Articles

Modification of Near Active Site Residues in Organophosphorus Hydrolase Reduces Metal Stoichiometry and Alters Substrate Specificity†

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ABSTRACT: Organophosphorus hydrolase (OPH, EC 8.1.3.1) is a dimeric, bacterial enzyme that detoxifies many organophosphorus neurotoxins by hydrolyzing a variety of phosphonate bonds. The histidinyl residues at amino acid positions 254 and 257 are located near the bimetallic active site present in each monomer. It has been proposed that these residues influence catalysis by interacting with active site residues and the substrate in the binding pocket. We replaced the histidine at position 254 with arginine (H254R) and the one at position 257 with leucine (H257L) independently to form the single-site-modified enzymes. The double modification was also constructed to incorporate both changes (H254R/H257L). Although native OPH has two metals at each active site (four per dimer), all three of these altered enzymes possessed only two metals per dimer while retaining considerable enzymatic activity for the preferred phosphotriester (P−O bond) substrate, paraoxon (5−100% kcat). The three altered enzymes achieved a 2−30-fold increase in substrate specificity (k_{cat}/K_m) for demeton S (P−S bond), an analogue for the chemical warfare agent VX. In contrast, the substrate specificity for diisopropyl fluorophosphonate (P−F bond) was substantially decreased for each of these enzymes. In addition, H257L and H254R/H257L showed an 11- and 18-fold increase, respectively, in specificity for NPPMP, the analogue for the chemical warfare agent soman. These results demonstrate the ability to significantly enhance the specificity of OPH for various substrates by site-specific modifications, and it is suggested that changes in metal requirements may affect these improved catalytic characteristics by enhancing structural flexibility and improving access of larger substrates to the active site, while simultaneously decreasing the catalytic efficiency for smaller substrates.

Organophosphorus hydrolase (OPH,[1] EC 8.1.3.1) is a bacterial enzyme encoded by the *opd* gene originating from two different plasmids of *Pseudomonas diminuta* MG and *Flavobacterium* ATCC 27551 (*1*−*3*). This enzyme breaks down a variety of neurotoxic organophosphorus (OP) compounds, including the chemical warfare agents sarin, soman, and tabun, and OP insecticides parathion, coumaphos, and diazinon (*4*, *5*). Cleavage of the P−O, P−F, or P−CN bonds of these neurotoxic compounds (Figure 1) renders them relatively nontoxic as the bond broken by hydrolysis is that involved in the irreversible inhibition of acetylcholinesterase by OP neurotoxins (*6*). The enzymatic hydrolysis of paraoxon, the preferred substrate of OPH, occurs at diffusion-controlled rates (10^8−10^9 M^{-1} s^{-1}). Recent studies have shown that OPH hydrolyzes organophosphorothiolates by direct cleavage of the phosphorothioester (P−S) bond (*7*−*9*). However, the rates of hydrolysis for members of this class of compounds, which includes the class V chemical warfare agents (VX and RVX) (*10*, *11*) and the insecticides malathion and acephate (*9*), are slow compared to hydrolytic rates for the phosphotriester and phosphonofluoridates, and even the phosphonocyanate tabun. Improvement of the catalytic turnover and specificity of OPH for this group of extremely toxic OP compounds is necessary for enzyme-based bioremediation.

Although the genes for two other OP-hydrolyzing enzymes have been cloned and their products purified (*12*, *13*), OPH has been the most extensively studied, and it is the only enzyme known to hydrolyze the P−S bond at significant rates (*8*, *9*). Detailed enzymatic characterization has suggested that

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[1] Abbreviations: OPH, organophosphorus hydrolase; OP, organophosphorus; VX, *o*-ethyl *S*-(2-diisopropylaminoethyl)methylphosphonothiolate; RVX or Russian VX, *o*-isobutyl *S*-(2-diethylamino)-methylphosphonothioate; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphonate; NPPMP, *p*-nitrophenyl-*o*-pinacolyl methylphosphonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; MES, 4-morpholinoethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 2-TP, 2,2′-dithiodipyridine; WT, wild type; FAAS, flame atomic absorption spectroscopy.

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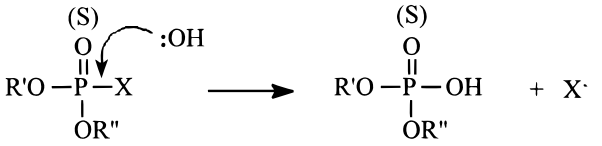

FIGURE 1: General scheme of hydrolysis for OP compounds. A hydroxyl radical attacks the phosphorus center of the substrate, displacing the leaving group. X may be O, F, C, or S; R is any alkyl group.

the catalytic mechanism proceeds via a S_N2 process in which an activated water molecule attacks the phosphoryl center, resulting in the displacement of the leaving group with an inversion of stereochemical configuration (*14*). X-ray crystallographic studies (*15*−*17*) have described OPH as a dimeric metalloenzyme that contains 2 equiv of zinc per monomer in its native form. The overall folding pattern of the monomer consists of an α/β barrel with eight strands of a parallel β-pleated sheet. Each active site has a binuclear metal center, and the native Zn^{2+} can be replaced by any of several metals, including Co^{2+}, Mn^{2+}, Cd^{2+}, or Ni^{2+}, with varying affects on rates of paraoxon hydrolysis (*18*, *19*). A careful thermodynamic analysis has revealed that OPH is a remarkably stable protein (G^{H_2O} = 40 kcal/mol) that folds in a three-state process through an inactive, dimeric intermediate (*20*).

The functional catalytic role and the coordination of the metal centers have been investigated by ^{113}Cd NMR (*21*) and site-directed mutagenesis (*22*−*24*), and the structure of OPH has been elucidated by X-ray crystallographic analyses (*15*−*17*). The apoenzyme, Cd^{2+}-substituted, and Zn^{2+}-substituted structures have been determined to 2.1, 2.0, and 2.1 Å, respectively, providing insight into the organization and nature of the enzyme active site. The highly positive character of the metal ions and histidines (His55, -57, -201, -230, -254, and -257) at the active site of the enzyme is counteracted by the presence of five aspartyl residues (Asp232, -233, -235, -253, and -301) and a carbamylated lysine (Lys169). The metal-coordinating ligands are four histidines (His55, -57, -201, and -230), one aspartate (Asp301), one carbamylated lysine (Lys169), and a bridging water molecule.

In this report, the effects of particular substitutions at positions 254 and 257 on enzymatic function are characterized by comparison of activity and specificity for a variety of substrates (Figure 2). In addition, metal chelation and reconstitution experiments were performed to evaluate the effect of changes in metal association. Alterations in metal content among these OPH variants were specifically correlated with altered substrate specificities.

EXPERIMENTAL PROCEDURES

Substrates. Paraoxon and DFP were obtained from Sigma (St. Louis, MO). Paraoxon was extracted five times in dichloromethane/10 mM CHES buffer (pH 9.0) (1:4) and dichloromethane/deionized water (1:4), and the dichloromethane was removed by evaporation in a 40 °C water bath. The purified paraoxon was dissolved in deionized water and stored at −20 °C. Demeton S (97% pure) was purchased from ChemService (Westchester, PA), and NPPMP was a gift from T.-C. Cheng (U.S. Army Edgewood Research, Development, and Engineering Center, Aberdeen Proving Ground, Aberdeen, MD).

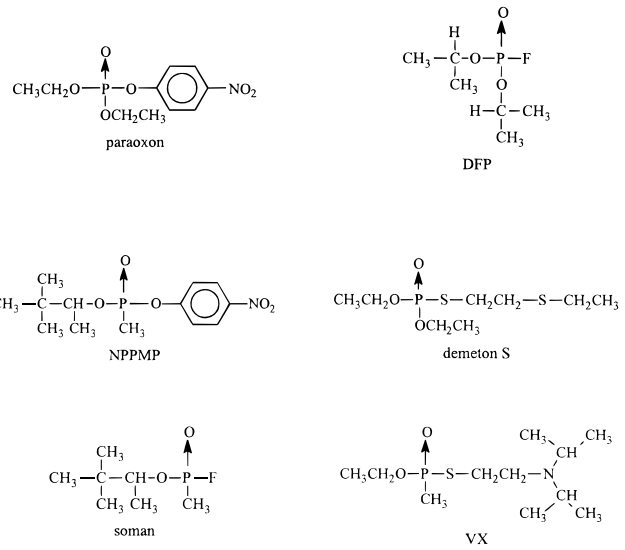

FIGURE 2: Selected substrates of OPH.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using the manufacturer's protocol (Clontech, Palo Alto, CA; *25*) directly in the plasmid expression vector template pOP419 (*23*, *24*). All primers were designed using the GENEWORKS program from IntelliGenetics (Mountain View, CA). One nanomole of denatured plasmid template was hybridized with 1 nmol of a phosphorylated selection primer HindIIIM (5′ CAT gCA AgC ATg gCg TAA TC 3′) and 10 nmol of a phosphorylated mutagenic primer. Mutagenic primer p254MUT was designed to replace the codon for His254 with that of Arg (5′ ACT gTg Cgg gAT gCg gTC TAg ACC gAT gAg 3′), and p257MUT was designed to replace the codon for His257 with that of Leu (5′ ACC AAT CgC ACT gAg Cgg gAT gTg gTC 3′). (The underlined bases represent the mismatches.) The resulting plasmids were designated pOP419-254R and pOP419-257L, respectively. Another mutagenic primer (H254RCo) was designed to replace the codon for His254 with that of Arg (5′ Cgg gAT gCg gTC TAg ACC gAT gAg 3′) directly in pOP419-257L. Since the *Hin*dIII site was replaced during the initial mutagenesis, the selection primer PstIM was used to remove a unique *Pst*I site in the plasmid pOP419-257L. The mutations were verified by double-stranded DNA sequencing using the Sequenase Version 2.0 DNA Sequencing Kit (U.S. Biochemical, Cleveland, OH) and by fluorescent tag sequencing on an ABI Prism 377 system (Applied Biosystems Division, Perkin-Elmer, Foster City, CA) by the Gene Technologies Laboratory at Texas A&M University (College Station, TX). The entire gene sequences were subsequently determined to check for secondary mutations.

Protein Expression and Purification. The OPH enzymes were produced and purified in the presence of cobalt chloride as previously described (*20*). The purity of OPH was determined by SDS−PAGE stained with Coomassie brilliant blue or with silver nitrate (*26*). The concentrations of the protein were estimated spectrophotometrically using the molar extinction coefficient of OPH (ϵ_{280} = 58 000 M^{-1} cm^{-1}).

Kinetic Characterization. A catalytic rate (k_{cat}) and Michaelis constant (K_m) for each substrate were determined by performing enzymatic assays on changing concentrations of substrate with a constant concentration of enzyme. The

Table 1: Kinetic Parameters of OPH Enzymes[a]

	paraoxon	NPPMP	DFP	demeton S
WT OPH				
k_{cat} (s^{-1})	15000 ± 300	23 ± 2	75 ± 6	4.2 ± 0.1
K_M (mM)	0.12 ± 0.01	1.3 ± 0.3	0.96 ± 0.1	4.8 ± 0.2
k_{cat}/K_M (M^{-1} s^{-1})	1.3×10^8	1.7×10^4	7.8×10^4	8.7×10^2
K_i (mM)	17 ± 1	−	23 ± 8	−
H254R				
k_{cat} (s^{-1})	680 ± 10	2.5 ± 0.1	0.41 ± 0.01	16 ± 0.7
K_M (mM)	0.015 ± 0.001	0.47 ± 0.07	0.087 ± 0.01	4.4 ± 0.4
k_{cat}/K_M (M^{-1} s^{-1})	4.4×10^7	5.3×10^3	4.7×10^3	3.6×10^3
K_i (mM)	1.8 ± 0.1	−	−	−
H257L				
k_{cat} (s^{-1})	16000 ± 600	260 ± 40	19 ± 1	3.3 ± 0.05
K_M (mM)	0.30 ± 0.03	1.4 ± 0.3	0.73 ± 0.1	1.9 ± 0.9
k_{cat}/K_M (M^{-1} s^{-1})	5.3×10^7	1.9×10^5	2.6×10^4	1.8×10^3
K_i (mM)	15 ± 3	4.4 ± 2	21 ± 7	−
H254R/H257L				
k_{cat} (s^{-1})	1400 ± 40	54 ± 3	0.79 ± 0.06	68 ± 5
K_M (mM)	0.036 ± 0.003	0.18 ± 0.02	0.20 ± 0.06	2.5 ± 0.5
k_{cat}/K_M (M^{-1} s^{-1})	4.0×10^7	3.0×10^5	4.0×10^3	2.7×10^4
K_i (mM)	4.4 ± 0.5	1.7 ± 0.2	−	−

[a] Assays were performed as described in Experimental Proceedures. See the text for details.

change in absorbance per minute at the appropriate wavelength was monitored on a Pharmacia Ultrospec 2000 UV/Visible spectrophotometer (Pharmacia LKB Biotechnology, Cambridge, U.K.) utilizing the accompanying Pharmacia Biotech Swift Applications software. Assays with substrates which yielded *p*-nitrophenol as a cleavage product (paraoxon and NPPMP) were monitored by following the appearance of the product ($\epsilon_{400} = 17\,000$ M^{-1} cm^{-1}) in a reaction mixture containing 50 mM CHES at pH 9.0 and 25 °C. Due to solubility limitations of the substrate, assays with NPPMP included 5% (v/v) methanol. Assays with demeton S, which yields free thiol groups upon hydrolysis, were monitored by the reaction of cleavage products (free thiols) with DTNB ($\epsilon_{412} = 14\,145$ M^{-1} cm^{-1}) (*27*) or 2-TP ($\epsilon_{343} = 7070$ M^{-1} cm^{-1}) (*28*) in tripartate buffer (50 mM MES, 25 mM *N*-ethylmorpholine, and 25 mM diethanolamine) with 1% (v/v) methanol at pH 8.0 and 25 °C. Assays with DFP, which yields free fluoride ion (F^-) upon hydrolysis, were monitored with an ion-selective electrode (Orion) in a reaction mixture containing 500 mM KCl and 50 mM HEPES at pH 7.2 and 25 °C. Plotting the concentration of a substrate versus reaction velocity provided a data set which was fit using KaleidaGraph (Synergy Software, Reading, PA). Preliminary data analyses were fit to the Michaelis−Menten equation. Some assays were best fit by a Michaelis−Menten equation modified to include substrate inhibition as a parameter, and k_{cat}, K_M, and K_i were determined directly from this curve fit.

Metal Chelation and Titration Experiments. Glassware and other laboratory materials were made metal-free as previously described (*29*, *30*); buffers and substrate solutions were rendered metal-free by utilizing Chelex-20 resin according to the protocol of the manufacturer (Bio-Rad Laboratories, Hercules, CA). Apoenzyme was prepared by dialysis of the enzyme (1−2 mg/mL) against several changes of chelation buffer [50 mM HEPES, 20 mM KCl, and 2.5 mM 1,10-phenanthroline (pH 8.1)] at 4 °C. Chelation was stopped when the enzyme had <1% residual activity against 3 mM paraoxon. The chelator was removed by extensive dialysis against the same metal-free buffer without 1,10-phenanthroline and monitored by the disappearance of absorbance in the dialysate at 327 nm (*18*, *19*). Residual metal content was determined by FAAS on a Perkin-Elmer 2380 atomic absorption spectrophotometer as previously described (*18*, *19*).

Metal titration was performed by dividing the apoenzyme into 10 aliquots and dialyzing each against metal-free buffer supplemented with 0−6 equiv of $CoCl_2$ for 48 h. Reconstituted enzymes were assayed for recovery of activity with metal-free substrates, and metal content was determined by FAAS after dialysis against metal-free buffer.

RESULTS

Kinetics of P−O Bond Hydrolysis. The two phosphotriester (P−O bond) substrates used in this study were paraoxon and the soman analogue NPPMP. Paraoxon, the preferred substrate of WT OPH, is hydrolyzed at a diffusion-controlled rate (1.3×10^8 M^{-1} s^{-1}). The single-site-substituted enzyme H257L had a rate of hydrolysis very similar to that of the WT enzyme ($k_{cat} = 16\,000$ and $15\,000$ s^{-1}, respectively), and a similar estimate for K_i (15 and 17 mM, respectively), but the K_M value for H257L was approximately 3 times that of the WT value of 120 μM. In comparison, both the catalytic rate and the K_M of H254R for paraoxon were lower than those values of the WT enzyme, and this enzyme was more sensitive to substrate inhibition. For each of these kinetic parameters, the values for the double mutant H254R/H257L were intermediate with respect to those of H254R and H257L. Kinetic saturation curves for WT OPH, H254R, H257L, and H254R/H257L with paraoxon indicated that none of the altered enzymes was better than the original enzyme in degradation of paraoxon (Table 1), and the specificity in each case was compromised by 50−70%.

The leaving group (*p*-nitrophenol) from the hydrolysis of NPPMP is identical to that of paraoxon, but the pinacolyl side chains of the phosphoric acid derivative are more bulky and hydrophobic. In addition, NPPMP has a more limited aqueous solubility than paraoxon, and quantitative assays used to evaluate the kinetic characteristics of the enzymatic degradation of NPPMP included 5% methanol (v/v). The maximal turnover rate by an OPH enzyme for NPPMP ($k_{cat-NPPMP}$) observed in these studies was 260 s^{-1}, observed

with H257L; this represented a 10-fold improvement over the rate of the WT enzyme (Table 1). In comparison, the H254R enzyme showed a 10-fold loss in the catalytic rate with NPPMP compared ti that of the WT enzyme. The $K_{M-NPPMP}$ value for H254R was also lower in comparison to that of WT (0.47 and 1.3 mM, respectively). H254R/H257L had approximately twice the catalytic rate of the WT enzyme, and the K_M value was further lowered to 0.18 mM. Under the experimental conditions described here, both H257L and H254R/H257L provided improved catalytic hydrolysis of NPPMP.

Kinetics of P−F Bond Hydrolysis. DFP is a much smaller substrate than either paraoxon or NPPMP. When the P−F bond is broken by either enzymatic or chemical hydrolysis, a fluoride ion is released. The solubility limit of DFP permitted substrate saturation assays which included up to 7 mM DFP without the addition of methanol or other organic solvent. Estimations of kinetic parameters showed that WT OPH was better at the degradation of DFP than H254R, H257L, or H254R/H257L (Table 1) with a k_{cat} of 75 s^{-1} and a K_M of 0.96 mM. The H257L enzyme retained about 25% of the catalytic rate of WT OPH, while its K_M value was slightly reduced. The catalytic rates of both H254R and H254R/H257L dropped to approximately 1% of the WT rate. Although both enzymes showed some improvement in the K_M value (e.g., the value for H254R was 10-fold lower than that for the WT enzyme), the specificity values (k_{cat}/K_M) were about 6% of WT.

Kinetics of P−S Bond Hydrolysis. Demeton S is an analogue of the V class chemical warfare agents VX and RVX. The long, hydrophobic leaving group of demeton S is attached to the phosphoryl center of the molecule through a P−S bond. Substrate saturation assays with up to 8 mM demeton S included 1% methanol. A methanol inhibition curve was derived to assess the impact of up to 20% methanol on demeton S hydrolysis by each of the enzymes included in this study (data not shown). Methanol inhibited each of the enzymes to the same degree and in a consistent fashion; therefore, direct comparison of the enzymes was appropriate.

The hydrolytic rates of H254R toward demeton S were improved compared to those of WT OPH ($k_{cat-demetonS} = 16$ and 4.2 s^{-1}, respectively) (Table 1). Although the catalytic rate of H257L was slightly lower than that of the WT enzyme, the H257L enzyme had a K_M value which was less than half that of the WT enzyme. Thus, the specificity constants of H254R and H257L were 4- and 2-fold better for demeton S than for the WT, respectively. The double mutant H254R/H257L demonstrated a quite significant improvement in both k_{cat} and K_M ($k_{cat} = 68$ s^{-1}, $K_M = 2.5$ mM), and the specificity of this enzyme for demeton S was more than 30-fold better than that of WT.

Metal Stoichiometry. WT OPH was shown by FAAS to contain 1.9 metals per monomer, or four per dimer, which agrees with earlier findings (*21*−*24*). However, each of the site-directed substitutions (H254R, H257L, and H254R/H257L) contained only two metals per protein dimer (Table 2).

Activity Assays with Metal-Depleted and Metal-Reconstituted Enzyme. Enzymatic activity assays for metal-depleted and metal-reconstituted enzymes were performed using both a rapidly hydrolyzed substrate (paraoxon) and a slowly reactive substrate (demeton S). The native, two metals per monomer, OPH enzyme was observed to hydrolyze paraoxon at diffusion-limited rates (1.3×10^8 M^{-1} s^{-1}), while the reaction rate for demeton S was shown to be 5 orders of magnitude slower (8.7×10^2 M^{-1} s^{-1}). Experimentally, this required a much higher concentration of enzyme for the demeton S assay to observe hydrolysis over a reasonable time of reaction. However, a higher concentration of enzyme in a reaction may minimize the effect of adventitious metals on the reaction rate by decreasing the ratio of metal to enzyme under some assay conditions (*31*). Maximal catalysis of paraoxon during reconstitution experiments occurred when the WT enzyme contained 2 mol of Co^{2+} per monomer. As shown in Figure 3A, the kinetic profile does not reach a plateau until 2 mol of Co^{2+} is provided per mole of monomer. The inclusion of additional Co^{2+} in the reconstitution buffer or the assay buffer did not affect the rates of catalysis. In contrast, when the Co^{2+}-reconstituted WT OPH was used in comparable kinetic saturation experiments with the phosphorothiolate substrate demeton S, the kinetic profile levels off at 1 mol of Co^{2+} per mole of monomer. Although the

Table 2: Metal Contents of OPH Enzymes[a]

enzyme	no. of Zn^{2+}/monomer	no. of Co^{2+}/monomer	total
WT OPH	0.57 ± 0.01	1.3 ± 0.03	1.9
H254R	0.37 ± 0.1	0.78 ± 0.05	1.1
H257L	0.68 ± 0.03	0.40 ± 0.01	1.1
H254R/H257L	0.82 ± 0.1	0.15 ± 0.06	1.0

[a] Metal content was determined by FAAS as described in Experimental Proceedures. See the text for details.

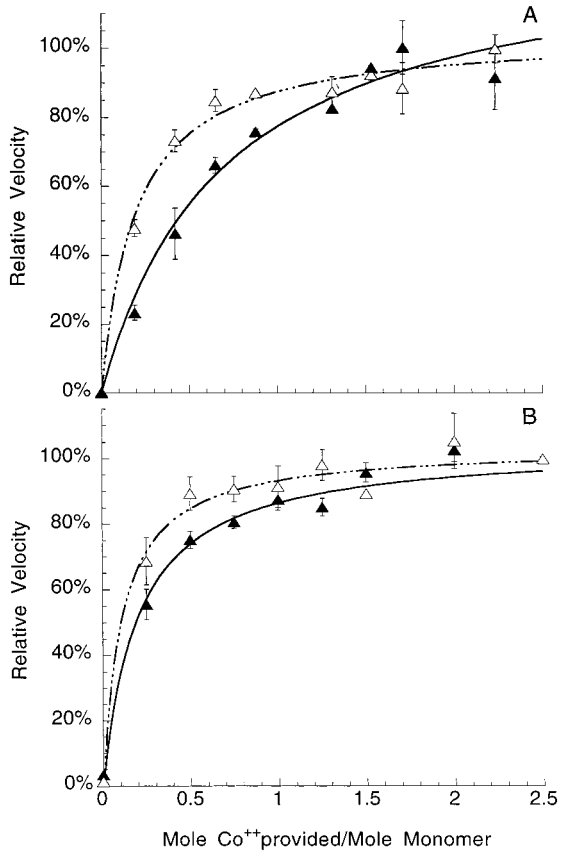

FIGURE 3: Relative activity of Co^{2+}-reconstituted enzymes with 3 mM paraoxon (▲) and 6 mM demeton S (△): (A) reconstituted WT OPH and (B) reconstituted H254R/H257L.

WT enzyme was shown by FAAS to sequester up to 2 mol of metal per monomer (Table 2), the association of a single Co^{2+} per mole of monomer produced maximal hydrolysis, and higher concentrations of metal did not enhance catalysis of demeton S. Despite the evidence that the active sites could accept more metal, additional metal did not contribute to an improved catalytic efficiency of the enzyme relative to the hydrolysis of demeton S.

Similar chelation and reconstitution experiments were performed with each of the altered enzymes, and the experimental data for the H254R/H257L enzyme are shown in Figure 3B and are representative of all OPH variants analyzed in this study. Although up to 3 equiv of Co^{2+} was provided for reconstitution of these enzymes, the kinetic profiles for both paraoxon and demeton S level off at 1 mol of metal per mole of monomer. Additional cobalt in the reconstitution or assay buffers did not improve activity against either substrate.

DISCUSSION

Evidence from the published crystallographic structures of OPH suggests that the histidinyl residues at positions 254 and 257 are not primary metal ligands directly involved in the active centers of the enzyme; however, these residues are involved in an extensive series of interactions with other residues which are directly in contact with substrate, and therefore, these two amino acids function as integral participants at the active site. His254 stacks with both His257 and primary metal ligand His230 in a manner which suggests that these three residues are involved in stabilizing van der Waals stacking interactions (Figure 4A). Varying atoms of His254 appear to participate in hydrogen bond interactions with components of Asp232, Asp233, and the primary metal ligand Asp301 (Figure 4B). The role of His254 as a secondary ligand for both metal ions and its other hydrogen bonding interactions leads us to postulate that the residue at position 254 serves as a key participant in defining the active site structure of OPH. In addition, His257 appears to be directly involved in formation of the hydrophobic substrate pocket. Evidence for this is provided by the Zn^{2+}-substituted structure which shows the interaction of His257 with the methylbenzyl group of the substrate analogue diethyl 4-methylbenzylphosphonate. (This catalytic inhibitor has been required for crystal formation of the two metal-containing enzymes.) Although neither His254 nor His257 is directly involved in metal ligation, various substitutions at positions 254 and 257 have resulted in losses of paraoxonase activity ranging from 10 to 90% (*23*, *24*). Amino acid substitutions at position 254 and/or 257 would be expected to disrupt the numerous hydrogen bonding and electrostatic interactions associated with the side chains of the native residues that are seen in the WT Zn^{2+}-substituted structure. These changes would be expected to impart a new flexibility to the active site that may be responsible for the enhanced catalysis of the altered enzymes for some larger substrates.

It has been proposed that the catalytic mechanism of OPH involves the activation of a nucleophilic water molecule through interaction with the binuclear metal center. For this proposed mechanism, both metals would be required for enzymatic function. Although data from FAAS consistently show that H254R, H257L, and H254R/H257L contain an

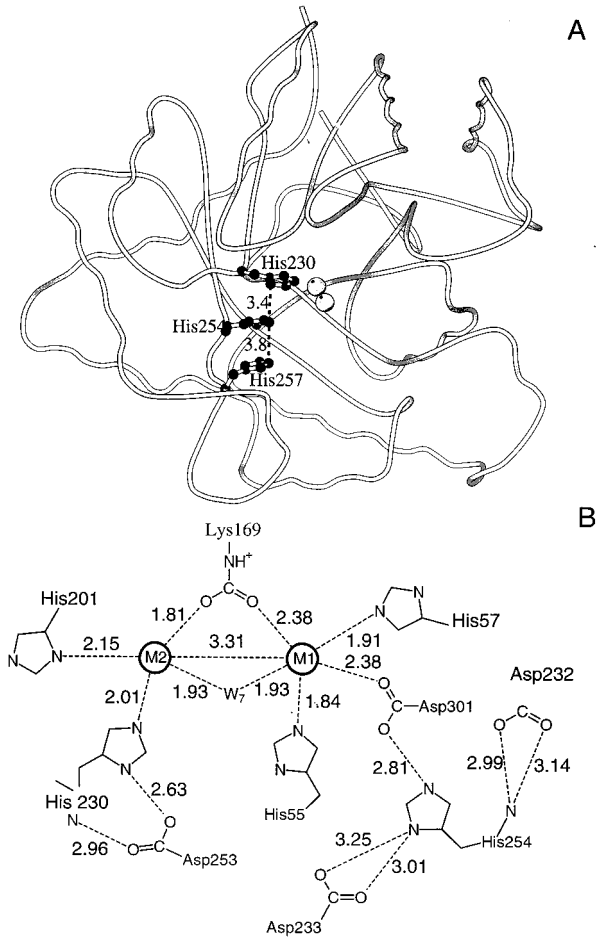

FIGURE 4: (A) Molscript diagram (*40*) depicting the stacking interactions of secondary ligand His254 with His257 and metal ligand His230. (B) Schematic diagram of the active site of WT OPH surrounding the zinc ions. Interatomic distances, which were determined by examination of the Zn^{2+}-containing structure (1DPM), are given in angstroms.

average of two metals per dimer, these enzymes would have severely diminished enzymatic activity if the proposed mechanism is correct. The residual activity of these enzymes would presumably result from an asymmetry in the homodimer such that one of the individual monomers would have two metals with enhanced catalytic capabilities and the other would have no metals. It would be assumed that monomers with a single metal or no metal would not contribute to the detected hydrolysis in keeping with the proposed mechanism. Alternatively, enzymatic variants that show an average of one metal per monomer may have a lower binding affinity for the metals, and under the experimental conditions, half of the metal content would be lost prior to FAAS analysis. Metal chelation and Co^{2+} reconstitution, immediately followed by kinetic characterization, were performed to address these concerns, and the WT enzyme, which is bimetallic, was included to provide an internal control.

Experimental chelation and metal reconstitution of the WT enzyme with various metals have been performed previously (*18*, *32*). During those experiments, the catalytic reactivation of OPH was analyzed by paraoxon hydrolysis as the metals were reintroduced into the enzyme. Comparison of the paraoxon and demeton S activity of Co^{2+}-reconstituted WT OPH (Figure 3A) demonstrated that the metal required by the WT enzyme for maximal hydrolysis is substrate-

dependent. As reported by others, OPH reached maximal paraoxonase activity with two metals provided per monomer, and additional cobalt was not inhibitory to paraoxonase activity (*18*, *32*). However, using the enzyme from the same titration experiment in a tandem assay against demeton S, maximal activity was reached with a single metal provided per monomer. The addition of more metal did not affect the enzymatic activity toward demeton S. The difference in metal requirements for catalysis of the two different reactions suggests that the catalytic mechanism for hydrolysis of paraoxon, and the alternative substrate, demeton S, may be different. This may explain the results of previously reported inhibition studies in which DFP was shown to be a competitive inhibitor of paraoxon hydrolysis (*33*), but demeton S was a noncompetitive inhibitor (*9*).

The altered OPH enzymes examined in this study share the characteristic that maximal hydrolysis of each substrate tested is achieved with only two metals provided per protein dimer (one metal per monomer). Despite this, these enzymes still have good specificity (k_{cat}/K_M) for the preferred substrate, paraoxon. Although the H254R substitution (present also in the double mutant) sacrifices catalytic efficiency in the turnover of paraoxon (k_{cat} values), the losses are compensated by improvements in binding (K_M values). In addition, H254R, H257L, and H254R/H257L show improvement in activity and specificity compared to the WT enzyme with the larger substrates NPPMP and/or demeton S. In contrast, each of the enzymes has diminished activity with the rapidly hydrolyzed, small substrate DFP. It is probable that the loss of one metal from each of the active sites of these enzymes causes subtle rearrangement and enlargement of the substrate binding pocket. If this did occur, the observed increase in rates of hydrolysis of larger substrates with the concomitant diminished specificity for smaller substrates would be expected.

Watkins et al. demonstrated an improved rate of catalysis of OPH for DFP (*34*). This study was stimulated by the Zn^{2+} structure of OPH (1DPM) with a substrate analogue bound at the active site. The active site pocket surrounding the potential leaving group is lined with hydrophobic residues (Trp131, Phe132, Leu271, Phe306, and Tyr309). Because DFP has a fluoride leaving group, replacement of one side chain with a residue capable of hydrogen bond formation and proton donation (His, Tyr, or Lys) was predicted to enhance catalysis. Mutations to active site residues Phe132 and Phe306, including some double mutations, succeeded in enhancing k_{cat} up to 10-fold. However, there was no improvement in K_M, and in fact, the higher K_M values caused the specificity for DFP to be improved only 2-fold compared to that of WT OPH. Our results demonstrated that it is possible to significantly alter the substrate specificity of OPH (up to 30-fold for the VX analogue, demeton S) by mutational substitution near the active site, and that these changes in catalytic characteristics are correlated with the metal content of the enzyme.

The observation that site-directed substitutions of amino acids near the active site of a binuclear, metal center could result in the loss of a single metal without significant loss of catalytic activity has limited precedence. Another enzyme that contains two metals in its native form which has been observed to function catalytically as a monometal enzyme is D-xylose isomerase (*35*). In this case, the bimetallic D-xylose isomerase catalyzes the interconversion of aldose and ketose sugars by sequential catalytic steps of ring opening and isomerization. A monometal variant, E180K, effectively catalyzes the ring opening step at 20% of the native rate but has no isomerase activity. In the case of the hydrolysis of organophosphorus neurotoxins by OPH, the stoichiometry of the metal constituency of the active site seems to be altered without significant change in the characteristics of enzyme catalysis. The changes in enzyme function seem to be related to the size of the active site, rather than to the catalytic efficiencies.

Although the enzymes described in detail in this communication retained kinetic competence for a multitude of substrates, other enzymes resulting from alternative substitutions for the original histidinyl residues at positions 254 and 257 have dramatically diminished activity (*23*, *24*). Alterations for histidinyl residues 55, 57, 201, and 230 (which serve as primary metal ligands in published structures of Zn^{2+}- and Cd^{2+}-substituted WT OPH) have all shown very limited catalytic capability (*23*, *24*, *36*, *37*). Interestingly, substitutions at positions 55 and 57, which serve as ligands for the more buried metal ion, have incurred less catalytic penalty than have substitutions at positions 201 and 230, which serve as ligands for the more solvent-exposed metal ion. This may suggest that the more solvent-exposed metal ion (M_1) is the essential catalytic metal.

Although there are significant functional differences between Cd^{2+}- and Zn^{2+}-substituted enzymes (*18*, *19*), the only noted difference between the Cd^{2+}- and Zn^{2+}-substituted structures was observed in the coordination sphere of the more solvent-exposed cation (*17*). In the Cd^{2+} structure, the ion is ligated in an octahedral arrangement, while the Zn^{2+} structure shows the ion ligated in a distorted tetrahedral environment. Kinetically, there is a larger difference between the Co^{2+}-substituted enzymes and other metal-substituted enzymes; therefore, less subtle structural differences might be expected between a Co^{2+}-substituted structure and the existing Zn^{2+}- and Cd^{2+}-substituted structures that have been published.

If the protein structure is considered to be the structural scaffolding of the enzyme, and the metal ions serve as separate or combined hubs around which much of the active site is anchored, it would logically follow that when one of the metal ions is lost, some of the physical rigidity surrounding the active site might be compromised. The contribution of a single hydrogen bond to the structural stability of proteins has been examined through selective mutagenesis in other proteins, and it will be informative to evaluate the contribution of a second metal ion to the structural stability of OPH. It has been observed that enhanced catalysis resulted from the presence of Co^{2+} or Zn^{2+} in the active site rather than Mn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, or Fe^{2+}. Since Co^{2+} and Zn^{2+} are metal ions with greater inherent coordination flexibility compared with other divalent cations (*38*, *39*), it is possible to propose that the enhanced catalysis of the monometal mutants H254R, H257L, and H254R/H257L might be attributable to enhanced flexibility at the active site due to the loss of one metal. We would suggest that protein engineering involving the loss of one metal binding site in OPH has permitted more flexibility in the active site and results in alterations to the kinetic characteristics of this enzyme over an inherently broad substrate specificity.

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REFERENCES

1. McDaniel, C. S., Harper, L. L., and Wild, J. R. (1988) *J. Bacteriol. 170*, 2306.
2. Mulbry, W. W., and Karns, J. S. (1989) *J. Bacteriol. 171*, 6740.
3. Serdar, C. M., and Gibson, D. T. (1985) *Bio/Technology 3*, 567.
4. Dave, K. I., Miller, C. E., and Wild, J. R. (1993) *Chem.-Biol. Interact. 87*, 55.
5. Dumas, D. P., Durst, H. D., Landis, W. G., Raushel, F. M., and Wild, J. R. (1990*) Arch. Biochem. Biophys. 277*, 155.
6. Shafferman, A., Ordentlich, A., Barak, D., Stein, D., Ariel, N., and Velan, B. (1996) *Biochem. J. 318*, 833.
7. Chae, M. Y., Postula, J. F., and Raushel, F. M. (1994) *Bioorg. Med. Chem. Lett. 4*, 1473.
8. Kolakowski, J. E., DeFrank, J. J., Harvey, S. P., Szafraniec, L. L., Beaudry, W. T., Lai, K., and Wild, J. R. (1997) *Biocatal. Biotransform. 15*, 297.
9. Lai, K., Stolowich, N. J., and Wild, J. R. (1995) *Arch. Biochem. Biophys. 318*, 59.
10. Hoskin, F. C. G., and Walker, J. E. (1997) *Bull. Environ. Contam. Toxicol. 59*, 9.
11. Rastogi, V. K., DeFrank, J. J., Cheng, T.-C., and Wild, J. R. (1997) *Biochem. Biophys. Res. Commun. 241*, 294.
12. Cheng, T.-C., Harvey, S. P., and Chen, G. L. (1996) *Appl. Environ. Microbiol. 62*, 1636.
13. Hassett, C., Richter, R. J., Humbert, R., Chapline, C., Crabb, J. W., Omiecinski, C. J., and Furlong, C. E. (1991) *Biochemistry 30*, 10141.
14. Lewis, V. E., Donarski, W. J., Wild, J. R., and Raushel, F. M. (1988) *Biochemistry 27*, 1591.
15. Benning, M. M., Kuo, J. M., Raushel, F. M., and Holden, H. M. (1994) *Biochemistry 33*, 15001.
16. Benning, M. M., Kuo, J. M., Raushel, F. M., and Holden, H. M. (1995) *Biochemistry 34*, 7973.
17. Vanhooke, J. L., Benning, M. M., Raushel, F. M., and Holden, H. M. (1996) *Biochemistry 35*, 6020.
18. Omburo, G. A., Kuo, J. M., Mullins, L. S., and Raushel, F. M. (1992) *J. Biol. Chem. 267*, 13278.
19. Omburo, G. A. (1992) Ph.D. Dissertation, Texas A&M University, College Station, TX.
20. Grimsley, J. K., Scholtz, J. M., Pace, C. N., and Wild, J. R. (1997) *Biochemistry 36*, 14366.
21. Omburo, G. A., Mullins, L. S., and Raushel, F. M. (1993) *Biochemistry 32*, 9148.
22. Kuo, J. M., and Raushel, F. M. (1994) *Biochemistry 33*, 4265.
23. Lai, K., Dave, K. I., and Wild, J. R. (1994) *J. Biol. Chem. 269*, 16579.
24. Lai, K. (1994) Ph.D. Dissertation, Texas A&M University, College Station, TX.
25. Deng, W. P., and Nickoloff, J. A. (1992) *Anal. Biochem. 200*, 81.
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) *Anal. Biochem. 94*, 75.
28. Grassetti, D. R., and Murray, J. F. (1967) *Arch. Biochem. Biophys. 119*, 49.
29. Auld, D. S. (1988) in *Metallobiochemistry* (Riordan, J. F., and Vallee, B. L., Eds.) pp 13−14, Academic Press, San Diego, CA.
30. Wagner, F. W. (1988) in *Metallobiochemistry* (Riordan, J. F., and Vallee, B. L., Eds.) pp 21−32, Academic Press, San Diego, CA.
31. Holmquist, B. (1988) *Methods Enzymol. 158*, 6.
32. Kuo, J. M. (1995) Ph.D. Dissertation, Texas A&M University, College Station, TX.
33. Dumas, D. P., Wild, J. R., and Raushel, F. M. (1989) *Biotechnol. Appl. Biochem. 11*, 235.
34. Watkins, L. M., Mahoney, H. J., McCulloch, J. K., and Raushel, F. M. (1997) *J. Biol. Chem. 272*, 25596.
35. Allen, K. N., Lavie, A., Glasfield, A., Tanada, T. N., Gerrity, D. P., Carlson, S. C., Farber, G. K., Petsko, G. A., and Ringe, D. (1994) *Biochemistry 33*, 1488.
36. diSioudi, B. (1998) Ph.D. Dissertation, Texas A&M University, College Station, TX.
37. Watkins, L. M., Kuo, J. M., Chen-Goodspeed, M., and Raushel, F. M. (1997) *Proteins 29*, 553.
38. Holland, D. R., Hausrath, A. C., Juers, D., and Matthews, B. W. (1995) *Protein Sci. 4*, 1955.
39. Vallee, B. L., and Williams, R. J. P. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 498.
40. Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946.

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