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Structural basis for thermostability revealed through the identification and characterization of a highly thermostable phosphotriesterase-like lactonase from *Geobacillus stearothermophilus*

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Abstract

A new enzyme homologous to phosphotriesterase was identified from the bacterium *Geobacillus stearothermophilus* (GsP). This enzyme belongs to the amidohydrolase family and possesses the ability to hydrolyze both lactone and organophosphate (OP) compounds, making it a phosphotriesterase-like lactonase (PLL). GsP possesses higher OP-degrading activity than recently characterized PLLs, and it is extremely thermostable. GsP is active up to 100°C with an energy of activation of 8.0 kcal/mol towards ethyl paraoxon, and it can withstand an incubation temperature of 60°C for two days. In an attempt to understand the thermostability of PLLs, the X-ray structure of GsP was determined and compared to those of existing PLLs. Based upon a comparative analysis, a new thermal advantage score and plot was developed and reveals that a number of different factors contribute to the thermostability of PLLs.

Keywords

Phosphotriesterase-like lactonase; Arrhenius; thermophile; extremophile; activation energy; bioremediation; thermal advantage score

Studies on enzyme and protein stability are ongoing and often involve investigations into which structural factors have the greatest effects on thermostability. These studies all employ

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Electronic Supplementary Materials

The plot constructed from the calculated B' values of the 4 enzymes analyzed in this work is included in the supplementary material in order to show which enzymes have the most amino acids with side-chain B' values equal to or greater than 2.

different strategies for analysis. For example, extensive comparisons of families of enzymes containing both mesophiles and thermophiles have been conducted, and trends which positively impact the most families have led to the conclusions that high polar surface area, a high number of hydrogen bonds, and greater side-chain branching are beneficial for thermostability [1; 2]. Additionally, primary sequence alignments of mesophiles and thermophiles within the same enzyme family have been analyzed in order to assess changes in sequence and overall amino acid composition that may have evolved to allow enzymes to adapt to different climates. These studies have indicated that an increase in charged residues and a decrease in polar, uncharged residues are beneficial for thermostability [3]. Site-directed mutagenesis has also been used to replace residues at influential positions in enzyme structures in order to determine the contribution of that residue to stability and such studies have concluded that prolines are beneficial and glycines are detrimental to thermostability [4], and that negatively charged residues and prolines positioned at the beginning of helices are both beneficial for stability [5; 6].

In any detailed study, it is important that a sampling of enzymes with various levels of thermostability is analyzed. To increase the accuracy of a comparison, the enzymes would ideally have similar functions. The phosphotriesterase from *Pseudomonas diminuta* (PTE) is the most widely studied and characterized organophosphate (OP)-degrading enzyme, catalyzing the hydrolysis of the OP pesticides ethyl and methyl paraoxon (Fig. 1A–B) with near diffusion rates [7]. However, because OP compounds are synthetic, clues about the natural function of PTE have been sparse until recently when it was demonstrated to have promiscuous lactone-degrading activity [8]. Following this discovery, other homologous enzymes such as that from *Deinococcus radiodurans* (Dr-OPH) and *Sulfolobus solfataricus* (SsoPox) have been isolated and shown to efficiently catalyze the hydrolysis of lactone compounds with a promiscuous OP-degrading activity [9; 10], placing them in a newly emerging group of enzymes termed phosphotriesterase-like lactonases (PLLs). It has been hypothesized that phosphotriesterase enzymes evolved from lactonases, however, so far there have been no studies directed at establishing the phenotypes of OP-degrading enzyme knockout strains that would directly support that the lactonase activity observed is indeed the natural function of the enzyme. One of the benefits of having enzymes possessing this dual substrate specificity is the potential for their development into practical applications involving both remediation of OP-based toxic compounds and inhibition of quorum-sensing, a process that relies on lactone signaling molecules. The ideal enzymes to be used for these practical applications possess high levels of solubility and thermostability [11].

We have identified and characterized an enzyme homologous to PTE from *Geobacillus stearothermophilus* strain 10 (GsP) and shown that it possesses a level of thermostability that greatly surpasses that of PTE. Additionally, we have characterized its hydrolytic activity toward OP compounds and a sampling of lactone compounds (Fig. 1C – E) in order to determine if it contains the same dual activity as known PLL enzymes. By determining the X-ray structure of GsP and comparing its primary sequence (Fig. 2) and structure to homologous PLLs, we developed a comparative ranking system of the structural features of each enzyme that are predicted to contribute to the thermostability of the PLL family, utilizing previously determined trends in thermostability. This information can be used in the future to engineer enzymes for better use in practical applications.

Materials and Methods

Ethyl paraoxon (diethyl-p-nitrophenyl-phosphate), methyl paraoxon (dimethyl-p-nitrophenyl-phosphate), and demeton-S (S-2-ethylthioethyl O,O-dimethyl phosphorothioate) were purchased from ChemService. N-hexanoyl homoserine lactone was purchased from Caymen Chemical. γ -nonanoic lactone and δ -undecanoic lactone were purchased from Sigma-Aldrich. All other chemical reagents were purchased from Sigma-Aldrich or Fisher Scientific.

Cloning and expression of GsP

A gene encoding a homologue of PTE was discovered in the genome *Geobacillus stearothermophilus* strain 10 [12; 13; 14; 15]. This strain was obtained from the Bacillus Genetic Stock Center (BGSC Accession No. [9A21](https://www.bgsc.org); www.bgsc.org). The oligonucleotides 5'-AACGTTCCATGGCTAAAACGTGCGAAACCGTCCTTG-3' and 5'-CTTGCAATCTAGATCAGTGATGGTGATGGTGATGAGCCGAGAACAGCG-3' were utilized to amplify the gene by PCR from whole cells. In addition to placing *NcoI* and *XbaI* restriction endonuclease sites upstream and downstream of the OPH gene, the PCR process directly fused a C-terminal (his)₆-tag coding sequence to the gene. The (his)₆-tag gene was digested with *NcoI* and *XbaI* and inserted into a pBR322-based vector in which the ampicillin resistance marker has been replaced with the neomycin resistance marker from pACYC177, creating pLybAC11 for expression in *E. coli* [16; 17]. Expression is driven from the *E. coli* rhamnose (*rhaBAD*) promoter (which is inducible with 0.2% L-rhamnose) and λ cII ribosome binding site [18; 19]. Upon induction, GsP was expressed to a level that was one-quarter of the total soluble protein.

Purification of GsP

The plasmid bearing the GsP gene was transformed into *E. coli* strain BW25113. A flask containing 2xYT media supplemented with 50 mg/mL kanamycin and 1 mM CoCl₂ was inoculated and allowed to grow for 4 h at 37°C. The culture was then transferred to a 1 L fed batch fermentor, in the same media, at pH 6.8. The fermentor was set at 37°C with agitation at 800 rpm and air flow of 1 volume/min. The pH was maintained by addition of NH₄OH or feed (10% glycerol, 10g/L yeast extract pH 5.5). The cells were induced by addition of 0.01% rhamnose once the OD₆₀₀ reached 15. Following fermentation, the cells were harvested by centrifugation and frozen at -80°C until used.

Frozen cell pellets (100 g) were thawed in 400 mL dilution buffer (50 mM Tris-HCl, pH 8.0, 100 μ M CoCl₂) supplemented with 0.01 mg/mL lysozyme. The solution was stirred at room temperature for 30 min and the cells were ruptured using a Parr pressure cell equilibrated at 1,500 psi with N₂ for 30 min (Parr Instrument Co.). The lysate was clarified by centrifugation and the volume of supernatant brought to 1 L with dilution buffer. The diluted supernatant was then applied to a 500 mL column of DEAE cellulose equilibrated with dilution buffer. The column was washed with 3 volumes of dilution buffer and the protein was eluted with 3 volumes of a linear gradient from 0 – 400 mM buffered KCl. Fractions containing OPH activity eluted between 230 and 250 mM KCl and were pooled. The pooled fractions were adjusted to 500 mM KCl and diluted by 3 volumes with loading buffer (50

mM Tris, pH 8.0, 100 μ M CoCl₂, 10 mM imidazole, and 500 mM KCl). The protein solution was applied to 100 mL of affinity resin loaded with Co²⁺ ions and equilibrated with loading buffer (GE Healthcare). The column was washed with 4 volumes of loading buffer and eluted with a linear gradient from 10 to 500 mM imidazole in loading buffer. Fractions with OPH activity were collected, pooled, dialyzed, and concentrated using a tangential flow dialysis membrane in dilution buffer. The dialyzed, concentrated protein was stored at 4°C until used. Fractions were analyzed using SDS-PAGE, which showed that the protein was purified to over 95%. The appearance of a band around 36 kDa (MW = 36,360) indicated the presence of GsP. Protein concentration was estimated by the Bradford method [20].

Kinetic characterization of GsP toward OP compounds

In order to determine kinetic parameters for GsP, kinetic measurements were performed in triplicate with all substrates by varying substrate concentrations from 0 to 10 mM. Before initiation of the reaction, the enzyme was incubated on ice with 500 μ M CoSO₄ for 15 min. Activity determination in the presence of various divalent metals showed that, while the active site of GsP can accommodate, i.e. be activated by, the same spectrum of divalent metals as PTE, like PTE, the activity of GsP is highest with Co²⁺ present in the active site [21] (data not shown). Therefore, Co²⁺ was used in all assays and for determination of kinetic parameters. A total reaction volume of 200 μ L was prepared in a 96-well plate (path length = 0.44 cm). The final concentration of enzyme in the reaction mix was held constant at 7 μ M. Control reactions were performed with no enzyme present to monitor the non-enzymatic background hydrolysis rates of the substrates that were subtracted from the enzymatic rates. All assays were performed on a SpectraMax 384 Plate Reader at a constant temperature of 35°C over a total elapsed time of 45 min.

Kinetic assays with ethyl and methyl paraoxon were conducted in a final concentration of 50 mM CHES, pH 9.0, and the rates of the reactions were monitored by the increase in absorbance at 400 nm due to the release of the product *p*-nitrophenol (extinction coefficient = 17 mM⁻¹cm⁻¹). Activity of demeton-S was monitored in 50 mM HEPES, pH 7.2, with a final concentration of 1 mM 5,5'-dithiobis(2-nitrobenzoate) (DTNB). DTNB reacts with free thiols formed during the breakdown of demeton-S and the product, 5'-thiol-2-nitrobenzoate, can be monitored at 412 nm with an extinction coefficient of 14.15 mM⁻¹cm⁻¹.

Specific activity and k_{cat} values were calculated using the protein concentration determined from the Bradford method. Michaelis-Menten plots were used to determine the kinetic parameters, V_{max} and K_{m} , using Equation 1 to fit the data using non-linear regression and the Enzyme Kinetics module of SigmaPlot (SYSTAT Software, Inc.).

$$\text{rate} = \frac{k_{\text{cat}} [E]_t}{1 + \frac{K_{\text{M}}}{[S]}} \quad (1)$$

A unit of activity is defined as one μ mol of product produced per min.

Kinetic characterization of GsP toward lactones

It has previously been shown that hydrolysis of lactone compounds by OPH occurs via a ring-opening [9; 22]. The assay used for determination of kinetic parameters toward these compounds relies on the fact that a proton is released during this ring-opening at pH values above the pK_a of the carboxylic acid. The OD_{577} of the pH indicator, cresol purple ($\epsilon = 47,300$ at 577 nm), decreases with decreasing pH, providing a direct method for monitoring proton release over time.

The protocol for this assay was adopted from Chapman and Wong [23]. Reactions were conducted in 96-well plates with each well containing final assay concentrations of 0.1 M NaCl, 1 M $CaCl_2 \cdot 2H_2O$, 2.5 mM Bicine, pH 8.3, and 0.3 mM Cresol Purple dissolved in DMSO. The assay was conducted in triplicate, varying substrate concentrations over the appropriate range. Each lactone compound was dissolved in DMSO and stock solutions were made in order to add a constant amount, 2 μ L, to each well, to ensure the amount of solvent was kept constant at 1% in each reaction. The reaction was initiated by addition of the enzyme, diluted in 50 mM Tris, pH 7.5, to a final assay concentration of 40 μ M for N-hexanoyl homoserine lactone, 1.7 μ M for γ -nonanoic lactone and 350 nM for δ -undecanoic lactone. Background hydrolysis was measured in a reaction with no enzyme present. The absolute value of the slope obtained for hydrolysis of lactone compounds was determined and used to calculate the velocity at each substrate concentration. The data were plotted in Michaelis-Menten format in order to obtain kinetic parameters as described earlier.

Thermostability of GsP

GsP was incubated at 60°C in the presence of 500 μ M $CoSO_4$ for time points ranging from 1 min to 24 h. After each time point, the remaining enzyme activity was measured using methyl paraoxon as a substrate as described above. The activity was determined at 25°C in order to accurately compare to studies performed previously with *Df*-OPH [24]. GsP was used at a final assay concentration of 7 μ M. Methyl paraoxon was used at concentrations varying from 10 to 150 μ M. Specific activity after 1 min of incubation was normalized to 100%, and values for each time point were plotted as the percent residual activity. Enzymatic activity at each time point was measured in triplicate and these values were used to calculate the standard deviation for each point.

Heat inactivation of GsP

GsP was incubated in the presence of 500 μ M $CoSO_4$ for 15 min at temperatures ranging from 25°C to 90°C. The enzyme was then cooled on ice and assayed at 25°C as described above. The final concentration of GsP used in the assays was 7 μ M. Activity of GsP after incubation at 25°C was normalized to 100% and these values at each temperature were plotted as percent residual activity. Enzymatic activity at each temperature was measured in triplicate and these values were used to calculate the standard deviation for each point.

Energy of activation of GsP

An aliquot of 56 μ M GsP was incubated on ice in the presence of 500 μ M $CoSO_4$. Assays were performed in triplicate using ethyl paraoxon as a substrate at concentrations ranging from 0 to 9 mM, and temperatures ranging from 40°C to 100°C. The assay mixtures were

allowed to pre-incubate for 5 min at the desired temperature, and the reactions were initiated by the addition of GsP taken directly from the ice. The total reaction volume was 1 mL and the final assay concentration of GsP was 2.5 μM . The background rate of hydrolysis of ethyl paraoxon at each temperature was measured and subtracted from the enzyme catalyzed rate. This data were then fit to the Michaelis-Menten equation (Equation 1) in order to determine the turnover number, k_{cat} , at each temperature. The k_{cat} values were then plotted in an Arrhenius plot as $\ln(k_{\text{cat}})$ versus $1/T$ where k_{cat} is in units of min^{-1} and T is the temperature in units of degrees Kelvin. From the Arrhenius equation (Equation 2)

$$\ln k = \frac{-E_a}{RT} + \ln A \quad (2)$$

The energy of activation, E_a , for the enzyme catalyzed reaction was then calculated from the resulting slope of the plot which is equal to $-E_a/R$ with the ideal gas constant, R , taken as $1.987 \text{ cal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$. Standard deviations were calculated from the triplicate values measured at each temperature.

Melting temperature of GsP

A MicroCal VP-DSC differential scanning calorimeter with 500 μL cells was used to determine the melting temperature of both OPH enzymes. The scan rate was $90^\circ\text{C}/\text{h}$ and a temperature range of 10°C to 125°C was scanned. A post-scan equilibration for 3 min at 125°C was conducted before the cells were cooled and equilibrated at 10°C for 15 min. Each sample was degassed for 5 min before being loaded into the cells. Control scans of phosphate buffer only were conducted prior to the scans with proteins in order to create a thermal reference for the machine. Once these reference scans were complete, only the sample cell was emptied and filled with degassed GsP. The enzyme was diluted to 0.5 mg/mL in 60 mM phosphate, $\text{pH } 7.0$. After each scan, the cells were washed out with degassed phosphate buffer in order to eliminate residual enzyme or precipitate which may have formed. Three different protein samples were used to perform individual scans. Data were analyzed by subtracting reference buffer scans from those of the enzyme sample and integrating from a progress baseline using the program Origin [25].

Crystallization of GsP

Crystallization trials were carried out at 16°C using the vapor diffusion method in hanging drop format with 4 μL drops. Each drop contained 2 μL of protein at a concentration of either 10 or 20 mg/mL and 2 μL of the 750 μL well solution. Coarse screening was carried out using Hampton Research Index, Crystal, Cryo, PEG/Ion, Salt, and Lite Screens as well as the Nextal MPD Screen. Out of a total of ~ 500 initial conditions, 5 hits were found and optimized by changing precipitant and cryoprotectant concentrations, as well as pH . A data set was collected on a crystal that formed in a condition found through optimization: 0.25 M ammonium acetate, 0.2 M sodium citrate, $\text{pH } 5.0$, 30% w/v PEG 4000. The crystal formed in a final concentration of 10 mg/mL *GsP* and was soaked in a cryoprotectant solution containing 0.3 M ammonium acetate, 0.2 M sodium citrate, $\text{pH } 5.0$, and 20% PEG 4000.

Data Processing and Refinement of GsP

Data collection was carried out at SER-CAT (sector 22) of the Advanced Photon Source at Argonne National Laboratory on the BM line with a Mar 225 detector. A 210° data set was collected at a wavelength of 1.0 Å with a 2 s exposure per 0.5° oscillation. Data were processed and scaled using HKL2000 [26]. Initial phases were obtained by molecular replacement using PHASER [27; 28] with *Dr*-OPH as the search model. GsP and *Dr*-OPH share 53.3% sequence identity. Positional and B-factor refinements were performed iteratively with the program CNS [29], using a maximum-likelihood target function and structure factor amplitudes. Manual rebuilding was performed with the program O [30]. All refinement steps were monitored by comparing R_{cryst} and R_{free} with 5% cross-validated data. Metal ions, water, and glycerol were modeled using an omit map contoured at 3σ .

Calculation of factors influencing the thermostability of PLL enzymes

The factors influencing the thermostability of 4 PLL enzymes, PTE, *Dr*-OPH, GsP, and that from *Sulfolobus solfataricus* (SsoPox) were analyzed. For simplicity, calculations were only performed for the monomeric structures of the enzymes, which are all dimers. For each enzyme, the two chains of the dimer align with all-atom RMSD values equal to or less than 0.25.

The numbers of interactions for each protein were calculated using their crystal structures and the Protein Interaction Calculator (PIC) [31]. According to PIC criteria, the residues Ala, Ile, Leu, Met, Phe, Pro, Trp, Tyr, and Val are considered to participate in hydrophobic interactions if they fell within the 5 Å range. A donor-acceptor distance cutoff of 3.5 Å was used to assign hydrogen bonds and the residues Arg, Asp, Glu, His, and Lys were considered to participate in ionic interactions if they fell within the 6 Å range. Pairs of phenyl ring centroids separated by a distance between 4.5 and 7 Å account for aromatic-aromatic interactions. Other factors influencing thermostability, such as cavity size and volume were calculated using the programs Putative Active Site with Spheres (PASS) and Voidoo [32; 33]. The PASS algorithm is designed to fill cavities in a protein structure with a set of spheres. The spheres that are the most buried account for cavities within the protein. Total enzyme volumes were calculated with Voidoo using probes with a 1.4 Å radius [33]. The polar surface area (PSA) was calculated using the program VEGA, considering O, S, N, P, and H atoms not bonded to C as polar atoms [34; 35; 36]. All other values were obtained by looking at the primary sequence or the overall structure in PyMol [37]. Radii of gyration were calculated using MOLEMAN [38].

The average B-factors for each side chain within each protein were normalized according to the method used by Parthasarathy and Murthy [39] in which B' is calculated as:

$$B' = \frac{(B - \langle B \rangle)}{\sigma B} \quad (\text{Equation 1})$$

where $\langle B \rangle$ is the average B value and σB is the standard deviation. When 0 is taken as the mean, positive B' values indicate residues with B-factors above the mean while negative B' factors indicate residues with B-factors below the mean.

Results

Cloning, expression, and purification of GsP

The phosphotriesterase gene from *G. stearothermophilus* strain 10 was over-expressed in *E.coli* and purified using a two-step procedure as described in the Materials and Methods. Using this method, we obtained 1 g of protein from 100 g of cells, and it was judged to be over 95% pure via SDS-PAGE. The purified and concentrated GsP enzyme was observed to be brown in color, in contrast to *Dr*-OPH, which has been demonstrated to have a bright purple color (Fig. 3) [9; 40] and PTE which is colorless. Like *Dr*-OPH, GsP can be concentrated to over 200 mg/mL without any noticeable precipitation [24]. Storage of GsP for after 1 month at 4°C indicates that the enzyme retains its full activity toward ethyl and methyl paraoxon (data not shown).

Kinetic characterization of GsP

The enzymatic activity of GsP towards the most widely utilized OP compounds, ethyl and methyl paraoxon and demeton-S (Fig. 1AB), was determined at 35°C in order to accurately compare to measured values for *Dr*-OPH [24], and the resulting values for the kinetic parameters are summarized in Table 1. The turnover number, k_{cat} , for the hydrolysis of ethyl paraoxon by GsP was found to be $6.9 \pm 1.1 \text{ min}^{-1}$, and the k_{cat} value for methyl paraoxon hydrolysis is $0.47 \pm 0.02 \text{ min}^{-1}$. Finally, *GsP* has a k_{cat} of $0.036 \pm 0.006 \text{ min}^{-1}$ towards demeton-S (Table 1). These values are higher than those measured for *Dr*-OPH and SsoPox [9; 10; 24], and about 4 orders of magnitude slower than those reported for PTE [7].

Additionally, kinetic parameters were determined against three lactone compounds: γ -nonanoic lactone, δ -undecanoic lactone, and N-hexanoyl homoserine lactone (Fig. 1C–E). The rates of turnover for these lactone compounds were found to be comparable to those for the OP compounds with k_{cat} values of $7.0 \pm 0.16 \text{ min}^{-1}$, $23.1 \pm 0.35 \text{ min}^{-1}$, and $0.73 \pm 0.02 \text{ min}^{-1}$, respectively. However, GsP is 10^2 to 10^5 -fold more specific toward lactone compounds, as seen with the high k_{cat}/K_m values toward these compounds. The best substrate for this enzyme was δ -undecanoic lactone with a k_{cat}/K_m of $9.3 \pm 1 \times 10^5 \text{ min}^{-1}\text{M}^{-1}$.

Time-dependent thermostability of GsP

The environmental conditions that *Geobacillus stearothermophilus* strain 10 is known to withstand suggests that its enzymes are likely thermostable. Therefore, we sought to first determine how long GsP could be incubated at high temperatures before losing activity. GsP retained 100% of its activity toward methyl paraoxon after incubation for 48 h at 60°C (Fig. 4A). In fact, the activity of GsP appears to increase after incubation at 60°C, indicating a heat induced activation of this thermophilic enzyme. Although we cannot directly ascribe this activation to specific physical process at this time, it could be do to a heat-induced conformational change that causes a transition of from an E to E* state. In addition, GsP also retained 72% of its activity after incubation at 90°C for 2 h (Fig. 4A). In contrast, *Dr*-OPH from the radiation-resistant extremeophile *D. radiodurans* loses significant activity after incubation for 3.5 h at 60°C, and shows no recordable activity after incubation at 90°C

[24]. The homologous enzyme from the hyperthermophile *S. solfataricus* (SsoPox) has denaturation half-lives of 4 h and 90 min at 95°C and 100°C [10].

Temperature-dependent heat inactivation of GsP

The ability of GsP to retain enzymatic activity after incubation for 15 min at a variety of temperatures ranging from 25°C to 90°C was tested by incubating the enzyme at the desired temperature and then measuring the remaining activity of the enzyme towards the substrate methyl paraoxon at 25°C. GsP was found to retain full activity after incubation at each of the temperatures tested (Fig. 4B). In contrast, *Dr*-OPH exhibits no detectable activity after incubation at temperatures over 70°C [24] and PTE loses activity above 40°C [41].

Energy of activation of GsP

Since GsP is considerably thermostable, we wanted to next test its enzymatic activity and determine its kinetic parameters, k_{cat} and K_m , with the substrate ethyl paraoxon over a range of increasing temperatures. The response of the enzyme to increasing concentrations of ethyl paraoxon was therefore measured at different temperatures ranging from 40° to 100°C and the data were fit to the Michaelis-Menten equation (Equation 1) to derive the kinetic parameters (Fig. 5A). The resulting k_{cat} values were observed to continually increase over the temperature range studied, while the K_m values were observed to remain virtually unchanged with an average value of approximately 1.8 mM. Therefore, the change in k_{cat} values, which also directly reflects the change in k_{cat}/K_m values with respect to the changing temperature, was plotted in Arrhenius format in Fig. 5B. The data were fit to the Arrhenius Equation (Equation 2) and a value for the energy of activation, E_a , was calculated to be 8.0 kcal/mol for the GsP catalyzed hydrolysis of ethyl paraoxon. The background rates of ethyl paraoxon hydrolysis at each temperature were also measured and the E_a was calculated to be 13.9 kcal/mol. This indicates that GsP is successful at lowering the amount of energy necessary for hydrolysis of ethyl paraoxon, thereby resulting in a rate of hydrolysis faster than the uncatalyzed reaction.

Melting temperature of GsP

Differential scanning calorimetry (DSC) was used to measure the temperature at which GsP ‘melts’ or denatures. Experiments were performed in triplicate by measuring the individual DSC curves for three separate protein samples. Integration of curves gave a melting temperature of $106.6 \pm 0.3^\circ\text{C}$ (data not shown). This value is about 20° C higher than that obtained for *Dr*-OPH under the same conditions [24]. These data indicate that the two enzymes possess very different stabilities or melting temperatures. In contrast, DSC scans for PTE showed no endothermic peaks but produced an exothermic peak at 76° C, indicating that the enzyme aggregates [41]. These data, and the results of thermal denaturation experiments using capillary electrophoresis, have established that the PTE enzyme is thermally unstable above 60° C [41].

X-ray structure of GsP

To gain a more detailed understanding into the structural basis for the thermostability of GsP, we determined its X-ray structure to 2.36 Å resolution. A summary of the data

collection and refinement statistics is presented in Table 2. X-ray coordinates have been deposited in the Protein Data Bank under PDB ID 3F4D. The apoenzyme was found to crystallize with two protein chains in the asymmetric unit resulting in a Matthews coefficient of 2.31 Da/Å³. Both protein chains were identified by molecular replacement using *Dr*-OPH (PDB ID 2ZC1) as a search model [40]. Similar to *Dr*-OPH, SsoPox, and PTE, GsP belongs to the amidohydrolase family of enzymes which utilize a binuclear metal center to catalyze the hydrolysis of a variety of substrates [42]. Enzymes in this family share the tertiary structure of an α/β barrel in which the active site is located at the C-terminal ends of the β-strands and in the loop regions connecting these strands to their subsequent helices (Fig. 6A). The active site of GsP contains the three substrate binding pockets found in other OPH enzymes: the small (F28, Y30, T70, C74, V268, W271), large (R230, I233, M236, V237, W289) and leaving group (Y100 and E103) pockets (Fig. 6B). The two metal ions in the active site of OPH have been named the α-metal, which is the more buried metal, and the β-metal, which is more solvent exposed metal [43] (Figs 6B and 7). Since GsP was purified and crystallized in the presence of Co²⁺, these ions are modeled into the active site. A water molecule is observed to bridge the active site metals and is believed to play a role in catalysis (Fig. 7) [44; 45].

The metal binding site of GsP contains a tyrosine residue at position 97 in close proximity to the β-metal, making it similar to that of *Dr*-OPH where the tyrosine directly coordinates to the β-metal (Fig. 7). The structural role of Y97 in *Dr*-OPH has previously been investigated and found to participate in a charge transfer relay with the β-metal, giving the enzyme its purple color [9; 40]. In the structure of *Dr*-OPH, this tyrosine is 2.2 Å away from the β-metal while in GsP it is 3.3 Å away (Fig. 7). This increased distance in GsP likely weakens the coordinating bond to the metal and hence alters the strength of the charge-transfer, thereby giving the enzyme more of a brown color instead of the purple color observed in *Dr*-OPH. PTE has a tryptophan residue at this homologous position (Fig. 8), and is therefore colorless.

Discussion

We have successfully demonstrated that the phosphotriesterase from *Geobacillus stearothermophilus* strain 10 (GsP) possesses activity toward both organophosphate (OP) and lactone compounds (Fig. 1), and can therefore be classified as a phosphotriesterase-like lactonase (PLL). We have also determined the X-ray structure of GsP and observed that it shares the same structural fold as the most widely studied phosphotriesterase PTE, as well as the recently characterized PLLs *Dr*-OPH and SsoPox [9; 10; 24; 40; 46] both which possess levels of thermostability higher than that of PTE. Our experimental data have revealed that GsP is a remarkably thermostable enzyme, remaining active at 60°C for 48 h, and increasing in activity from 40 to 100°C. This is comparable to the level of thermostability of SsoPox which exhibits increasing activity at temperatures up to 95°C and retains 50% activity after 1.5 h incubation at 100°C [46] and greater than the levels of PTE, which is inactive at 60°C [47], and *Dr*-OPH, which remains active for 3.5 h at 60°C and exhibits no activity above 70°C [24]. Because the X-ray structures of these 4 homologous enzymes are available (PTE, 1PSC; *Dr*-OPH, 2ZC1; GsP, 3F4D; and SsoPox, 2VC5) and the enzymes share similar substrate specificities with varying levels of thermostability, we were able to conduct a

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detailed analysis of the structural features contributing to the thermostability of this class of enzymes. As discussed earlier, a number of factors can contribute to the stability of an enzyme, including, but not limited to: intraprotein interactions, polar surface area, stability of secondary structural elements, cavity size, and amino acid composition [48; 49]. In addition, slight differences among homologous enzymes can account for differential thermostability within a superfamily [50]. For this study we used a combination of secondary and tertiary structural elements as well as primary sequence analysis (Fig. 2). Each enzyme is functional as a dimer, and the two chains of their dimers align with an all-atom, RMSD equal to or less than 0.25 Å (data not shown). Therefore, for simplicity, calculations were performed only for the monomers of the enzymes unless otherwise noted. Moreover, since future predictions of thermostability would be made based on the primary amino acid sequence of the monomer, we proceeded accordingly.

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In a protein with over 300 residues that are folded into different secondary structural elements, many networks of interactions between amino acids can form and can additively contribute to the overall stability of a protein. The number of interactions throughout each monomeric enzyme structure, as well as those at their dimer interfaces, were calculated using the Protein Interaction Calculator (PIC) [31] and the results are summarized in Table 3. An amino acid composition consisting of a low number of polar, uncharged residues, and a high number of charged residues, has been correlated to high thermostability because it suggests a greater likelihood for hydrophobic, aromatic-aromatic, and ionic interactions within the protein, all of which have been demonstrated to have positive effects on protein stability [3; 11; 51; 52; 53; 54]. The results presented in Table 3 show that SsoPox and GsP have the highest number of charged residues, and in turn, the largest number of stabilizing ionic interactions throughout their structures. In addition, both of these highly thermostable enzymes have the least amount of polar residues, and GsP has a high number of hydrophobic and aromatic-aromatic interactions. SsoPox has the largest number of overall subunit interface interactions, followed by GsP, *Dr*-OPH, and PTE, indicating stabilization of the protein in its active dimeric form.

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Besides a high number of intraprotein interactions, a large polar surface area (PSA) has been correlated to high thermostability because of its potential for interactions with solvent [11; 55; 56]. The program VEGA [34; 35; 36] was used to calculate the PSA for each protein and the results are presented in Table 3. GsP has the highest PSA value, followed by *Dr*-OPH, PTE, and SsoPox. It is interesting to note that unexpectedly, SsoPox has the lowest PSA value among the enzymes even though it is near the top in terms of thermostability. We also calculated the PSA values for each of the dimers (Table 3) and the results show a similar trend with the exception that SsoPox now has a larger PSA value than PTE, remaining below that of GsP and *Dr*-OPH, and suggesting other factors also contribute to its thermostability.

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In addition to the surface being highly polar, experimental evidence shows that the length of exposed surface loops can also dictate thermostability, with shorter loops often found in thermophilic enzymes [57; 58]. One of the most noticeable differences setting PTE apart from the other 3 phosphotriesterases is the relative size of loop 7 (Figs. 2 and 8) [24]. In GsP and *Dr*-OPH, loop 7 is 15 residues long, and in SsoPox it is 11 residues long. However, loop 7 of PTE is 26 residues long and contains a small helix that is not found in the other

enzymes. The additional length of this loop causes it to swing out as much as 11 Å further into the solvent accessible region. The differences in the lengths and orientations of this loop region have been used to form a hypothesis about the evolution of these enzymes from lactonases to OP-hydrolases [8] and can also be used to understand their relative levels of thermostability. Thermophilic enzymes have been shown to exhibit low radii of gyration which can indicate less conformational movement away from the center point [59]. The radii of gyration for loop 7 of the enzymes analyzed here were found to directly correlate with the size of this loop, being lower for the more thermostable enzymes and highest for PTE (Table 3).

The stability of another secondary structural element, the α -helices, can also affect the overall thermostability of a protein. A single mutation at the normally positive N-terminus of a helix to a negatively charged residue has been shown to increase thermostability by increasing electrostatic interactions with the helix dipole [5]. The structural analysis presented in Table 3 indicates that GsP has by far the largest number of negatively charged residues in these favorable N-terminal helix positions. Additionally, because of their rigidity, prolines at these positions have been shown to positively affect thermostability [6] and once again, GsP has the highest number of proline residues at these beneficial positions.

The importance of β -branched amino acids (Ile, Thr, Val) in protein stabilization has been emphasized because of their tighter packing capability due to the fewer side-chain conformations available [60]. A high number of these stabilizing residues has therefore been correlated to increased thermostability. However, these residues have also been shown to be detrimental to thermostability when found in α -helices [61]. Although GsP has the lowest number of these stabilizing amino acids overall, it has the lowest percentage within its helices, indicating that the majority are contributing to packing and overall stability rather than destabilization of α -helices. Thermophiles also tend to have higher alanine content in their helices [62] and the change from valine to alanine in a helix has been shown to increase thermostability [63]. As a result, a high ratio of alanine to β -branched amino acids within helices should be most beneficial for thermostability. The ratio of alanine to β -branched amino acids in helices is summarized in Table 3 and shows that GsP and PTE have the highest ratios.

Another approach for assessing the rigidity of proteins is by evaluating the atomic displacement parameters (B-factors) within the structure, as they can indicate movement and disorder around a specific structural region. The average B-factors for each side chain within each protein were normalized according to the method used by Parthasarathy and Murthy [39] in which B' is calculated by the relationship; $B' = (B - \langle B \rangle) / \sigma B$, where $\langle B \rangle$ is the average B-factor and σB is the standard deviation in B-factors. When 0 is taken as the mean, positive B' values indicate residues with B-factors above the mean while negative B' factors indicate residues with B-factors below the mean. Assessing the B' values reveals that *Dr*-OPH and PTE have more residues with B-factors well above the mean, possessing B' values of 2 and higher (Supplemental Figure S1). Thus, the higher B' values appear to correlate with the lower thermostability of *Dr*-OPH and PTE compared to the other two enzymes.

The size of cavities within a protein has also been shown to play a role in thermostability of a protein, with smaller cavities being more beneficial [64; 65]. Using the program PASS [32] to calculate the number of cavities and their volumes, we find that *Dr*-OPH has the fewest number of cavities and that PTE has the highest number of cavities taking up the highest percentage of its total volume (Table 3). The total volume for each protein was calculated using the program Voidoo [33].

Focusing on one cavity in particular, the active site, provides further insight into the increased thermostability of SsoPox, GsP, and *Dr*-OPH over PTE. Using the active sites defined by Vanhooke et al. for PTE [66], Hawwa et al. for *Dr*-OPH [24] and Elias et al. for SsoPox [10], the active site volume of each enzyme was calculated using the program PASS [32]. The calculations show that PTE has the largest volume, followed by SsoPox, GsP, and finally *Dr*-OPH (Table 3). While a larger active site can be beneficial for catalytic activity [67], it can also be detrimental to the thermostability of a protein as it decreases the propensity of side chain interactions and therefore, protein stabilization. The fact that SsoPox, GsP, and *Dr*-OPH all have smaller active site cavities than PTE could provide evidence for their lower OP-degrading activity but higher thermostability. Also noteworthy is the fact that GsP has the most β -branched amino acids in its active site, supporting tighter packing [60].

The primary sequence alone can also give an indication of the level of thermostability of an enzyme. Proline and glycine residues have been shown to have opposite effects on stability, since these are the most rigid and flexible amino acids in terms of conformational flexibility. A single amino acid change to a proline or away from a glycine has been shown to increase thermostability [4; 49; 50; 68]. The number of prolines and glycine residues for each enzyme are summarized in Table 3. It is noteworthy that both GsP and SsoPox have the lowest number of glycines indicating that they should be more thermostable. Additionally, GsP has a high number of prolines also suggesting increased thermostability. The SsoPox enzyme however has about the same number of prolines to that of PTE, indicating that this factor may not be as important for imparting the increased thermostability over PTE.

From the analysis presented above, it is clear that a number of factors can contribute to enzyme thermostability within a structural family and that attributing a single structural property to increased or decreased thermostability is not straightforward and is likely unrealistic in most general cases. Therefore, we have developed a graphical representation that compares the various factors contributing to thermostability and have applied it to the family of OP and lactone degrading enzymes analyzed here (Fig. 9). Each enzyme has been assigned a “thermal advantage score” (TAS) for each of the contributing factors. The TAS is defined as the relative value for each beneficial trait as compared to the protein with the lowest desirable thermostability, in this case the mesophilic PTE. All values from Table 3 have been normalized against PTE which is given a score of ‘1’ for each category in which a higher number is beneficial, and a score of ‘-1’ for each category in which a higher number is detrimental. Positive thermal advantage scores indicate characteristics that are favorable for high thermostability while negative scores indicate characteristics detrimental to thermostability. After PTE, *Dr*-OPH has the next highest level of thermostability, followed by the highly thermostable PLLs GsP and SsoPox.

The thermal advantage plot shows that GsP has fewer negative characteristics in every category as compared to PTE, clearly an indication of its high thermostability. SsoPox as well, has fewer negative characteristics in all categories other than β -branched amino acids in helices. In addition, GsP has a substantial advantage over the other enzymes in 4 categories: the amount of β -branched amino acids in its active site; the number of prolines at the beginning of its helices, the number of negatively charged residues at the beginning of its helices, and the number of aromatic-aromatic interactions it possesses throughout the structure. To a lesser extent, GsP has an advantage in PSA, the number of polar residues, the number of β -branched amino acids in helices, and hydrophobic interactions. SsoPox has an advantage over all three enzymes in six categories: the number of subunit interactions, the number of charged residues, the number of ionic interactions, the number of areas with high B' values, the number of glycines, and the radius of gyration of loop 7. The thermostability calculations presented in Figure 9 are therefore in very good agreement with experimental data for these four enzymes [24; 41; 46]. Our analysis utilizes previously determined trends to examine the direct effects on one enzyme family, allowing for a more in-depth comparison and determination of factors specifically influencing the thermostability of this family. The construction of a thermal advantage plot for a family of enzymes offers a general and quick approach to visually comparing enzyme thermostability, and provides a means of using the 'winning' categories to predict the features of an unknown enzymatic structure within the family. Additionally, this data can serve as a guide when mining databases for enzymes with the proper balance of activity and stability. We support previous assertions that thermostability is not based solely on one characteristic but rather the additive effect of many and observe that the two most thermostable phosphotriesterase enzymes studied to date, GsP and SsoPox, possess many of the characteristics widely accepted as common among thermostable enzymes.

While analyzing the categories in which the most thermostable enzymes have a clear advantage is valuable in determining factors that contribute to high thermostability, it is also interesting to comment on the categories in which those enzymes are at a disadvantage. As compared to *Dr*-OPH, the more thermostable GsP and SsoPox enzymes have disadvantages in the number and sizes of cavities including the active site, the number of proline residues, and the number of hydrogen bonds. However, these two enzymes exhibit increased activity over *Dr*-OPH [10; 24]. Therefore, it is apparent that enzymes within this family have developed a balance between the properties that contribute to higher thermostability and the properties that contribute to enzymatic activity, and certain structural factors shown to increase thermostability have been compromised in order to obtain increased hydrolytic activity. The fact that PTE, which is the most active OP-degrading enzyme analyzed here, has a thermal disadvantage in most categories, supports this idea. Additionally, it has previously been asserted that the high OP-degrading activity exhibited by PTE is the result of evolution from a PLL ancestor. We now see that it is possible that some of the structural features that gave the original PLL enzymes their high thermostability may have been compromised for the evolution of OP-degradation.

Since high thermostability has been linked to resistance to harsh conditions frequently used in the development of practical applications [46], with its high thermostability and OP and lactone degrading activities, GsP, may have potential for further development in industrial

applications. Additionally, the knowledge gained through the studies presented above should now serve as a guide to help direct enzyme engineering efforts aimed at improving the thermostability of different PLLs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

OP	organophosphate
GsP	phosphotriesterase-like lactonase from <i>Geobacillus stearothermophilus</i>
PTE	phosphotriesterase from <i>Pseudomonas diminuta</i>
Dr-OPH	phosphotriesterase-like lactonase from <i>Deinococcus radiodurans</i>
SsoPox	phosphotriesterase from <i>Sulfolobus solfataricus</i> ;
PLL	Phosphotriesterase-like lactonase
RMSD	root-mean-square-deviation

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Fig. 1.

GsP substrates used for kinetic studies. These include the OP compounds ethyl (R = CH₂CH₃) and methyl (R = CH₃) paraoxon (**A**) and demeton-S (**B**) and the lactone compounds γ -nonanoic lactone (**C**), δ -undecanoic lactone (**D**), and N-hexanoyl homoserine lactone (**E**).

PTE	MQTRRVVLKSAAAAGTLLGGLAGCASVAGSIGTGDRINTVRGPITISEAGFTLTHEHICG	60
Dr-OPH	-----MTAQTVTGAVAAAQLGATLPHEHVIF	26
GsP	-----MAKTIVETVLGPVPVEQLGKTLIHEHFLF	28
SsoPox	-----MRIPLVGKDSIESKDIGFTLIHEHLRV	27
PTE	SSAGFLRAWP--EFFGSRKALAEKAVRGLRRARAAGVRTIVDVSTFDIGRDVSLLAEVSR	118
Dr-OPH	GYPGYAGDVT--LGPFDHAAALASCTETARALLARGIQTVVDATPND CGRNPAFLREVSE	84
GsP	GYPGFQGDVT--RGTFREDEALRVAVEAAEKMKRHGIQTVVDPTPND CGRNPAFLRRVAE	86
SsoPox	FSEAVRQQWP---HLYNEDEEFRNAVNEVKRAMQFGVKTIVDPTVMGLGRDIRFMEKVVK	84
PTE	AADVHIVAATGLWFDPP---LSMRLR-----SVEELTQFFLREIQYGIEDTGIRAGII	168
Dr-OPH	ATGLQILCATGFYYEGEGATTYFKFR--ASLGDAESEIYEMMRTEVTEGIAGTGIRAGVI	142
GsP	ETGLNIICATGYYYEGEGAPPYFQFR--RLLGTAEDDIYDMFMAELTEGIADTGIKAGVI	144
SsoPox	ATGINLVAGTGIYIYID-LPFYFLNR-----SIDEIADLFIHDIKEGIQGTLNKAGFV	136
PTE	KVATTG-KATPFQELVLKAAARASLATGVPVTTHTAASQRDGEQQAAIFESEGLSPSRVC	227
Dr-OPH	KLASSRDAITPYEQLFFRAAARVQRETGVPIITHTQEG-QQGPQQAELLTSLGADPARIM	201
GsP	KLASSKGRITEYEKMFRAAARAQKETGAVIITHTQEG-TMGPEQAAYLLEHGADPKKIV	203
SsoPox	KIAADEPGITKDVEKVIRAAAIANKETKVPIITHSNHNNTGLEQQRILTEEGVDPGKIL	196
Loop 7		
PTE	IGHSDDTDDL SYLTALAARGYLIGLDHIPHSAIGLEDNASASALLGIRSWQTRALLIKAL	287
Dr-OPH	IGHMDGNTDPAYHRETLRHGVSIAFDRIG-----LQGMVGTPPTDAERLSVLTTL	250
GsP	IGHMCGNTDPDYHRKTLAYGVYIAFDRFG-----IQGMVGAPTDEERVRTLAL	252
SsoPox	IGHLGDTDNIDYIKKIADKGSFIGLDRYG-----LDLFLPVDK---RNETTLRL	242
PTE	IDQGYMKQILVSNWDLFGFSSYVTNIMDVM DRVNP DG-MAFIPLRVIPFLREKGV PQETL	346
Dr-OPH	LGEYADRLLLSHDSIWHWLGRPPAIP EAALPAVKDWHPLHI SDDILPDLRRRGITEEQV	310
GsP	LRDGYEKQIMLSHDTVNVWLGRPFTLPEPFAEMMNWHVEHLFVNIIPALKNEGIRDEVL	312
SsoPox	IKDGYSDKIMISHD-YCCTIDWGTAKPEYKPKLAPRWSITLIFEDTIPFLKRNNGVNEEVI	301
PTE	AGITVTNPARFLSPTLRAS	365
Dr-OPH	GQMTVGNPARLFG-----	323
GsP	EQMFIGNPAALFSA-----	326
SsoPox	ATIFKENPKKFFS-----	314

Fig. 2.

Primary sequence alignment of phosphotriesterase enzymes used in thermostability comparisons. Polar residues are highlighted in blue and charged residues are highlighted in red. Loop 7 is indicated with a bar over the sequence for PTE, GsP, and Dr-OPH and a bar under the sequence for SsoPox.

Fig. 3. Color differences between purified and concentrated GsP and *Dr*-OPH. In the presence of Co^{2+} , GsP is brown in color while *Dr*-OPH is purple in color. Pure PTE is colorless.

Fig. 4.

The effect of temperature on the stability of GsP. (A) Residual activity of GsP after incubation at 60°C and 90°C for various amounts of time. Open circles represent GsP at 60°C, and open triangles represent GsP at 90°C. (B) Residual activity of GsP after being incubated at various temperatures for 15 min.

Fig. 5.

Plots used to determine the energy of activation, E_a , for GsP catalyzed hydrolysis of ethyl paraoxon. **(A)** The effect of increasing temperature on the specific activity of GsP toward ethyl paraoxon. The top curve represents the assay conducted at 100°C and the bottom curve represents the assay conducted at 40°C. **(B)** The Arrhenius plot of $\ln k_{cat}$ vs. $1/T$. The slope is equal to $-E_a/R$ where R is the ideal gas constant ($1.987 \text{ cal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$). This data were best fit to give an E_a of 8.0 kcal/mol for GsP catalyzed hydrolysis of ethyl paraoxon.

Fig. 6.

X-ray structure of GsP (A) The overall structure of GsP colored by secondary structural elements (α -helices in red, β -sheets in yellow, loops in green) shows that it possesses the α / β fold. Metal ions are shown in gray. (B) Stereoview of the active site of GsP. Metals are shown in cyan with metal binding residues (H23, H25, Y99, Lcx145, H178, H206, D266) shown in red. Small binding pocket residues (F28, Y30, T70, C74, V268, W271) are shown in blue, large binding pocket residues (R230, I233, M236, V237, W289) are shown in green, and leaving group residues (Y100, E103) are shown in yellow.

Fig. 7.
Schematic diagram of the metal binding site of GsP.

Fig. 8. Overlay of the loop 7 regions of GsP (green), *Dr*-OPH (red), SsoPox (orange), and PTE (blue). Loop 7 of PTE is 11 residues longer than that of both GsP and *Dr*-OPH, and 14 residues longer than that of SsoPox, and also contains an extra helix. Also highlighted in this figure is the difference in the residue at position 97, which is a tyrosine in GsP, *Dr*-OPH, and SsoPox, but a tryptophan in PTE. The metal ions are found in the same relative position in each structure and are shown as spheres in the color corresponding to the enzyme.

Fig. 9.

Thermal Advantage Scores for phosphotriesterase enzymes. Thermal advantage score is defined as the relative value for each trait contributing to an enzyme's thermostability. Values from Table 3 are normalized to those of PTE, which is given a score of 1 for those traits in which a higher number is beneficial and a score of -1 for those traits in which a higher number is detrimental. Positive thermal advantage scores indicate characteristics which are favorable for high thermostability while negative advantage scores indicate

characteristics detrimental to thermostability. This graph provides a comparison of the thermal advantage scores by category for GsP, *Dr*-OPH, and PTE.

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Table 1

Summary of the kinetic parameters for GsP against the substrates ethyl and methyl paraoxon and demeton-S^a.

	Substrate	k_{cat} (min ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (min ⁻¹ mM ⁻¹)
GsP	Ethyl paraoxon	6.9 ± 1.1	2.1 ± 0.32	3.28 ± 0.28
	Methyl paraoxon	0.47 ± 0.02	0.27 ± 0.031	1.74 ± 0.08
	Demeton-S	0.036 ± 0.006	6.1 ± 1.9	0.0059 ± 0.002
	γ-nonanoic lactone	7.0 ± 0.16	0.23 ± 0.03	30.4 ± 3.0
	δ-undecanoic lactone	23.1 ± 0.35	0.025 ± .003	924 ± 100
	N-hexanoyl homoserine lactone	0.73 ± 0.02	2.7 ± 0.21	0.27 ± 0.02

^a All assays were conducted at 35° C unless otherwise noted.

Table 2

Summary of the X-ray data processing and refinement statistics for GsP

Space Group	P2 ₁
Unit cell Å (a, b, c)	47.2, 156.7, 50.2
Unit cell° (α = γ, β)	90.000, 116.207
Resolution Range Å	20 – 2.36
Number of Observations	83,314
Redundancy	3.2 (2.6) ^a
R _{merge} (%)	5.6 (22.6)
χ ²	1.2 (0.81)
I/σI	24.5 (4.1)
Completeness (%)	96.4 (79.2)
Number of Chains	2
R _{cryst}	17.0
R _{free}	24.9
FOM	0.809
Reflections in Working Set	24,326
Reflections in test set	1,233
Total number of reflections	25,559
r.m.s.d. bond lengths (Å)	0.009
r.m.s.d. bond angles (Å)	1.5
r.m.s.d. dihedral angles (Å)	23.3
r.m.s.d. improper angles (Å)	0.92
Overall mean B (Å ²)	40.8
Number of residues	648
Number of metal ions	4
Number of water molecules	510
<i>Ramachandran Plot</i>	
Favored Regions (%)	86.9
Allowed Regions (%)	13.1
Disallowed Regions (%)	0

^aValues in parenthesis represent data for the last resolution shell (2.44 – 2.36 Å).

Table 3

Values calculated for factors determining thermostability of PLL enzymes

Beneficial Trait	PTE	Dr-OPH	GsP	SsoPox
Polar Surface Area (Å ²) ^a Monomer	6468	6758	7374	5468
Polar Surface Area (Å ²) ^a Dimer	11,826	12,619	13,217	12,182
Interactions				
Hydrophobic Interactions ^a	310	274	314	280
Aromatic Interactions ^a	10	12	17	12
Hydrogen Bonds ^a	725	733	675	647
Ionic Interactions ^a	42	39	46	53
Subunit Interactions				
Hydrophobic Interactions	18	22	21	33
Aromatic Interactions	4	4	7	11
Hydrogen Bonds	42	40	54	70
Ionic Interactions	6	6	10	3
TOTAL	70	72	92	117
Secondary Structural Elements				
Negatively Charged Residues at Beginning of Helices ^a	9	11	18	10
Prolines at Beginning of Helices ^a	2	5	8	2
Ala/Ile-Thr-Val Ratio in Helices ^a	1.08	0.81	1	0.35
Radius of gyration for Loop Regions (Å ²) ^b	8.8	6.9	6.9	6.4
Residues with B' 2	15	14	6	2
Molecular Volume				
Active Site (Å ³)	1003	395	682	801
Number of Cavities ^b	73	34	58	63
Cavity Size (Å ²) (% of total volume) ^b	6259 (19)	3648 (11)	5832 (18)	4412 (13)
Amino Acid Composition				
Number of Charged Residues ^a	72	63	79	88
Number of Polar Residues ^b	72	59	46	50
β-branched amino acids				
Overall ^a	78	65	64	75
In active site ^a	1	3	4	3
In helices (% of overall) ^b	25 (32)	27 (42)	19 (30)	37 (49)
Number of prolines ^a	14	18	17	13

Beneficial Trait	PTE	<i>Dr-OPH</i>	GsP	SsoPox
Number of glycines ^b	33	34	31	23

^aHigh numbers have been shown to be beneficial for thermostability. [2; 4; 5; 6; 11; 40; 42; 43; 44; 45; 47; 54; 56; 65]

^bLow numbers have been shown to be beneficial for thermostability.[3; 4; 55; 59; 60]