Contribution of the active-site metal cation to the catalytic activity and to the conformational stability of phosphotriesterase: temperature- and pH-dependence

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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 pI 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 $T \leq 35^\circ C$, followed by an activation phase near $45\text{--}48^\circ C$ and then inactivation which was completed at $60^\circ C$. 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 $60.5\text{--}67^\circ C$, $58\text{--}64^\circ C$ and $53\text{--}64^\circ C$ 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 detoxification 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, $T_m \approx 75^\circ C$. 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 $T \approx 45^\circ C$, followed by an inactive state ($T_m \approx 58^\circ C$), 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 $\approx 18\text{ M}\Omega \cdot \text{cm}$) 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-PTE_m. pET17b was from Novagen as described previously [13]. The PTE_m 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); and (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]:

k_{obs} is the first-order rate constant of denaturation:

$$k_{\text{obs}} = \frac{k_3}{1 + 1/K_U} \quad \text{with } K_U = \frac{[U]}{[N]} = \frac{k_1}{k_2}$$

The two-state and reversibility assumptions for protein unfolding apply for $k_3 \approx 0$ [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. Paraaxon 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 A_{400} due to the release of *p*-nitrophenol ($\epsilon_{400} = 17\,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) from paraaxon. One unit of activity (U) is defined as 1 µmol of paraaxon 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 ($\approx V_{\text{max}}$) of paraaxon (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 pK_a 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

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.

(> 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

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

Metal	$K_m \pm \text{S.D. (mM)}$	$k_{\text{cat}} \pm \text{S.D. (s}^{-1}\text{)}$	$\text{p}K_a \pm \text{S.D.}^*$
Zn^{2+}	0.056 ± 0.001	1960 ± 40	7.83 ± 0.04
Co^{2+}	0.113 ± 0.002	7800 ± 600	7.4 ± 0.1
Cd^{2+}	0.152 ± 0.004	3900 ± 300	7.81 ± 0.05

* V_{max} versus pH.

Metal ion	Mn^{2+}	Zn^{2+}	Cd^{2+}	Ni^{2+}	Co^{2+}
Ion electro-negativity	-1.55	-1.65	-1.69	-1.91	-1.88
Ionic radius (Å)	0.80	0.74	0.97	0.69	0.72
pI	Nd	7.2	7.1	7.5	7.5
K_m (mM)	0.080 ± 0.006	0.045 ± 0.005	0.370 ± 0.050	0.150 ± 0.010	0.110 ± 0.010
k_{cat} (s^{-1})	1750 ± 40	2200 ± 300	4900 ± 1300	6000 ± 150	8400 ± 1300

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 ($\diamond$) and K_m values ($\blacklozenge$) are shown in the upper panel; changes in ion electronegativity ($\circ$), pI ($\blacktriangle$) and k_{cat} ($\bullet$) 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

Figure 3 Changes in structure and catalytic activity of PTE induced by the presence of different active-site metal cations

(A) A Cd^{2+} -PTE monomer from the co-ordinates of 1JGM is shown by a grey tube with the two Cd^{2+} shown by red balls. The four significant substitutions of a mutant, 25-fold more active than the wild-type PTE [29], and the substitutions of the triple mutant with turnover rate enhanced by three orders of magnitude [30] are highlighted in purple and blue respectively: A80 (opposite side), K185 and I274 have peripheral location; H254, H257 and L303 are solvent-accessible. (B) Close-up view of superimposed metal centres of PTE containing Zn^{2+} (1HZY), Mn^{2+} (110B) or Cd^{2+} (1JGM). The deepest Cd^{2+} (red ball) and the solvent-exposed Zn^{2+} and Mn^{2+} (blue and green balls respectively) are highlighted; the solvent-exposed Cd^{2+} is indicated by a red circle for allowing visualization of the other metals at the corresponding position. The local structural changes induced by the cation type at the binuclear metal centre are shown for the residues co-ordinated to metal ions (thin sticks).

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 $\text{pH} < \text{pI}$, 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 $\text{Co}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+}$, 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 $2200 \pm 300 \text{ s}^{-1}$ 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 pI 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 pI 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 pI, 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 pI 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_pSC -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

Figure 4 pH profiles of activity of the three metal-substituted PTEs

Normalized activities of purified PTEs containing Zn^{2+} (∇), Co^{2+} ($\bullet$) or Cd^{2+} ($\blacklozenge$) with paraoxon (1 mM) as the substrate.

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^{2+} -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 ΔH 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

Figure 5 Effect of temperature on the catalytic activity of the three metal-substituted PTEs

Temperature-dependence profiles are shown for Zn^{2+} -PTE (A), Co^{2+} -PTE (B) and Cd^{2+} -PTE (C), at pH 8.0, 8.5, 9.0 and 9.5. Activities are normalized (maximum activity = 100%) and corrected according to the pH-dependence shown in Figure 4. The results represent the mean value of three independent experiments.

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- 4 Benning, M. M., Kuo, J. M., Raushel, F. M. and Holden, H. M. (1994) Three-dimensional structure of phosphotriesterase: an enzyme capable of detoxifying organophosphate nerve agents. *Biochemistry* **33**, 15001–15007
- 5 Benning, M. M., Shim, H., Raushel, F. M. and Holden, H. M. (2001) High resolution X-ray structures of different metal-substituted forms of phosphotriesterase from *Pseudomonas diminuta*. *Biochemistry* **40**, 2712–2722
- 6 Omburo, G. A., Kuo, J. M., Mullins, L. S. and Raushel, F. M. (1992) Characterization of the zinc binding site of bacterial phosphotriesterase. *J. Biol. Chem.* **267**, 13278–13283
- 7 Hong, S. B. and Raushel, F. M. (1996) Metal–substrate interactions facilitate the catalytic activity of the bacterial phosphotriesterase. *Biochemistry* **35**, 10904–10912
- 8 Hong, S. B. and Raushel, F. M. (1999) Stereochemical constraints on the substrate specificity of phosphotriesterase. *Biochemistry* **38**, 1159–1165
- 9 Chen-Goodspeed, M., Sogorb, M. A., Wu, F., Suk-Bong, H. and Raushel, F. M. (2001) Structural determinants of the substrate and stereochemical specificity of phosphotriesterase. *Biochemistry* **40**, 1325–1331
- 10 Chen-Goodspeed, M., Sogorb, M. A., Wu, F. and Raushel, F. M. (2001) Enhancement, relaxation, and reversal of the stereoselectivity for phosphotriesterase by rational evolution of active site residues. *Biochemistry* **40**, 1332–1339
- 11 Grimsley, J. K., Scholtz, J. M., Pace, C. N. and Wild, J. R. (1997) Organophosphorus hydrolase is a remarkably stable enzyme that unfolds through a homodimeric intermediate. *Biochemistry* **36**, 14366–14374
- 12 Rochu, D., Ducret, G., Ribes, F., Vanin, S. and Masson, P. (1999) Capillary electrophoresis with optimized temperature control for studying thermal denaturation of proteins at various pH. *Electrophoresis* **20**, 1586–1594
- 13 Rochu, D., Beaufet, N., Renault, F., Viguié, N. and Masson, P. (2002) The wild type bacterial $\text{Co}^{2+}/\text{Co}^{2+}$ -phosphotriesterase shows a middle-range thermostability. *Biochim. Biophys. Acta* **1594**, 207–218
- 14 Serdar, C. M., Murdock, D. C. and Rohde, M. F. (1989) Parathion hydrolase gene from *Pseudomonas diminuta* MG: subcloning, complete nucleotide sequence, and expression of the mature protein of the enzyme in *Escherichia coli*. *Bio/Technology* **7**, 1151–1155
- 15 Lumry, R. and Eyring, H. (1954) Conformation changes of proteins. *J. Phys. Chem.* **58**, 110–120
- 16 Sanchez-Ruiz, J. M. (1992) Theoretical analysis of Lumry-Eyring models in differential scanning calorimetry. *Biophys. J.* **61**, 921–935
- 17 Curdel, A. and Iwatsubo, M. (1968) Biosynthetic incorporation of cobalt into yeast alcohol dehydrogenase. *FEBS Lett.* **1**, 133–136
- 18 Speckhard, D. C., Wu, F. Y.-H. and Wu, C.-W. (1977) Role of the intrinsic metal in RNA polymerase from *Escherichia coli*. *In vivo* substitution of tightly bound zinc with cobalt. *Biochemistry* **16**, 5228–5234
- 19 Dumas, D. P., Caldwell, S. R., Wild, J. R. and Raushel, F. M. (1989) Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*. *J. Biol. Chem.* **264**, 19659–19665
- 20 Petrikovics, I., Hong, K., Omburo, G., Hu, Q. Z., Pei, L., McGuinn, W. D., Sylvester, D., Tamulinas, C., Papahadjopoulos, D., Jaszberenyi, J. C. et al. (1999) Antagonism of paraoxon intoxication by recombinant phosphotriesterase encapsulated within sterically stabilized liposomes. *Toxicol. Appl. Pharmacol.* **156**, 56–63
- 21 Caldwell, S. R., Newcomb, J. R., Schlecht, K. A. and Raushel, F. M. (1991) Limits of diffusion in the hydrolysis of substrates by the phosphotriesterase from *Pseudomonas diminuta*. *Biochemistry* **30**, 7438–7444
- 22 Kolakovski, J. E., DeFrank, J. J., Harvey, S. P., Szafraniec, L. L., Beaudry, W. T., Lai, K. and Wild, J. R. (1997) Enzymatic hydrolysis of the chemical warfare agent VX and its neurotoxic analogues by organophosphorus hydrolase. *Biocatal. Biotransform.* **15**, 297–312
- 23 Kuo, J. M. and Raushel, F. M. (1994) Identification of the histidine ligands to the binuclear metal center of phosphotriesterase by site-directed mutagenesis. *Biochemistry* **33**, 4265–4272
- 24 Di Sioudi, B., Grimsley, J. K., Lai, K. and Wild, J. R. (1999) Modification of near active site residues in organophosphorus hydrolase reduces metal stoichiometry and alters substrate specificity. *Biochemistry* **38**, 2866–2872
- 25 Omburo, G. A., Mullins, L. S. and Raushel, F. M. (1993) Structural characterization of the divalent cation sites of bacterial phosphotriesterase by ^{113}Cd NMR spectroscopy. *Biochemistry* **32**, 9148–9155
- 26 Watkins, L. M., Mahoney, H. J., McCulloch, J. K. and Raushel, F. M. (1997) Augmented hydrolysis of diisopropyl fluorophosphate in engineered mutants of phosphotriesterase. *J. Biol. Chem.* **272**, 25596–25601
- 27 Shim, H., Hong, S. B. and Raushel, F. M. (1998) Hydrolysis of phosphodiester through transformation of the bacterial phosphotriesterase. *J. Biol. Chem.* **273**, 17445–17450
- 28 Shim, H. and Raushel, F. M. (2000) Self-assembly of the binuclear metal center of phosphotriesterase. *Biochemistry* **39**, 7357–7364
- 29 Cho, C. M.-H., Mulchandani, A. and Chen, W. (2002) Bacterial cell surface display of organophosphorus hydrolase for selective screening of improved hydrolysis of organophosphate nerve agents. *Appl. Environ. Microbiol.* **68**, 2026–2030
- 30 Hill, C. M., Li, W. S., Thoden, J. B., Holden, H. M. and Raushel, F. M. (2003) Enhanced degradation of chemical warfare agents through molecular engineering of the phosphotriesterase active site. *J. Am. Chem. Soc.* **125**, 8990–8991
- 31 Donarski, W. J., Dumas, D. P., Heitmeyer, D. H., Lewis, V. E. and Raushel, F. M. (1989) Structure–activity relationships in the hydrolysis of substrates by the phosphotriesterase from *Pseudomonas diminuta*. *Biochemistry* **28**, 4650–4655
- 32 Caldwell, S. R. and Raushel, F. M. (1991) Detoxification of organophosphate pesticides using an immobilized phosphotriesterase from *Pseudomonas diminuta*. *Biotechnol. Bioeng.* **37**, 103–109
- 33 Lai, K., Dave, K. I. and Wild, J. R. (1994) Bimetallic binding motifs in organophosphorus hydrolase are important for catalysis and structural organization. *J. Biol. Chem.* **269**, 16579–16584
- 34 Munnecke, D. M. (1978) Detoxification of pesticides using soluble or immobilised enzymes. *Process Biochem.* **13**, 14–31
- 35 Weingand-Ziadé, A., Ribes, F., Renault, F. and Masson, P. (2001) Pressure and heat-induced inactivation of butyrylcholinesterase: evidence for multiple intermediates and remnant inactivation process. *Biochem. J.* **356**, 487–493
- 36 Rochu, D., Georges, C., Répition, J., Viguié, N., Saliou, B., Bon, C. and Masson, P. (2001) Thermal stability of acetylcholinesterase from *Bungarus fasciatus* as investigated by capillary electrophoresis. *Biochim. Biophys. Acta* **1545**, 216–226
- 37 Siksnis, V. A., Galkantaite, N. Z., Melik-Nubarov, N. S., Levitsky, V. Yu., Slepnev, V. I. and Mozhaev, V. V. (1990) An unusual (zig-zag) temperature dependence of the rate of irreversible thermoinactivation of enzymes. *Biokhimiya* **55**, 1347–1355
- 38 Villaverde, J., Cladera, J., Padrós, E., Rigaud, J.-L. and Duñach, M. (1997) Effect of nucleotides on the thermal stability and on the deuteration kinetics of the thermophilic F_0F_1 ATP synthase. *Eur. J. Biochem.* **244**, 441–448

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