 95% pure and that the activity was associated with a band at ∼31 kDa. Protein concentrations were determined using an $\epsilon_{280} = 38600\ M^{-1}\ cm^{-1}$. To determine the molecular weight of GpdQ, a Superdex column was calibrated using a gel filtration molecular weight marker kit (Sigma) consisting of thyroglobulin (669 kDa), apo-ferritin (443 kDa), *β*-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

Kinetic Measurements and Data Analysis. All kinetic measurements were performed on a SPECTRAmax-340 plate reader (Molecular Devices Inc., Sunnyvale CA). The assays contained 0.1 mM $MnCl_2$ and 25 mM CHES buffer, pH 9.0. The release of *p*-nitrophenol ($\epsilon_{400} = 17000\ M^{-1}\ cm^{-1}$) was monitored at 25 °C for the enzymatic hydrolysis of compounds **1**−**8**. Hydrolysis of compounds **9**−**15** was monitored by coupling product formation to alcohol dehydrogenase (ADH). The assay contained 10 units of ADH, 1.0 mM NAD^+, 25 mM CHES, pH 9.0, and 0.1 mM $MnCl_2$, and the production of NADH ($\epsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$) was monitored at 25 °C. GpdQ-catalyzed hydrolysis of *O*-isopropyl methyl phosphonate (**12**) and *O*-isobutyl methyl phosphonate (**13**) was also measured by ^{31}P NMR spectroscopy. Hydrolysis of compounds **20**, **26**, and **27** was assayed using Ellman's reagent, DTNB, and the release of the 2-nitro-5-thiobenzoate anion ($\epsilon_{412} = 14150\ M^{-1}\ cm^{-1}$) was followed at pH 8.0 (*20*).

Kinetic parameters were obtained by fitting the data to eq 1, where v is the initial velocity, k_{cat} is the turnover number, [A] is the substrate concentration, and K_m is the Michaelis constant. First-order rate constants for product formation and substrate decay from changes in the NMR spectra were obtained by fitting the data to eqs 2 or 3, respectively, where A_t and P_t are the substrate and product concentration at any time t and k is the first-order rate constant.

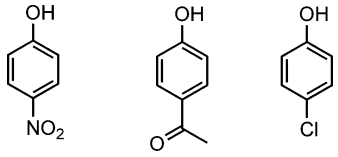
$$v/E_t = k_{cat}[A]/(K_m + [A]) \quad (1)$$

$$P_t = A_0(1 - e^{-kt}) \quad (2)$$

$$A_t = A_0(e^{-kt}) \quad (3)$$

Growth Assays. E. coli BL21(DE3) competent cells were transformed with the GpdQ-containing plasmid and allowed

Scheme 4



to grow on LB plates containing 0.05 mg/mL kanamycin, resulting in GpdQ+ cells. Overnight cultures in LB medium were inoculated from fresh transformations and washed three times with minimal media to remove any traces of the rich medium. To assess the ability of the GpdQ+ cells to grow on organophosphate and organophosphonate esters, the growth rate was monitored under phosphate-limiting conditions using liquid MOPS phosphate-free minimal medium, pH 7.4 (*21*). The minimal medium was supplemented with 0.5 mM $MnCl_2$, 0.1% glucose as the carbon source, and 0.1 μg/mL thiamin. Substrates were added to the cultures to a final concentration of 1.0 mM. A 20 mL culture was inoculated with a 1% fresh, previously washed, overnight culture. As a control, GpdQ$^-$ BL21(DE3) cells were grown in the presence and absence of inorganic phosphate. Cell growth was monitored by measuring the OD_{600} for 4−5 days at 30 °C.

The choice of organophosphate triester substrates for an in vivo selection protocol under phosphate-limiting conditions is limited by the ability of *E. coli* to survive the release of a phenol or alcohol product upon hydrolysis of initial substrate. The *E. coli* BL21(DE3) cells were tested for their ability to grow in the presence of various phenols at a concentration of 1.0 mM (Scheme 4). Methanol was added to a final concentration of 1.0% to enhance the solubility of some of these phenols. The growth rate was monitored every 20 min at 37 °C for the first 12 h and then periodically after that.

Two different forms of the bacterial phosphotriesterase were tested for their ability to complement the phosphate deficiency in minimal medium: wild-type PTE cloned in the low copy number plasmid, pZA32-luc, and a mutant of PTE (designated as pET20b-5S). The catalytic constants for the hydrolysis of paraoxon by this mutant (K185R/D208G/R319S) are nearly the same as the wild-type PTE, but the net protein expression is significantly enhanced, relative to the native enzyme, because of an apparent increase in the stability of the apoenzyme (*22*). Cells coexpressing PTE and GpdQ were tested for their ability to grow on the paraoxon and rVX analogues (**24** and **25**) as the sole phosphorus source. Phosphate-free MOPS minimal medium was supplemented with 1.0 mM **24** or **25**, 0.1% glucose, 0.1 μg/mL thiamin, 50 μg/mL kanamycin, and 100 μg/mL ampicillin. A 20 mL culture was inoculated with 5% (v/v) from the overnight culture. Additional metal was found to have an inhibitory effect.

RESULTS

Expression and Purification of Glycerophosphodiester Diesterase. The gene for GpdQ was cloned from the genomic DNA of *E. aerogenes* into a pET30 plasmid and expressed in *E. coli* BL21(DE3). GpdQ was purified using size exclusion and anion-exchange column chromatography. The overall yield obtained from a 1 L culture was approximately 30−40 mg of purified protein. The diesterase activity is associated with a ~31 kDa band on SDS−PAGE, consistent with the calculated mass of 30.84 kDa for an individual subunit. GpdQ elutes from an equilibrated Superdex gel filtration column at a volume equivalent to a molecular mass of 200 kDa. These data suggest that the protein oligomerizes as a hexamer, consistent with the recently published crystal structure (*23*). The specific activity of GpdQ toward the hydrolysis of 1.0 mM **1** was enhanced 2-fold and 4-fold when the assay contained 0.1 mM $ZnCl_2$ or $MnCl_2$, respectively. Atomic absorption analysis of the purified protein indicated that GpdQ contained a mixture of 1.0 and 0.6 equiv per subunit of Mn^{2+} and Zn^{2+}, respectively. As a member of the metal-dependent family of phosphodiesterases, GpdQ is expected to utilize a binuclear metal center for catalysis (*24*). The crystal structure of the Zn^{2+}- and Co^{2+}-substituted GpdQ showed two divalent metal ions in each subunit (*23*). Metal analysis revealed that the occupancy of the β-site is 80% of the occupancy at the α-site (*23*).

Table 1: Kinetic Parameters for the Hydrolysis of Organophosphate Diesters and Methyl Phosphonate Monoesters by GpdQ

substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
1	6.0 ± 0.2	0.9 ± 0.1	(6.7 ± 0.5) × 10^3
2	2.0 ± 0.05	0.9 ± 0.1	(2.1 ± 0.1) × 10^3
3	0.5 ± 0.01	0.4 ± 0.03	(1.2 ± 0.1) × 10^3
4	1.6 ± 0.04	1.3 ± 0.1	(1.2 ± 0.1) × 10^3
5	7.3 ± 0.6	2.6 ± 0.3	(2.8 ± 0.2) × 10^3
6	1.0 ± 0.04	6.5 ± 0.5	(1.7 ± 0.2) × 10^2
7	4.0 ± 0.1	1.8 ± 0.1	(2.2 ± 0.2) × 10^3
8	2.8 ± 0.29	13 ± 2.1	(2.2 ± 0.4) × 10^2
9	(5.0 ± 0.1) × 10^{-3}	0.06 ± 0.01	82 ± 14
10	(7.4 ± 0.5) × 10^{-3}	0.5 ± 0.06	15 ± 1
11	(1.2 ± 0.1) × 10^{-2}	0.74 ± 0.13	16 ± 3
12	(1.0 ± 0.1) × 10^{-2}	19 ± 2	0.51 ± 0.02
13	(1.0 ± 0.1) × 10^{-1}	3.3 ± 0.6	33 ± 4
14	nd[a]	nd	<0.01
15	0.31 ± 0.02	27 ± 3	11 ± 1
16	nd	nd	5 ± 1
17	nd	nd	<1
18	nd	nd	2 ± 1
19	nd	nd	13 ± 1
20	nd	nd	<2
21	nd	nd	(1.2 ± 0.1) × 10^4
22	1.1 ± 0.03	0.61 ± 0.04	(1.8 ± 0.1) × 10^3
27	0.94 ± 0.02	0.42 ± 0.03	(2.2 ± 0.2) × 10^3

[a] Not determined.

Substrate Specificity of GpdQ. The substrate specificity of GpdQ toward the hydrolysis of various organophosphate diesters and organophosphonate monoesters was investigated using the compounds shown in Scheme 3. This list of potential substrates includes analogues of toxic organophosphonate nerve agents for which the corresponding phosphonate diester would be a potential substrate for the bacterial phosphotriesterase (PTE). Compounds **11**, **12**, **13**, **14**, and **15** are the primary hydrolysis products of the nerve agents VX, sarin, rVX, soman, and GF, respectively. The kinetic parameters for the hydrolysis of compounds **1**−**22** are summarized in Table 1. The best overall substrate tested in this investigation for GpdQ is bis(*p*-nitrophenyl) phosphate (**1**) with a k_{cat}/K_m of 6.7 × 10^3 M^{-1} s^{-1} and a k_{cat} of 6 s^{-1}.

The substrate specificity of GpdQ followed two major trends. Organophosphate diesters are better substrates than their corresponding methyl phosphonate analogues. For example, the k_{cat}/K_m for the hydrolysis of *O*-ethyl-*p*-NPP (**2**)

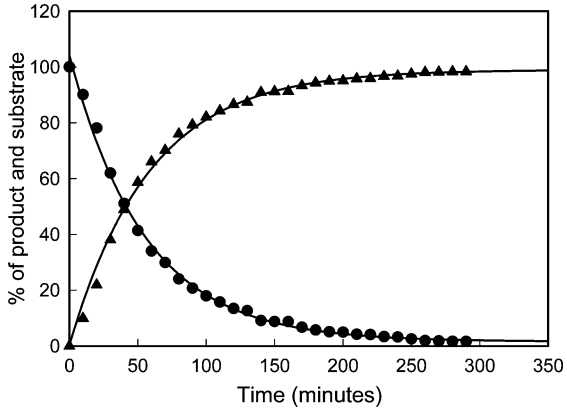


FIGURE 1: GpdQ-catalyzed hydrolysis of 10 mM isobutyl methyl phosphonate (**13**) as measured by ^{31}P NMR spectroscopy. The amount of enzyme was 9.3 μM, and the turnover number was calculated as 0.22 s^{-1}.

is 2-fold higher than the corresponding ethyl-*p*NPP (**3**). This feature was more pronounced in the case of **7** where the catalytic efficiency is 10-fold higher than for compound **8**. The second feature that emerged from the substrate specificity profile is that the turnover numbers for organophosphate diesters with a *p*-nitrophenol leaving group are significantly higher than the turnover numbers for the corresponding aliphatic diesters. However, the K_m values are lower for the alkyl diesters, resulting in higher k_{cat}/K_m values. For example, the k_{cat}/K_m for the hydrolysis of **2** is 140-fold higher than the k_{cat}/K_m value for **10**.

Nuclear magnetic resonance (NMR) was employed to monitor the enzymatic hydrolysis of **13** at an initial concentration of 10 mM. The decrease in the signal intensity at 27.1 ppm for the substrate was accompanied by an increase in the resonance for the methyl phosphonate product at 21.2 ppm. The areas for both resonances were integrated, and the data corresponded to a k_{cat} of 0.22 s^{-1} (Figure 1). Similarly, the enzymatic hydrolysis of compounds **10**, **11**, and **12** was monitored using NMR, and the rate constants were in general agreement with the catalytic parameters obtained using the ADH coupling assay (data not shown). GpdQ did not exhibit any measurable activity for the hydrolysis product of soman (**14**). When 10 mM **14** was incubated with 48 μM enzyme at pH 8.0, less than 10% of the substrate was hydrolyzed after a 5 day incubation as measured by ^{31}P NMR spectroscopy.

GpdQ was tested for its ability to hydrolyze the organophosphate triesters paraoxon (**17**), **18**, and **19** (Table 1). The activity toward paraoxon was more than 3 orders of magnitude lower than that of the diester analogue, *O*-ethyl-*p*NPP (**2**). However, GpdQ exhibited a noticeable preference toward the hydrolysis of methyl-substituted triesters. The rate of *O*-ethyl-*O*-methyl-*p*NPP (**18**) hydrolysis was measurably faster than the rate of paraoxon hydrolysis and 7-fold lower than the rate of methyl paraoxon (**19**) hydrolysis. However, saturation with these compounds could not be achieved, and thus the kinetic characterization is limited to the measurement of k_{cat}/K_m. When tested for the hydrolysis of *p*-nitrophenyl phosphate (**16**), GpdQ exhibited a low level of monoesterase activity.

Hydrolysis of Methyl Demeton-S. The GpdQ-catalyzed hydrolysis of methyl demeton-S (**20**) was monitored spectrophotometrically using 0.4 mM substrate at different

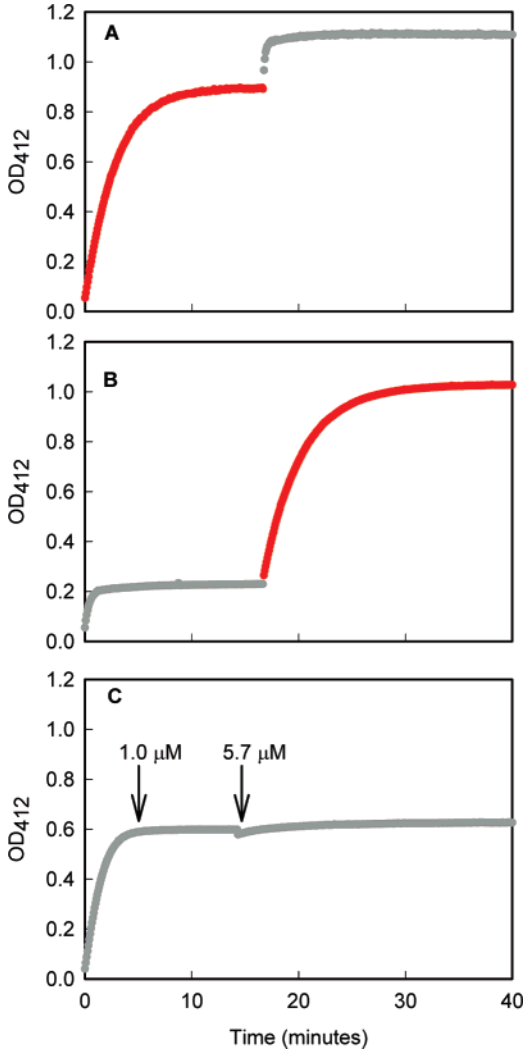


FIGURE 2: Hydrolysis of methyl demeton-S using a combination of PTE and GpdQ. In panel A, the total concentration of the triester was determined using 6.0 μM PTE (red), followed by the addition of 2.5 μM GpdQ (gray). In panel B, the order of adding the enzymes was reversed. The GpdQ time course (gray) was fit to eq 3, and an approximate value for k_{cat}/K_m of 1.2×10^4 M^{-1} s^{-1} was obtained for the hydrolysis of the diester contaminant. In panel C, the first addition of 1.0 μM GpdQ to 0.5 mM methyl demeton-S was followed by the addition of 5.7 μM enzyme. No hydrolysis of methyl demeton-S could be detected.

enzyme concentrations ranging from 0.12 to 1.0 μM. Regardless of the enzyme concentration, the reaction stopped after approximately 25% of the total substrate was hydrolyzed (data not shown). A ^{31}P NMR spectrum of the commercial substrate indicated the presence of a significant amount of the diester contaminant (**21**). The hydrolysis of methyl demeton-S was subsequently monitored using a combination of the bacterial phosphotriesterase (PTE) and GpdQ. In the first assay, the total concentration of the methyl demeton-S triester was determined using 5 μM PTE. After complete hydrolysis of the triester by PTE, 2.5 μM GpdQ was added to monitor the hydrolysis of the diester contaminant (Figure 2A). Similar results were obtained by reversing the order of the two enzymes (Figure 2B). A first-order rate constant of 1.8 $min^{-1} \pm 0.1$ was obtained by fitting the time course for the hydrolysis of the diester contaminant by GpdQ shown in Figure 2B to eq 3. Dividing the first-order rate constant by the enzyme concentration (2.5 μM), an ap-

proximate value for k_{cat}/K_m of 1.2×10^4 M^{-1} s^{-1} was obtained assuming that the concentration of the diester was below the value of K_m. These results demonstrate that the commercial methyl demeton-S contains a significant contaminating concentration of the diester. In an attempt to measure the rate for the GpdQ-catalyzed hydrolysis of the demeton-S triester, 0.5 mM compound was incubated with 1.0 μM enzyme (Figure 2C). After the hydrolysis of the diester contaminant was complete, an additional 5.7 μM enzyme was added, but there was no evidence that the remaining demeton-S triester could be hydrolyzed by this enzyme.

Because of the impurities in the commercial phosphorothiolate substrates, methyl demeton-S (**20**), ethyl demeton-S (**26**), and the diester (**27**) were synthesized to obtain potential substrates of high purity. The measured value of k_{cat}/K_m for the hydrolysis of **27** by GpdQ is 2.2×10^3 M^{-1} s^{-1} (Table 1). To measure the rate of the GpdQ-catalyzed hydrolysis of methyl demeton-S (**20**), 1.0 mM substrate was incubated with 1.0 μM enzyme for 6 h. The change in the absorbance at 412 nm due to the reaction of DTNB with the enzyme was subtracted from the total change in the absorbance at this wavelength. The upper limit for the turnover obtained in this experiment for the hydrolysis of methyl demeton (**20**) by GpdQ is 0.1 min^{-1}. Similarly, the hydrolysis of 1.0 mM ethyl demeton-S (**26**) using 1.0 μM enzyme was monitored, and the turnover was found to be less than 0.1 min^{-1}. These findings demonstrate that GpdQ exhibits minimal activity toward the hydrolysis of the phosphorothiolate triesters **20** and **26**.

Hydrolysis of an Analogue of EA 2192. The hydrolysis of compound **22** by GpdQ was investigated as a model for the hydrolysis of EA 2192 (**23**), the most toxic degradation product of the nerve agent VX. This substrate was varied over the concentration range 0.03−2.5 mM. A fit of the data to eq 1 provided the kinetic constants of 0.61 mM and 1.1 s^{-1} for K_m and k_{cat}, respectively.

Growth of GpdQ+ Cells on Organophosphates/Phosphonates. Cells expressing GpdQ were tested for their ability to grow on several organophosphate diesters and organophosphonate monoesters under phosphate-limiting conditions (compounds **9**−**15**). Figure 3 shows a graphical representation for the growth of GpdQ+ cells in the presence of various compounds at an initial concentration of 1.0 mM. As positive and negative controls, GpdQ+ cells were grown in the presence and absence of 1.0 mM potassium phosphate, respectively. *E. coli* cells lacking the diesterase gene were unable to utilize any of the substrates as a source of phosphorus and were able to grow only when the minimal medium was supplemented with inorganic phosphate or methyl phosphonate (data not shown). The GpdQ+ cells were able to grow on six of the seven substrates tested. As anticipated, the growth rate was proportional to the catalytic efficiency of GpdQ toward each substrate. For example, cells growing on *O*-isopropyl methyl phosphonate (**12**) as the phosphorus source experienced a lag time of nearly 3 days. The value of k_{cat}/K_m for this substrate is 0.5 M^{-1} s^{-1}. In contrast, cells growing on *O*-isobutyl methyl phosphonate (**13**) displayed a lag phase of approximately 30 h, while the corresponding value of k_{cat}/K_m for this compound is 33 M^{-1} s^{-1}. Not surprisingly, the soman analogue (**14**) did not support the growth of GpdQ+ cells, which is consistent with the inability of GpdQ to hydrolyze this substrate using in vitro assays.

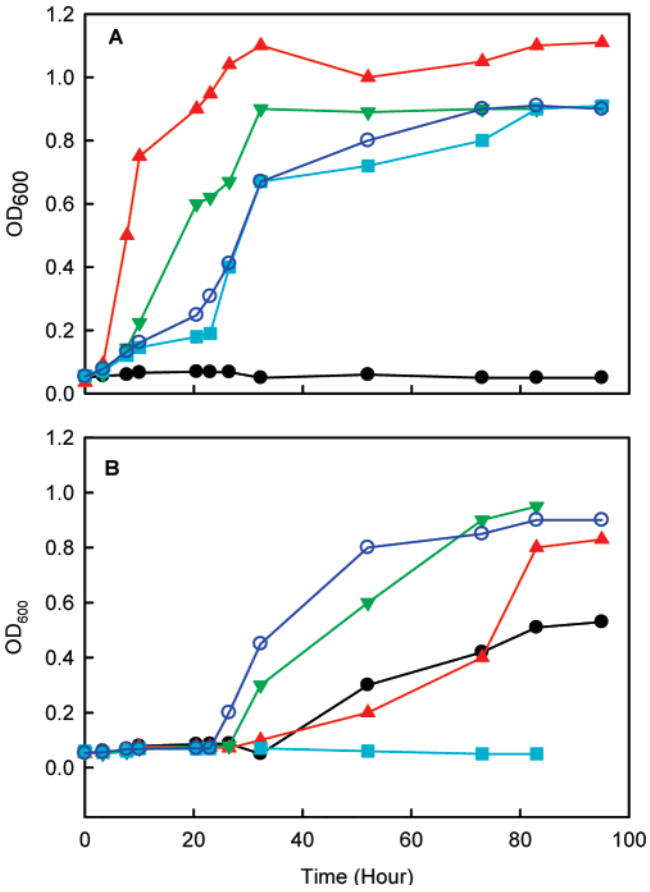


FIGURE 3: Growth of GpdQ+ cells on various compounds in the phosphate-limiting conditions. (A) The added compounds are inorganic phosphate (▲), methyl phosphonate (▼), dimethyl phosphate (■), diethyl phosphate (○), and no phosphate source (●). (B) The added compounds are ethyl methyl phosphonate (●), isopropyl methyl phophonate (▲), isobutyl methyl phosphonate (▼), pinacolyl methyl phosphonate (■), and cyclohexyl methyl phosphonate (○). The substrate concentration was 1.0 mM in phosphate-free MOPS minimal media, pH 7.4.

Effect of Various Phenols on the Growth Rate of E. coli. Since the in vivo hydrolysis of specific organophosphate triesters (compounds **17**−**19**) releases a phenolic product in the growth culture, *E. coli* was tested for its tolerance to the presence of different phenols (Figure 4). Of the three phenols tested, *p*-nitrophenol was the most toxic to *E. coli* and significantly inhibited the growth of these cells. It has been previously demonstrated that *p*-nitrophenol suppresses bacterial growth rate (*25*). In contrast, *p*-chlorophenol and *p*-hydroxyacetophenone displayed a slight inhibitory effect. Therefore, organophosphate triesters with a *p*-hydroxyacetophenone leaving group are more suitable for the in vivo selection compared to substrates containing *p*-nitrophenol.

Growth of E. coli Coexpressing PTE and GpdQ. The ability of *E. coli* cells that coexpress PTE and GpdQ to grow on the organophosphate triesters **24** and **25** as the sole phosphorus source was tested. The kinetic constant k_{cat}/K_m for the hydrolysis of **24** by wild-type PTE is 2×10^6 M^{-1} s^{-1}. The time course for the hydrolysis of the rVX analogue (**25**) by wild-type PTE is biphasic due to the differential rates of hydrolysis for the R_P- and S_P-enantiomers. The measured

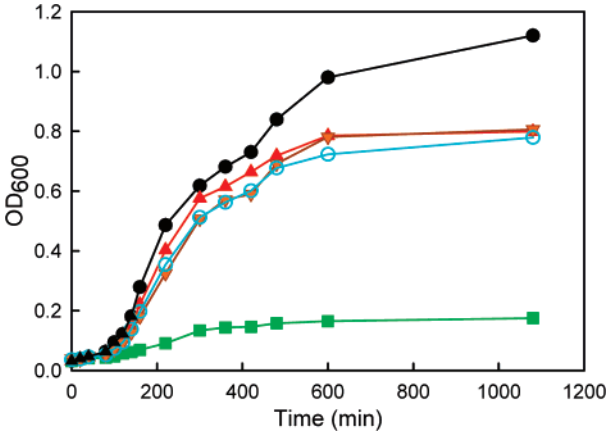


FIGURE 4: Time course for the growth of *E. coli* BL21(DE3) in the presence of added phenols. The phenols were *p*-nitrophenol (■), *p*-chlorophenol (▼), and *p*-hydroxyacetophenone (○). As a control, the cells were grown in the absence of added phenol (●) and in the presence of 1% methanol (▲). The concentrations of the phenols were 1.0 mM.

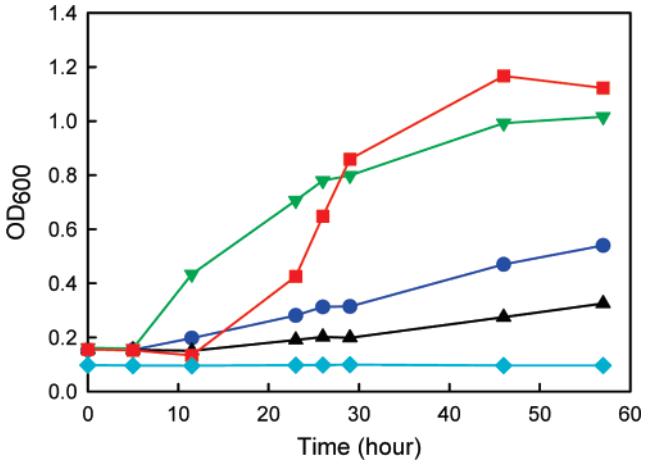


FIGURE 5: Time courses for the growth of GpdQ+/wild-type PTE in the presence of **24** (●), GpdQ+/wild-type PTE in the presence of **25** (▲), GpdQ+/pET20b-5S in the presence of **24** (▼), GpdQ+/pET20b-5S in the presence of **25** (■), and GpdQ+ cells with no additional phosphorus source (◆). The substrate concentration was 1.0 mM in phosphate-free MOPS minimal medium, pH 7.4.

values of k_{cat}/K_m for the R_P- and S_P-enantiomers of the racemic mixture for compound **25** are 6×10^5 M^{-1} s^{-1} and 1.3×10^4 M^{-1} s^{-1}, respectively. GpdQ+-containing cells were transformed with the wild-type PTE and the K185R/D208G/R319S mutant. A graphical representation of the growth rates under various conditions is shown in Figure 5. The length of the lag time for *E. coli* growing under phosphate starvation is dependent on the rate of hydrolysis of the triester substrates by PTE. The substantial difference in the net protein expression levels between the wild-type PTE and the K185R/D208G/R319S mutant was recognizable in the growth rate as the cells expressing the K185R/D208G/R319S mutant grew faster than the wild-type PTE in the presence of either the paraoxon analogue (**24**) or the rVX analogue (**25**). In addition, the 10-fold difference in the values of k_{cat}/K_m for the hydrolysis of compounds **24** and **25** influenced the apparent rate of formation of the diester product and the subsequent growth rate. With either the wild-type PTE or the K185R/D208G/R319S mutant, the *E. coli* cells grew faster in the presence of **24** than **25**.

DISCUSSION

Substrate Specificity of GpdQ. When applicable, in vivo selection is a powerful tool for identifying mutants with enhanced catalytic activity in directed evolution experiments. The use of PTE coexpressed with GpdQ to enable *E. coli* to utilize organophosphate triesters as the sole phosphorus source is potentially limited by the substrate promiscuity of the diesterase. Therefore, the substrate specificity of GpdQ was determined using a series of structurally diverse organophosphate diesters and phosphonate monoesters. Consistent with previously published data (*9*, *11*), the predominant activity of GpdQ is as a diesterase with relatively minimal monoesterase activity. GpdQ displayed the best overall activity toward the hydrolysis of bis-*p*NPP with a k_{cat}/K_m value of 6.7×10^3 M^{-1} s^{-1}. It is not unusual for bacterial diesterases to be able to hydrolyze bis-*p*NPP (*26*, *27*). A comparison of the k_{cat}/K_m values for *p*-nitrophenyl phosphates with their corresponding *p*-nitrophenyl phosphonates shows that GpdQ preferentially hydrolyzes the phosphate diesters over the phosphonate monoesters. For example, the k_{cat}/K_m value for the hydrolysis of *O*-phenyl *p*-nitrophenyl phosphate (**7**) is an order of magnitude larger than the k_{cat}/K_m for phenyl *p*-nitrophenyl phosphonate (**8**). The same trend is observed when comparing the k_{cat}/K_m values for *O*-ethyl *p*-nitrophenyl phosphate (**2**) with ethyl *p*-nitrophenyl phosphonate (**3**). A direct comparison for the alkaline hydrolysis of compounds **2** and **3** with KOH indicated that the phosphonate ester is eight times more reactive than the corresponding phosphate ester (data not shown).

The catalytic efficiency for the hydrolysis of alkyl phosphodiesters and phosphonate monoesters is significantly lower when compared to the substrates containing a *p*-nitrophenol leaving group. For example, the k_{cat}/K_m for the hydrolysis of diethyl phosphate (**10**) is a relatively low value of 15 M^{-1} s^{-1}. However, it is sufficient to support the growth of *E. coli* under phosphate-limiting conditions. The dependence of growth rate on the turnover of relatively slow enzymatic transformations has been observed previously. In their efforts to better understand the role of substrate promiscuity in divergent evolution, Gerlt and co-workers designed the D297G mutant of the L-Ala-D/L-Glu epimerase (AEE) to catalyze the reaction of *o*-succinylbenzoate synthase (OSBS) (*28*). OSBS is required for the biosynthesis of the electron transfer cofactor menaquinone, which is required for anaerobic growth of *E. coli*. Although the OSBS activity of the D297G mutant was significantly low ($k_{cat} = 0.013$ s^{-1} and $k_{cat}/K_m = 7.4$ M^{-1} s^{-1}), it enabled an OSBS-deficient *E. coli* strain to grow under anaerobic conditions.

GpdQ is thought to be the first enzyme isolated with the ability to hydrolyze alkyl diesters. A second enzyme that was isolated from *Delftia acidovorans* (PdeA) was shown to enable *E. coli* to utilize diethyl phosphate as the sole phosphorus source (*29*). The enzyme had a k_{cat}/K_m value of 5.0×10^3 M^{-1} s^{-1} for the hydrolysis of bis-*p*NPP (*30*). The *D. acidovorans* diesterase was used to construct a synthetic operon composed of PTE, PdeA, and alkaline phosphatase to enable *Pseudomonas putida* to utilize paraoxon as the sole phosphorus and carbon source (*25*). The second product from paraoxon hydrolysis, *p*-nitrophenol, was degraded using the gene products of a 13.8 kb region cloned from *Pseudomonas* sp. strain ENV 2020 (*25*).

GpdQ was also able to utilize methyl phosphonate monoesters as substrates to support the growth of *E. coli* under phosphate-starvation conditions. This is the first reported activity of GpdQ with this class of compounds. The highest catalytic efficiency ($k_{cat}/K_m = 33\ M^{-1}\ s^{-1}$) was obtained for *O*-isobutyl methyl phosphonate (**13**), which is the phosphonate monoester hydrolysis product of the nerve agent rVX. GpdQ did not possess any measurable activity toward the hydrolysis of compound **14**, which is the phosphonate hydrolysis product of the nerve agent soman.

Initial characterization of GpdQ by Ollis and colleagues indicated that the enzyme hydrolyzes methyl demeton-S (**20**) with a 10-fold lower specific activity than bis-*p*NPP (**1**) at pH 8.0 (*10*). The findings of our investigation strongly suggest that the previously observed hydrolysis of methyl demeton-S is in fact due to the presence of a substantial thiodiester contaminant in the commercial material and that the enzyme does not actually hydrolyze methyl demeton-S. The apparent activity is due entirely to the thiodiester contaminant (**21**). In support of this argument, we synthesized compound **27** and subsequently tested its hydrolysis by GpdQ. The k_{cat}/K_m value for the hydrolysis of **27** is $2.2 \times 10^3\ M^{-1}\ s^{-1}$, comparable to the k_{cat}/K_m value obtained for the hydrolysis of the diester contaminant in the commercial methyl demeton-S (Figure 2B). Furthermore, we tested GpdQ for the hydrolysis of compound **22**, the *O*-ethyl analogue of EA 2192, the principal and most toxic degradation product of the nerve agent VX at pH 8.0 (*17*). GpdQ displayed a k_{cat}/K_m value of $1.8 \times 10^3\ M^{-1}\ s^{-1}$ for the hydrolysis of **22**. As observed from the substrate specificity profile of GpdQ, the enzyme preferentially hydrolyzes methyl-substituted diesters. Therefore, the enzymatic hydrolysis of EA 2192 (**23**) is anticipated to occur at approximately a 2-fold higher rate than observed with compound **22**.

In Vivo Growth Assays. E. coli BL21(DE3) cells expressing GpdQ were examined for their ability to utilize various organophosphate diesters and methyl phosphonate monoesters as an alternate source for inorganic phosphate. The uptake of dimethyl phosphate and diethyl phosphate is mediated by the bacterial outer-membrane pore protein, PhoE. In *E. coli*, PhoE is expressed under phosphate-starvation conditions and is specific for the transport of negatively charged compounds such as phosphates and polyphosphates (*31*). As anticipated, the observed growth rate of these cells was faster for substrates with the higher rates of hydrolysis by GpdQ. For example, cells growing on *O*-isopropyl methyl phosphonate (**12**) showed a longer lag time than cells growing on *O*-diethyl phosphate (**10**) or *O*-isobutyl methyl phosphonate (**13**). Similarly, compound **14** did not support the growth of *E. coli* since it is not a substrate for GpdQ. The lag time of bacterial growth can be related to the bioavailability of the growth limiting factor. Keasling and co-workers demonstrated that a genetically engineered strain of *P. putida* growing in the presence of 1 mM paraoxon as the sole phosphorus source exhibited a lag time of approximately 40 h. Supplementing the growth medium with 1 mM K_2PO_4 significantly reduced the lag time to 20 h (*25*). The correlation between bacterial growth rate and catalytic efficiency was previously reported by Ollis and co-workers in their attempt to enhance the overexpression of OpdA in *E. coli* using the GpdQ-dependent in vivo selection strategy. When methyl paraoxon was utilized as the sole phosphorus source, cells coexpressing GpdQ with OpdA grew to ~1 mm after 2−3 days, while cells coexpressing GpdQ with PTE grew ~0.2 mm after the same period of time (*32*). The k_{cat}/K_m value of purified OpdA is 12-fold higher than PTE for the hydrolysis of methyl paraoxon (*33*).

The results from the in vivo assays for cells coexpressing GpdQ with PTE appear promising. Two variables were considered: (i) the intracellular concentration of PTE and (ii) the catalytic efficiency of PTE toward the hydrolysis of the substrates tested. When GpdQ was coexpressed with PTE, it was clear that a faster growth rate was obtained for the faster substrate, compound **24** relative to compound **25**. In the case of pET20b-5S, the net overexpression of the PTE mutant is enhanced by greater than an order of magnitude (*22*). This difference in the net intracellular concentration of PTE was evident as a significantly shorter lag time for the growth on compound **24** was observed. A comparison of the growth rates for GpdQ+/pET20b-5S cells using compounds **24** and **25** clearly demonstrates that the lag phase is dependent on the catalytic activity of PTE. This bacterial construction is currently being used to identify mutants of PTE that are enhanced in their ability to recognize and hydrolyze the most toxic of the organophosphate triesters and the organophosphonate diesters.

In conclusion, the *E. aerogenes* diesterase exhibits broad substrate specificity and enables *E. coli* to utilize methyl phosphonate monoesters as the sole phosphorus source. The methyl phosphonate esters represent the primary hydrolysis product of the corresponding PTE substrates. This investigation demonstrated that GpdQ exhibits minimal activity toward the hydrolysis of the phosphorothiolates, methyl demeton-S and ethyl demeton-S. In addition, the first enzymatic hydrolysis of a close analogue of EA 2192, the most toxic degradation product of the nerve agent VX, is described.

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SUPPORTING INFORMATION AVAILABLE

The synthetic schemes and physical constants for the preparation of compounds **2**−**9**, **17**−**22**, and **24**−**27**. This material is available free of charge via the Internet at http://pubs.acs.org.

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