Phosphotriesterase (PTE) detoxifies nerve agents and organophosphate pesticides. The two zinc cations of the PTE active centre can be substituted by other transition metal cations without loss of activity. Furthermore, metal-substituted PTEs display differences in catalytic properties. A prerequisite for engineering highly efficient mutants of PTE is to improve their thermostability. Isoelectric focusing, capillary electrophoresis and steady-state kinetics analysis were used to determine the contribution of the active-site cations Zn$^{2+}$, Co$^{2+}$ or Cd$^{2+}$ to both the catalytic activity and the conformational stability of the corresponding PTE isoforms. The three isoforms have different pl values (7.2, 7.5 and 7.1) and showed non-superimposable electrophoretic titration curves. The overall structural alterations, causing changes in functional properties, were found to be related to the nature of the bound cation: ionic radius and ion electronegativity correlate with $K_{m}$ and $k_{cat}$ respectively. In addition, the pH-dependent activity profiles of isoforms were different. The temperature-dependent profiles of activity showed maximum activity at $T \leq 35 \, ^\circ\text{C}$, followed by an activation phase near 45–48 $^\circ\text{C}$ and then inactivation which was completed at 60 $^\circ\text{C}$. Analysis of thermal denaturation of the PTEs provided evidence that the activation phase resulted from a transient intermediate. Finally, at the optimum activity between pH 8 and 9.4, the thermostability of the different PTEs increased as the pH decreased, and the metal cation modulated stability (Zn$^{2+}$-, Co$^{2+}$- and Cd$^{2+}$-PTE showed different $T_{m}$ values of 60.5–67 $^\circ\text{C}$, 58–64 $^\circ\text{C}$ and 53–64 $^\circ\text{C}$ respectively). Requirements for optimum activity of PTE (displayed by Co$^{2+}$-PTE) and maximum stability (displayed by Zn$^{2+}$-PTE) were demonstrated.

Key words: capillary electrophoresis, metalloenzyme, phosphotriesterase, protein stability, thermal unfolding.

INTRODUCTION

Enzymes that detoxify OPs (organophosphorus compounds) are of potential interest for skin protection, prophylaxis, decontamination, treatment and remediation. PTE (phosphotriesterase, EC 3.1.8.1) from Pseudomonas diminuta, a metalloenzyme that catalyses the hydrolysis of a broad range of OPs, including the chemical warfare agents [1,2], is the best-characterized PTE [3]. The rate constants of OP hydrolysis by the wild-type enzyme are high, suggesting that PTE could be utilized for detection and detoxication of nerve agents. The natural PTE is a dimeric zinc enzyme [4,5]. Interestingly, substitution of the two Zn$^{2+}$ ions in the binuclear metal centre by Co$^{2+}$, Cd$^{2+}$, Mn$^{2+}$ or Ni$^{2+}$ preserves catalytic activity, but $k_{cat}$ and $K_{m}$ are different. The Co$^{2+}$ enzyme is the most active isoform [6]. The ionic radius of cations and the distance between the two cations in the different metal-substituted PTEs are different. Although the less buried cation is at more than 7 Å from the surface of each subunit (mean radius of monomer is approx. 25 Å), its nature modulates both substrate binding and catalytic activity [7]. Highly resolved X-ray structures of metal-substituted PTEs provided information on the roles of cations in catalysis [5]. To be a leading candidate for detoxification of OPs, PTE has to be structurally modified to improve its catalytic potential. An interest in the structural constraints prompted us to study the stereochemical determinants governing the substrate specificity of PTE [8,9], and to design mutations for altering both the specificity and the catalytic rate [10].

Engineering mutants with improved efficiency and stability require detailed analysis of the protein stability. A thermodynamic analysis showed that Zn$^{2+}$-PTE is a remarkably heat-stable protein with an apparent denaturation mid-point, $T_{m} \approx 75 \, ^\circ\text{C}$. Denaturation is irreversible, leading to aggregates at high temperatures [11]. $T_{m}$ values are often determined using differential scanning calorimetry. A CE (capillary electrophoresis)-based method was proposed as an alternative approach [12]. We used CE to study the thermal stability of Co$^{2+}$-PTE; it was observed that the temperature-induced denaturation process can be described by a consecutive reaction model, including formation of an active intermediate with enhanced activity at $T \approx 45 \, ^\circ\text{C}$, followed by an inactive state ($T_{m} \approx 58 \, ^\circ\text{C}$), leading to aggregates. Thus the wild-type Co$^{2+}$-PTE was shown to display a middle-range thermostability [13]. The enzyme source, its history from bacterial expression to purification and storage and the technique used for denaturation study account for the discrepancies in the literature data. The aim of the present study was to analyse the effects of an associated metal cation on the catalytic properties and stability of PTE.

EXPERIMENTAL

Chemicals

$N,N$-Dimethylformamide as the electro-osmotic flow marker and the BCA (bicinchoninic acid) kit were obtained from Pierce (Rockford, IL, U.S.A.). Buffer components and other chemicals were of analytical grade. Water was highly purified (resistivity $\approx 18 \, \Omega \cdot \text{cm}$) on a Milli-Q apparatus (Millipore, Molsheim, France). Catalytic parameters were determined in 50 mM CHES

Abbreviations used: BCA, bicinchoninic acid; CE, capillary electrophoresis; CHES, 2-(N-cyclohexylamino)ethanesulphonic acid; IEF, isoelectric focusing; OP, organophosphorus compounds; PTE, phosphotriesterase; RMSD, root mean square deviation.

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[2-(N-cyclohexylamino)ethanesulphonic acid] buffer (pH 9). The pH-dependence of the activity was examined in 200 mM potassium phosphate/sodium tetraborate over the range pH 6–9, and for 200 mM CHES, pH 8.6–10. The temperature-dependence of activity was analysed in 200 mM boric acid/sodium tetraborate over the pH range 8–9.4. The running electrolytes for CE were 200 mM boric acid/sodium tetraborate buffers (pH 7.6–9.2) and sodium tetraborate/sodium hydroxide (pH 9.3–10.5). Buffers and samples for CE were filtered through 0.45 µm disposable filters (Schleicher and Schuell, Dassel, Germany).

PTE production, purification and controls

PTE was expressed in Escherichia coli HMS 174 (DE3) pLysS (Novagen, Madison, WI, U.S.A.) carrying the plasmid pET17b-PTEom. pET17b was from Novagen as described previously [13]. The PTEom gene (kindly provided by D. Fournier, Université de Toulouse, Le Mirail, France) was without the 29-amino-acid leader; it was found to improve significantly the enzyme expression level [14]. The metal cation was introduced by a biosynthetic method; bacterial cells were grown in a culture medium supplemented with the desired cation (final concentration 0.1 mM). Cations were added at the induction step to enhance the expression of active PTE. Subsequent steps were performed at 4 °C in 50 mM sodium borate (pH 8.5; adjusted with NaOH), containing 0.1 mM of the appropriate salt (ZnCl2, CoCl2 or CdCl2). The wild-type Zn2+,-, Co2+ - and Cd2+ -PTE were isolated from biomass by the method described in [13]. Briefly, the enzymes were purified as follows: (i) ion-exchange chromatography on Fast Flow DEAE-Sepharose (Amersham Biosciences, Uppsala, Sweden); (ii) affinity chromatography on Green 19-Agarose gel (Sigma); (iii) size-exclusion chromatography on Ultrogel AcA54 (Biosepra, Cergy-Saint-Christophe, France). The protein concentration was determined using the BCA method with BSA as the standard. Purity factor and homogeneity of PTEs were checked by measuring the specific activity, and performing SDS/PAGE and CE. Purity of the enzymes was > 95%.

Electrophoretic analysis

Using a pI calibration kit, pI of PTEs was determined by IEF (isoelectric focusing) in polyacrylamide gel over a pH gradient 3–9. Electrophoretic titration curves were performed over the wide pH range (3–9). Both techniques were performed on PhastGels using a PhastSystem apparatus (Amersham Biosciences). The protein bands in gels were stained with Coomassie Brilliant Blue or silver nitrate. PTEs were stained for activity in gels as described previously [13]. CE analyses were performed with a P/ACE 5510 system (Beckman, Gagny, France), equipped with a diode array detector and a P/ACE Station 1.21 software for system control, data collection and data analysis. This system was modified for precise temperature control over a wide range of temperatures (20–95 °C) [12]. Uncoated fused-silica capillaries (50 µm inner diameter, variable effective length) were used. Sample vials and the capillary were independently maintained thermostatically. Electrophoretic migrations were performed at constant current with the usual polarity conditions (the capillary injection terminal was next to the anode). Sequential electrophoreses were performed over the range of temperatures 25–70 °C. Before sample injection, the capillary was flushed with five times the capillary volume of the running buffer. The protein samples (2 mg · ml⁻¹), supplemented with N,N-dimethylformamide (final concentration 0.01 %, v/v), were injected hydrodynamically. Injection times were corrected for the change in viscosity of samples with temperature, using CE Expert 1.0 software (Beckman). Absorbance changes were recorded over the range 195–300 nm.

Thermal stability analysis

Irreversible protein denaturation involves at least two steps: (i) reversible unfolding of the native protein (N) and (ii) irreversible changes in the unfolded protein (U) to denatured state (D) [15]:

\[
N \xrightarrow{k_{1}} U \xrightarrow{k_{2}} D
\]

\[k_{obs} = \frac{k_{1}}{1 + 1/K_{U}} \quad \text{with} \quad K_{U} = \frac{[U]}{[N]} = \frac{k_{1}}{k_{2}}\]

The two-state and reversibility assumptions for protein unfolding apply for \( k_{i} \approx 0 \) [15,16]. Owing to rapid interconversion between the N and U states, U peaks were not well separated from N peaks. Unfolding was expected to show either a sigmoid transition or a break at \( T_{m} \) in the linear regression plot of the \( T \)-dependence of mobility [12].

Activity assay and determination of catalytic parameters

The PTE assays were performed at constant enzyme concentration ([E] = [S]), in 50 mM sodium borate buffer (pH 8.5) at 25 °C. Paraoxon as substrate (S), was used at more than ten different concentrations (0.01–1 mM). Activity was measured spectrophotometrically (Uvikon 941, Kontron, Zurich, Switzerland) by recording the increase in A₅₀⁰ due to the release of \( p \)-nitrophenol (ε₅₀⁰ = 17 000 M⁻¹ · cm⁻¹) from paraoxon. One unit of activity (U) is defined as 1 µmol of paraoxon hydrolysed per min. The steady-state parameters \( K_{m} \) and \( k_{cat} \) were determined by the Lineweaver–Burk plot using KaleidaGraph 3.6 (Synergy Software, Reading, PA, U.S.A.).

pH- and temperature-dependence of the catalytic activity of PTE

The pH profile for the rate (≈ \( V_{max} \)) of paraoxon (1 mM) hydrolysis by the three PTEs was determined in the pH range 6–10 at 25 °C. The buffers (200 mM) used were potassium phosphate/sodium tetraborate (pH 6–9) and CHES (pH 8.6–10). The apparent pKₐ values were determined from the plots of \( V_{max} \) versus pH. For heat-induced inactivation experiments, samples were heated at the desired temperature for 15 min (a time close to the CE migration time) and then cooled to 25 °C before the enzyme assay. Reversibility of the thermal inactivation was followed through the time course of activity recovery of PTE samples previously incubated at 60 °C for 15 min. Controls in which enzymes were not preincubated at 60 °C were performed.

RESULTS AND DISCUSSION

Metal content of substituted PTEs

Metal-substituted PTEs were produced by the biosynthetic method. Biosynthetic incorporation of various metal cations into the zinc sites of enzymes has been known for a long time [17,18]. The efficiency of metal substitution was studied by synchrotron radiation-induced X-ray fluorescence spectroscopy, flame atomic absorption spectroscopy and inductively coupled plasma-atomic emission spectroscopy. These methods indicated incomplete metal substitution. Several reasons could explain this failure: (i) the experimental errors affecting these methods.
Metal-dependence of activity and thermostability of phosphotriesterase

Figure 1  Electrophoretic titration curves of Zn$^{2+}$, Co$^{2+}$ and Cd$^{2+}$-PTE in the pH range 3–9

Intersection of curves with the sample loading line indicates the pI. pI values were more accurately determined by performing IEF in the same pH range, using pI markers. The lowermost picture shows superimposed titration curves of the three PTEs.

Table 1  Catalytic parameters of substituted PTEs for hydrolysis of paraoxon in 50 mM CHES buffer (pH 9.0) at 25 °C

<table>
<thead>
<tr>
<th>Metal</th>
<th>$K_m$ ± S.D. (mM)</th>
<th>$k_{cat}$ ± S.D. (s$^{-1}$)</th>
<th>$pK_a$ ± S.D.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn$^{2+}$</td>
<td>0.056 ± 0.001</td>
<td>1960 ± 40</td>
<td>7.83 ± 0.04</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>0.113 ± 0.002</td>
<td>7800 ± 600</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td>Cd$^{2+}$</td>
<td>0.152 ± 0.004</td>
<td>3900 ± 300</td>
<td>7.81 ± 0.05</td>
</tr>
</tbody>
</table>

* $V_{max}$ versus pH.

Figure 2  Correlation between physicochemical properties of the active-centre metal and the catalytic properties and pI of the corresponding PTE

The different metal-substituted enzymes are listed in the table at the bottom of the figure, and on the x-axis of the graphs after increasing enzymic activity for hydrolysis of paraoxon, as estimated from literature data. Changes in ionic radius (Δ) and $K_m$ values (∆) are shown in the upper panel; changes in ion electronegativity (○), pl (▲) and $k_{cat}$ (●) are shown in the lower panel.

For allowing comparison, the physicochemical parameters characterizing the metal ions and the catalytic constants of the enzymes containing these metals are indicated using relative units.

absorption spectroscopy in different substituted PTEs, the available three-dimensional structures of Zn$^{2+}$-, Cd$^{2+}$- and Mn$^{2+}$-PTE showed complete metal substitutions [5]. Thus the biosynthetic method was assumed to provide full metal substitution.

The pI and the electrophoretic titration curve of PTE show overall structural differences

The pI values of Zn$^{2+}$-, Co$^{2+}$- and Cd$^{2+}$-PTE determined by IEF were 7.2, 7.5 and 7.1 respectively. Each purified enzyme showed a single protein band exhibiting the whole enzymic activity. This is the first assessment of the pI of mature PTEs. pI values of 8.3 and 8 were reported for a Zn$^{2+}$-PTE expressed without excision

(> 15%); (ii) wide-ranging contamination by Zn$^{2+}$ of materials (chemicals and equipments); (iii) the binding of Zn$^{2+}$ to peripheral sites (e.g. on H123). Although Zn$^{2+}$ was found by flame atomic
of the signal peptide [19] and for an enzyme in which the first 33 amino acids of the precursor were replaced by MITNS [20]. Electrophoretic titration curves confirmed significant differences in electric charge between the three enzymes (Figure 1). Zn$^{2+}$- and Cd$^{2+}$-PTE exhibited shallow titration curves in the pH range 7–9. In the same pH region, the slope of the curve for Co$^{2+}$-PTE was steeper. This indicates that at pH < pI, Co$^{2+}$-PTE displays more solvent-exposed positively charged residues than the other two isoforms. In addition, the fact that pH mobility profiles depend on the nature of the cation bound to the active site indicates that the PTE conformations are slightly altered by the associated metal.

**Kinetic analysis of metal-substituted PTEs**

Paraoxon was used for comparing the catalytic properties of PTEs. As shown in Table 1 and Figure 2, Zn$^{2+}$-PTE had the highest affinity and Cd$^{2+}$-PTE the lowest affinity. Co$^{2+}$-PTE displayed the highest $k_{cat}$ values, the natural Zn$^{2+}$ cation showed the lowest $k_{cat}$ values. The relative magnitude of $k_{cat}$ values confirmed the cation ranking Co$^{2+}$ > Cd$^{2+}$ > Zn$^{2+}$, as estimated from literature data. Studies with enzymes produced and assayed under conditions similar to those used in the present work, allowed one to determine $k_{cat}$ values with large fluctuations (8400 ± 1300, 4900 ± 1300 and 2200 ± 300 s$^{-1}$ for Co$^{2+}$-, Cd$^{2+}$- and Zn$^{2+}$-PTE respectively) [6–8,19,21–28].

The Co$^{2+}$-enzyme, containing one of the most electronegative metal cations and being the most active PTE, displays the highest pl values and the steepest titration curve. Electronegativity of Ni$^{2+}$ is similar to that of Co$^{2+}$ and also provides a high activity to Ni$^{2+}$-PTE. Moreover, Ni$^{2+}$-PTE was found to exhibit a pl of 7.5, similar to that of Co$^{2+}$-PTE (D. Rochu, F. Renault and J. Collonge, unpublished work). The activity of PTE is related to the pl, which in turn correlates with the metal electronegativity. The binding of substrates appears to be correlated with the ionic radius of the cation. This is in agreement with reported affinity modulation by the type of metal cation [9]. Discrete differences between cation properties provide significant changes in the catalytic behaviour of PTE. Differences in pl and titration curve may reflect competition between H$^+$ and the most solvent-exposed cation (β-metal) for binding to nucleophilic amino-acid ligands in the active centre. Substitution of Zn$^{2+}$ by Cd$^{2+}$ or Mn$^{2+}$ was supposed to cause limited structural changes in the vicinity of the β-metal. The α-carbon traces for the Zn$^{2+}$- and Cd$^{2+}$-PTE superimpose with an RMSD (root mean square deviation) of 0.2 Å [3]. This RMSD reflects a practically indistinguishable change at the backbone level. However, alteration of the overall PTE conformation cannot be ruled out. Indeed, a Co$^{2+}$-PTE mutant with enhanced activity containing eight mutations has been described in [29]. In this mutant, three mutations were silent, one was conservative and the four significant ones were solvent-exposed residues (Figure 3). More recently, the turnover of a triple mutant of Zn$^{2+}$-PTE on solvent-exposed residues (Figure 3), for S$_7$S$_7$-Soman analogue, was found to be enhanced by nearly three orders of magnitude, compared with the wild-type enzyme [30]. Although the overall α-carbons between the wild-type enzyme and the triple mutant superimpose with an RMSD of only 0.47 Å, significant differences between the two forms of the enzyme occurred, via propagation, at the binuclear metal centre. Our results are in agreement with these observations. Metal exchange modulates the activity of PTE by altering the local structure, i.e. both the active-site region and the solvent-exposed surface. Then the metal radius affects both the substrate binding ($K_m$) and the rate of hydrolysis ($k_{cat}$) (Figure 2).

**Dependence on the active centre metal of thermoactivity and of optimum pH activity**

Figure 4 shows that the pH-dependent activity profiles of the three metal-substituted PTEs differ slightly. Conversely, the common
features are that the three PTEs (i) are inactive below pH 6, (ii) become active at pH > pI, (iii) exhibit 50% of their maximal activity at pH > 7.5 and (iv) their optimum activity is for pH 9–10 (above pH 10 the substrate is too labile to give reliable estimates of activity). However, a thorough comparison of our results with the literature data showed that PTE displays a maximal activity at pH ∼ 9 for the hydrolysis of paraoxon [3,23,31,32].

A fit of the pH-dependence of activity (at $V_{max}$) gave apparent $pK_a$ values of 7.83 ± 0.04, 7.41 ± 0.11 and 7.81 ± 0.05 for Zn$^{2+}$-, Co$^{2+}$- and Cd$^{2+}$-PTE respectively (Table 1). The reported $pK_a$ values (5.8–6.1, 5.6–6.5 and 8.1–8.5 for the corresponding enzymes [6,23,25,31,33]) were obtained under different buffer conditions for PTEs displaying modified or unprocessed N-terminal sequences that did not match with the mature PTEs studied in the present work. The $pK_a$ values determined in our study are high for catalytic histidine residue(s). However, high $pK_a$ values of histidine residues have been reported. Salt effects, due to specific binding of buffer components, might account for the increase in $pK_a$ values. However, it may be assumed that local conformational change in mature enzyme compared with unprocessed enzyme had altered the local dielectric constant and polarizability of histidine groups so that these groups became more solvent-exposed and more basic.

To determine the optimum temperature for stability, samples were preincubated over the temperature range 25–60 °C (pH 8–9.5), where enzyme activity was optimal, and then assayed at 25 °C. Figure 5 shows that (i) maximal stability is at $T \leq 35$ °C; (ii) heat inactivation occurs in the temperature range 35–60 °C; (iii) a transient activation phase is observed at $T \sim 45–48$ °C (denoted $T_a$), and it is followed by an abrupt loss of activity with complete inactivation at 60 °C. The three PTEs were inactive above approx. 60 °C. This is in agreement with an early observation showing that parathion hydrolase activities from Pseudomonas, Brevibacterium, Azotomonas and Xanthomonas are stable up to 45–50 °C [34]. Similarly, the half-life of purified Zn$^{2+}$-PTE was found to be 16 min at 55 °C and pH 9 [32]. A transient activation phase was reported for the Co$^{2+}$-PTE at pH 9.4 [13]. Transitory increase in activity during thermal inactivation was also reported for other enzymes [35–38]. Finally, Zn$^{2+}$-PTE appeared to be the most thermostable enzyme and Cd$^{2+}$-PTE the least (Figure 5). In addition, at pH 8, the temperature-dependence of PTE activity was found to depend on the type of metal cation.

Metal-cation-dependence of the structural stability of PTE

We previously reported $T_m = 58$ °C for Co$^{3+}$-PTE unfolding in 100 mM borate buffer at pH 9.4 [13]. In the present study, CE mobility of PTE associated with three different cations was investigated between pH 8 and 9.4 at different temperatures (between 30 and 70 °C). This provided substantial information about the role of the metal and the effect of pH on the stability of PTE. Under favourable conditions, the N and U states of PTE were populated at different temperatures, allowing $T_m$ and $\Delta H$ values to be estimated after peak area deconvolution [13] (Figure 6A). Under less alkaline conditions and/or other bound cations, U was not well separated from N. Then the unfolding conformational changes showed a break in the temperature-dependent plot of enzyme mobility (Figure 6B). In spite of this, the behaviour
of $T_m$ was observed for the three PTEs in the investigated pH range. Moreover, a second break in the curve at a lower temperature was observed, suggesting a second structural change. This would correspond to a populated, partially unfolded intermediate state, I. This second transition occurs at a temperature in agreement with $T_s$ at which transient activated intermediates (I) were observed (Figure 5). Although soluble aggregates of Co$^{2+}$-PTE were favoured at pH > 9.5 [13], we noticed that Cd$^{2+}$-PTE heated at 80 °C (pH 8.0) did not clog the capillary tube in which electrophoresis was performed. The dependence of the PTE stability on the active-site metal ion is shown in Figure 7. Thus three-dimensional structural differences between PTE substituted with different metals cannot explain the large discrepancy between thermal stability results. We provided evidence that, above approx. 60 °C, the enzyme loses its functional native conformation (N). Accordingly, the temperature-induced denaturation process of PTE dimers ($N_2$) can be described by an expanded form of the Lumry–Eyring model [15]:

\[ N_2 \rightarrow 2N^* \rightarrow 2U \rightarrow D_N \]

in which the intermediate ($I^*_2$ or $I^*$) displaying enhanced activity is populated at $T \approx 45–50$ °C, and the unfolded state ($U_2$ or $U$) leads to denatured aggregates ($D_N$).

The present study indicates that stability of PTE is correlated with substrate affinity: the most stable enzyme (Zn$^{2+}$-PTE) displays the lowest $K_m$ value, whereas the less stable one (Cd$^{2+}$-PTE) has the lowest affinity. Co$^{2+}$-PTE, the most efficient PTE, exhibits an intermediate thermostability. However, its unexpected behaviour at pH 8 has to be correlated with a particular structural feature. The changes in stability and activity of Zn$^{2+}$-, Cd$^{2+}$- and Co$^{2+}$-PTE can be partially compensated by the pH-dependence of both properties. Thus the type of the metal cation is essential for balancing stability and flexibility. Stability change with pH indicated that the less stable enzyme could be potentially usable under physiological conditions for skin protection and/or decontamination. The toxicity of Cd$^{2+}$ disqualifies Cd$^{2+}$-PTE.

**Conclusion**

The behaviour of PTE associated with different metal cations, over the pH range favourable for optimal enzymic activity, was analysed by CE, IEF and steady-state kinetics. Our study allowed one to (i) discriminate the role of the associated metal in stabilizing PTE; (ii) show that the optimal pH conditions for activity and stability are different; (iii) detect transient intermediates populated during heat-induced denaturation; and (iv) demonstrate the opposite requirements for optimum activity and maximum stability. This approach provides a rationale for choosing the 'good compromise' among engineered mutants with enhanced activity and substrate specificity. Investigation of the contribution of the metal cation towards stability will help us in designing highly active and stable mutants of PTE for skin protection against chemical warfare agents and detoxification of these compounds.

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