# Organophosphate hydrolase — an enzyme catalyzing degradation of phosphorus-containing toxins and pesticides

*E. N. Efremenko* and V. S. Sergeeva*

*Department of Chemistry, M. V. Lomonosov Moscow State University, Leninskie Gory, 119899 Moscow, Russian Federation. Fax: +7 (095) 939 5417. E-mail: efremenko@enzyme.chem.msu.ru*

The available investigations on the structure and properties of the enzyme organophosphate hydrolase exhibiting catalytic activity with respect to orthophosphates are reviewed. Recent data on the mechanism of enzymatic hydrolysis of paraoxon are surveyed. The role of two metal ions involved in the enzyme active site is considered. The substrate specificity and the influence of various inhibitors on the kinetic characteristics of the enzyme are discussed.

**Key words:** organophosphate hydrolase, mechanism of enzymatic catalysis, organophosphorus neurotoxins, amines, substrate specificity, inhibitors.

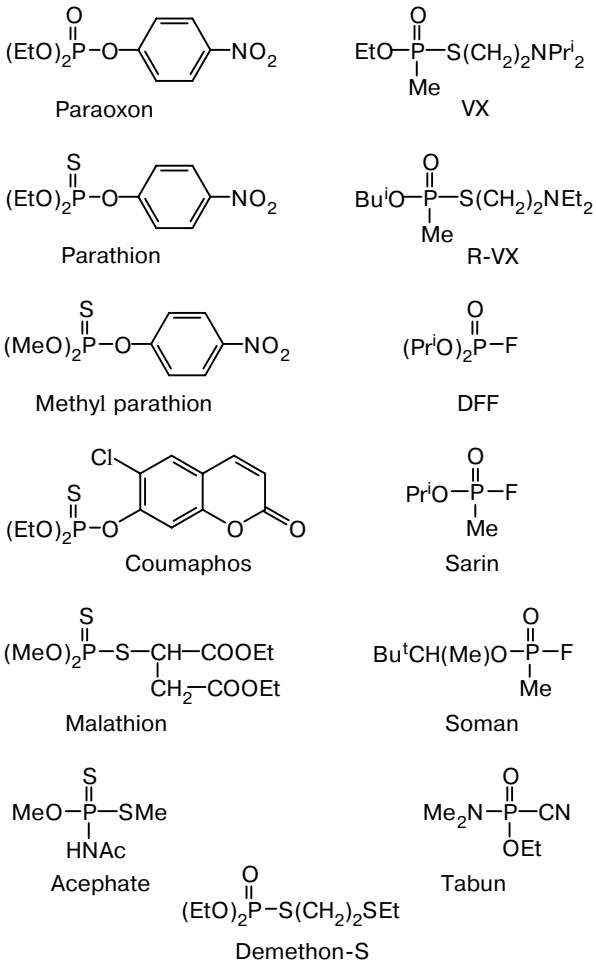
## Introduction

Biological procedures for degradation of organophosphorus (OP) neurotoxins have attracted growing interest in recent years. This group of compounds consists predominantly of pesticides, which are widely used in agriculture, and chemical warfare agents whose reserves must be destructed according to the Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction. Extensive studies are being carried out in a search for microorganisms capable of performing complex degradation of such OP compounds. Organophosphate hydrolase (OPH or phosphotriesterase, EC 3.1.8.1) appeared to be the key enzyme in the biodestruction of the above-mentioned compounds. This enzyme catalyzes hydrolysis of the phosphoester bond in orthophosphates. Originally, the enzyme was isolated from microorganisms *Pseudomonas diminuta* and *Flavobacterium* sp.[1—7] inhabiting in soils contaminated by OP pesticides.

The present review surveys the properties of this enzyme, which assumes currently ever increasing topicality.

## Kinetic characteristics and the substrate specificity of organophosphate hydrolase

Organophosphate hydrolase exhibits activity with respect to a rather broad spectrum of OP neurotoxins. The structural formulas of selected widespread OP pesticides and chemical warfare agents[8,9] which are potential substrates for OPH are given below.




Organophosphate hydrolase represents a dimer with the molecular weight of 72 kDa. The enzyme consists of two identical subunits containing 336 amino acid residues each.[10,11] For OPH, the free energy of formation of the protein globule is ~44 kcal mol$^{-1}$,[12] which is approximately twice as high as those for other dimeric

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**Table 1.** Kinetic parameters of paraoxon hydrolysis catalyzed by organophosphate hydrolase containing different metal ions in the active site[17]

| Metal ion | $k_{cat}$ /$s^{-1}$ | $K_m$ /mmol $L^{-1}$ | $(k_{cat}/K_m) \cdot 10^7$ /$mol^{-1}$ L $s^{-1}$ |
|---|---|---|---|
| $Zn^{2+}$ | 1520±40 | 0.045±0.005 | 3.4 |
| $Co^{2+}$ | 4870±90 | 0.13±0.01 | 3.7 |
| $Cd^{2+}$ | 2460±150 | 0.36±0.05 | 0.68 |
| $Mn^{2+}$ | 1800±40 | 0.065±0.004 | 2.8 |
| $Ni^{2+}$ | 1990±30 | 0.097±0.004 | 2.1 |

proteins.[13,14] It is assumed that interactions between the monomeric subunits exceed entropy losses in the formation of the oligomeric structure.

Organophosphate hydrolase is the metal-dependent enzyme and contains two $Zn^{2+}$ or $Co^{2+}$ ions per subunit. The effect of metal ions on the catalytic activity of OPH was examined in detail.[15—17] It was demonstrated that the enzyme in which the $Zn^{2+}$ or $Co^{2+}$ ions are replaced by the $Cd^{2+}$, $Mn^{2+}$, or $Ni^{2+}$ ions also exhibits catalytic activity. The constants $k_{cat}$ and $K_m$ for the enzyme containing various metal cations in the active site which have been determined for paraoxon (diethyl 4-nitrophenyl phosphate, **1**) as a model substrate of OPH are given in Table 1. It can be seen that the $Co^{2+}$-containing enzyme shows the highest catalytic activity with respect to phosphotriesters.

Investigations into the substrate specificity of OPH[10,11,18—24] have demonstrated that the enzyme catalyzes hydrolysis of the P—O, P—F, and P—S bonds to a different extent. The kinetic parameters of OPH-catalyzed hydrolysis of various OP neurotoxins are given in Table 2. It can be seen that OPH exhibits the lowest specificity with respect to the P—S bond.

Phosphodiesters are generally more stable to hydrolysis than triesters.[25] For example, the observed rate constant for spontaneous hydrolysis of ethyl 4-nitrophenyl hydrogenphosphate at pH 8.0 and 25 °C is

**Table 2.** Kinetic parameters of hydrolysis of organophosphorus pesticides and chemical warfare agents catalyzed by organophosphate hydrolase

| Substrate | Bond type | $k_{cat}$ /$s^{-1}$ | $K_m$ /mmol $L^{-1}$ | $k_{cat}/K_m$ /$mol^{-1}$ L $s^{-1}$ |
|---|---|---|---|---|
| Paraoxon | P—O | 3170 | 0.058 | $5.5 \cdot 10^7$ |
| Parathion | P—O | 630 | 0.24 | $2.6 \cdot 10^6$ |
| Methyl parathion | P—O | 76 | 0.84 | $0.9 \cdot 10^5$ |
| Coumaphos | P—O | 600 | 0.39 | $1.5 \cdot 10^6$ |
| Malathion | P—S | $6.7 \cdot 10^{-3}$ | 0.14 | 47.8 |
| Acephate | P—S | 2.8 | 160 | 17.5 |
| Demethon-S | P—S | 1.2 | 0.78 | $1.5 \cdot 10^3$ |
| VX | P—S | 0.3 | 0.44 | $6.8 \cdot 10^2$ |
| DFF | P—F | 74.7 | 0.96 | $7.8 \cdot 10^4$ |
| Sarin | P—F | 56 | 0.7 | $8.0 \cdot 10^4$ |
| Soman | P—F | 5 | 0.5 | $1.0 \cdot 10^4$ |

$\sim 10^{-10}$ $s^{-1}$, whereas the rate constant for hydrolysis of paraoxon (triester) under the same conditions is $10^{-7}$ $s^{-1}$. It was demonstrated that OPH catalyzes hydrolysis of phosphodiesters, the constants $k_{cat}$ being $\sim 10^5$ times smaller than $k_{cat}$ for hydrolysis of **1**. The $k_{cat}/K_m$ ratio for OPH-catalyzed hydrolysis of diesters can be increased by adding alkylamines to the reaction medium or by introducing positively charged amino acid residues (the replacement of the residue Met137 by Lys or Arg) into the microenvironment of the active site. It is assumed that the amine neutralizes the negative charge on phosphate, thus increasing the electrophilicity of the phosphorus atom.

Later on, the specificity of hydrolysis of a series of chiral and achiral derivatives of **1** containing various combinations of substituents (Me, Et, $Pr^i$, and Ph) was examined.[26,27] Apparently, the investigations of hydrolysis of these compounds were stimulated by the facts that *O*,*S*-esters of thiophosphoric acids, including VX and alkyl phosphonofluoridates (sarin and soman), contain the chiral phosphorus atom, and that the individual isomers of these compounds differ in the inhibiting action on acetylcholinesterase (AChE). For example, all four stereoisomers of soman are potential inhibitors, however, inactivation of the enzyme by the ($S_P$) enantiomers proceeds 2—4 orders of magnitude more rapidly (depending on the source of AChE) than inactivation by the ($R_P$) enantiomers. The kinetic parameters of hydrolysis of different substrates catalyzed by $Zn^{2+}$-dependent OPH are given in Table 3. The data for the $Co^{2+}$- and $Cd^{2+}$-dependent enzyme are also available in the literature.[27] The constant $k_{cat}$ decreases for all substituted phosphotriesters in the series Me > Et > $Pr^i$.

As can be seen from Table 3, the $k_{cat}$ and $k_{cat}/K_m$ values for the $S_P(-)$ enantiomers containing bulky substituents are larger than the analogous values for the $R_P(+)$ isomers, which indicates that the orientation of the *S* isomer in the active site is more favorable for the subsequent nucleophilic attack. The $R_P(+)$ enantiomers inactivate AChE more rapidly, but they are subjected to slower hydrolysis under the action of OPH compared with their less toxic analogs, *viz.*, the *S* enantiomers.[26,27]

## The structure of the active site and the mechanism of action of organophosphate hydrolase

It was established[28] that OPH-catalyzed hydrolysis of OP compounds follows the $S_N2$ mechanism with inversion of the configuration of the phosphorus atom. Studies using the site-directed mutagenesis demonstrated that the replacement of six out of seven His residues in the polypeptide chain of OPH by Asp residues led to a sharp decrease in the catalytic activity of the enzyme.[29,30] In the studies cited, the first model of the active site has been proposed according to which one of the metal ions is coordinated by the residues His55, His57, and His201 and another metal ion is coordinated by the residues His254 and His257. It was suggested[29] that imidazole of

**Table 3.** Rate constants of alkaline hydrolysis and hydrolysis catalyzed by $Zn^{2+}$-dependent organophosphate hydrolase[27] for optically active and optically inactive phosphotriesters of the general formula $p$-$NO_2$—$C_6H_4$—O—P(O)($OR^1$)($OR^2$)

| Substrate | | | $k_{obs}$[a] /$min^{-1}$ | $k_{cat}$ /$s^{-1}$ | $K_m$[b] | ($k_{cat}/K_m$)[c] |
|---|---|---|---|---|---|---|
| $R^1$ | $R^2$ | Chirality | | | | |
| Me | Me | | 2.2 | 7500 | 1050 | $7.2 \cdot 10^6$ |
| Et | Et | | 0.69 | 2280 | 35 | $6.5 \cdot 10^7$ |
| $Pr^i$ | $Pr^i$ | | 0.07 | 170 | 47 | $3.6 \cdot 10^6$ |
| Ph | Ph | | 35 | 3700 | 230 | $1.6 \cdot 10^7$ |
| Et | Me | $R_P$-(+) | | 4000 | 160 | $2.5 \cdot 10^7$ |
| | | $S_P$-(−) | 1.4 | 5800 | 160 | $3.6 \cdot 10^7$ |
| | | (±) | | 4900 | 180 | $2.7 \cdot 10^7$ |
| $Pr^i$ | Me | $R_P$-(+) | | 180 | 490 | $3.6 \cdot 10^5$ |
| | | $S_P$-(−) | 0.47 | 2000 | 43 | $4.7 \cdot 10^7$ |
| | | (±) | | 2000 | 150 | $1.4 \cdot 10^7$ |
| Ph | Et | $R_P$-(+) | | 310 | 170 | $1.8 \cdot 10^6$ |
| | | $S_P$-(−) | 4.3 | 3100 | 18 | $1.8 \cdot 10^8$ |
| | | (±) | | 2700 | 34 | $8.0 \cdot 10^7$ |
| Ph | $Pr^i$ | $R_P$-(+) | | 100 | 110 | $9.1 \cdot 10^5$ |
| | | (±) | 1.5 | 820 | 15 | $5.5 \cdot 10^7$ |

[a] 1.0 *M* KOH.
[b] μmol $L^{-1}$.
[c] $mol^{-1}$ L $s^{-1}$.

the His residue which is not directly involved in the coordination bond with the metal ion serves as a general base catalyst, thus activating the water molecule which acts as a nucleophile. One of the metal ions is involved in the coordination bond with the substrate molecule, thus reducing the electron density on the reaction site.

X-ray diffraction studies of OPH[31—33] demonstrated that six His residues are proximal to the enzyme active site and four of them (His55, His57, His201, and His230) serve as ligands for the metal ions in the active site. In addition, analysis of the apo and holo forms of the enzyme revealed substantial differences in their structures. The structural model of the active site in the $Cd^{2+}$-containing enzyme is shown in Fig. 1.

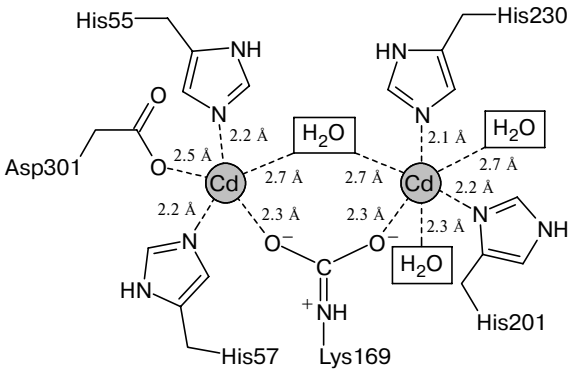



**Fig. 1.** Structural model of the active site of $Cd^{2+}$-containing organophosphate hydrolase.[32]

Two metal ions are linked to each other through the $N^{\varepsilon}$-carboxylated residue Lys169. In the apo form of the enzyme, the residue Lys169 is not modified. It was shown that the presence of hydrogencarbonate at high concentrations accelerates the formation of the enzyme active site on going from the apo form to the holo form in the presence of metal ions. The $^{13}C$ NMR spectroscopic studies established that carbon dioxide is involved in the formation of the carboxylated Lys residue.

The water molecule serves as the second bridging ligand between the metal ions. The coordination sphere about one metal ion has a trigonal-bipyramidal configuration, and that about another metal ion adopts an octahedral configuration. The distance between the metal ions in the active site is 3.8 Å. The residue His254, which is not coordinated to the metal ions, is located at a distance of 7 Å from the active site.

It appeared that the residue Asp253 (it is omitted in the scheme) also plays an important role in the formation of the OPH active site.[32] This residue forms a hydrogen bond with imidazole of the residue His55 in the apo form of the enzyme. In the holo form, the carboxy group of Asp253 is involved in a hydrogen bond with the residue His230, thus providing its favorable spatial orientation. The replacement of the latter residue by Asn leads to a decrease in the $k_{cat}$ value by a factor of 1000.

In addition to the functional groups of the enzyme directly participating in catalysis, three hydrophobic regions were assumed to influence the binding of the substrate, thus governing the specificity of the enzyme.[27,34] The arrangement of the amino acid residues involved in the microenvironment of the active site and the presumed orientation of the substrate analog in the active site of OPH are shown in Fig. 2. The benzyl group of the nonhydrolyzable analog of paraoxon is located in the cavity formed by the residues His257, Leu271, Phe306, and Met317. One of the ethoxy groups interacts with a group of residues consisting of Trp131, Ile106, Leu303, Phe306, and Ser308 and another ethoxy group interacts with the region containing Trp131, Phe132, Leu271, Phe306, and Tyr309. The role of the residue Gly60 is unclear. The residue His254 assists in maintaining the active conformation of the enzyme. It was suggested that the first of the above-described groups of the amino acid residues is better adapted to interactions with bulky and branched hydrophobic fragments, whereas the ethoxy substituents at the phosphorus atom are preferential for two other groups. Since the contribution of Coulomb interactions to binding of orthophosphates in the enzyme active site is insignificant, it is, probably, these two groups of amino acid residues that are responsible for the specificity and stereoselectivity of hydrolysis under the action of OPH.

The kinetic studies of interactions of organic amines introduced into the reaction medium with the functional groups in the enzyme active site elucidated the role of the metal ions in the catalysis performed by OPH.[35]

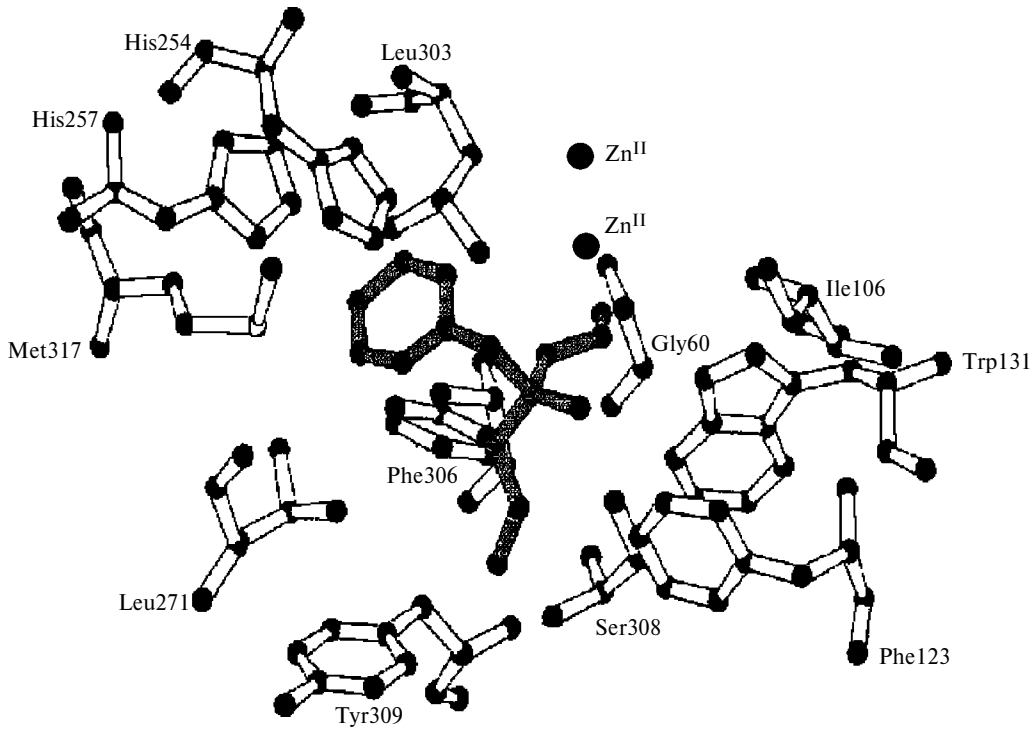



**Fig. 2.** Active site of organophosphate hydrolase in the presence of the coordinated nonhydrolyzable substrate analog ($(EtO)_2P(O)CH_2Ph$).[26] The amino acid residues which are directly bound to the metal ions are omitted.

Examination of the influence of some amines which are characterized by different geometric parameters and basicities on the rate of enzymatic hydrolysis of **1** demonstrated (Fig. 3) that amines can be divided into two groups according to their effect on OPH, *viz.*, those activating OPH (curves *1*—*5*) and those inhibiting the enzyme or exerting no substantial effect on its catalytic activity (curves *6*—*8*).

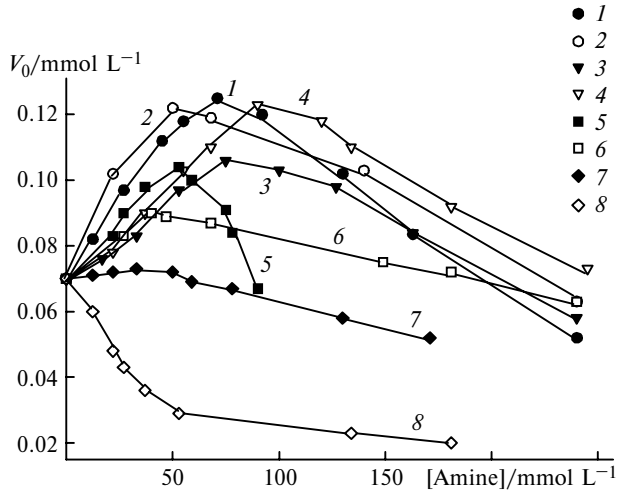



**Fig. 3.** Influence of amines on the initial rate of paraoxon hydrolysis catalyzed by organophosphate hydrolase:[35] *1*, $Pr^i_2NH$ ($pK_a$ 10.96); *2*, $Et_2NH$ ($pK_a$ 10.49); *3*, $Et_3N$ ($pK_a$ 11.01); *4*, $Bu^tNH_2$ ($pK_a$ 10.83); *5*, piperidine ($pK_a$ 11.23); *6*, $NH_3$ ($pK_a$ 9.25); *7*, morpholine ($pK_a$ 8.33), *8*, pyridine ($pK_a$ 5.23).

It was found that in the range of the pH optimum of OPH functioning (pH 8.5—9.5), the enzyme activation is proportional to the concentration of the free form rather than to the concentration of the protonated form of the amine (Fig. 4).

The dependence of $V_m$/[E], which was calculated according to the Michaelis—Menten equation, on the concentration of the free forms of various amines is shown in Fig. 5. Comparison of the slopes of the dependences obtained leads to the conclusion that the

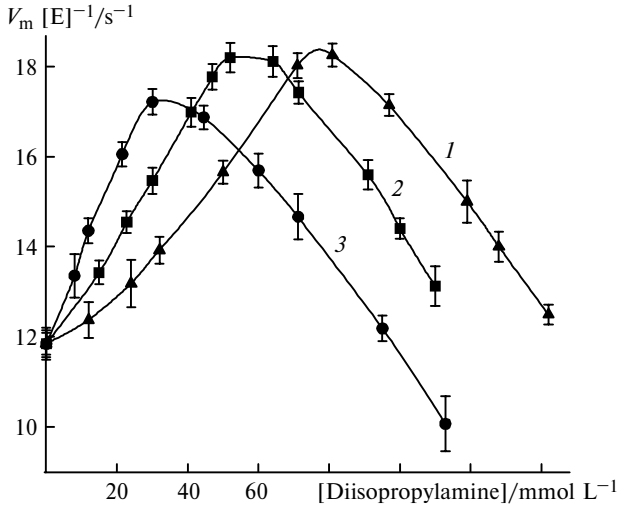



**Fig. 4.** Enzymatic hydrolysis of paraoxon in the presence of diisopropylamine at pH 8.6 (*1*), 9.0 (*2*), and 9.4 (*3*).[35]

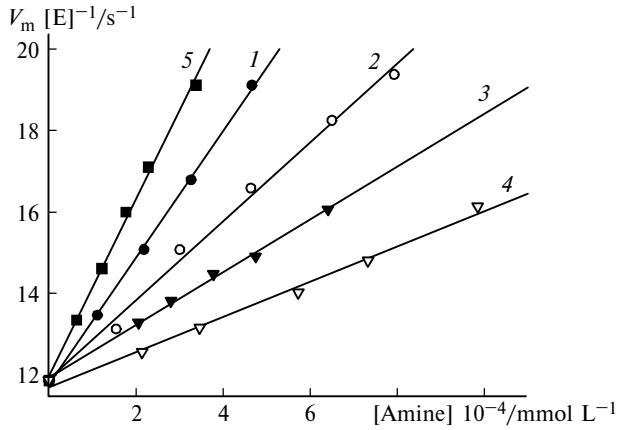



**Fig. 5.** Dependence of $V_m$/[E] for paraoxon hydrolysis catalyzed by organophosphate hydrolase on the concentration of the free form of amine:[35] *1*, $Pr^i_2NH$ ($pK_a$ 10.96); *2*, $Et_2NH$ ($pK_a$ 10.49); *3*, $Et_3N$ ($pK_a$ 11.01); *4*, $Bu^tNH_2$ ($pK_a$ 10.83); *5*, piperidine ($pK_a$ 11.23).

activating action of amines is determined by both $pK_a$ of the amine (decreases in the series piperidine > diisopropylamine > diethylamine) and its structural characteristics (tertiary amines (triethylamine) and amines containing branched radicals (*tert*-butylamine) have lower activating action). It was found that the activation of OPH by amines is noncompetitive.[35]

The fact that OPH is activated by amines made it possible to elucidate some details of the mechanism of the substrate conversion in the enzyme active site. Previously, a possible mechanism of the action of OPH has been discussed[25,32,33] based on the data from X-ray diffraction analysis, but the role of the second metal ion in the active site of OPH has remained an open question.

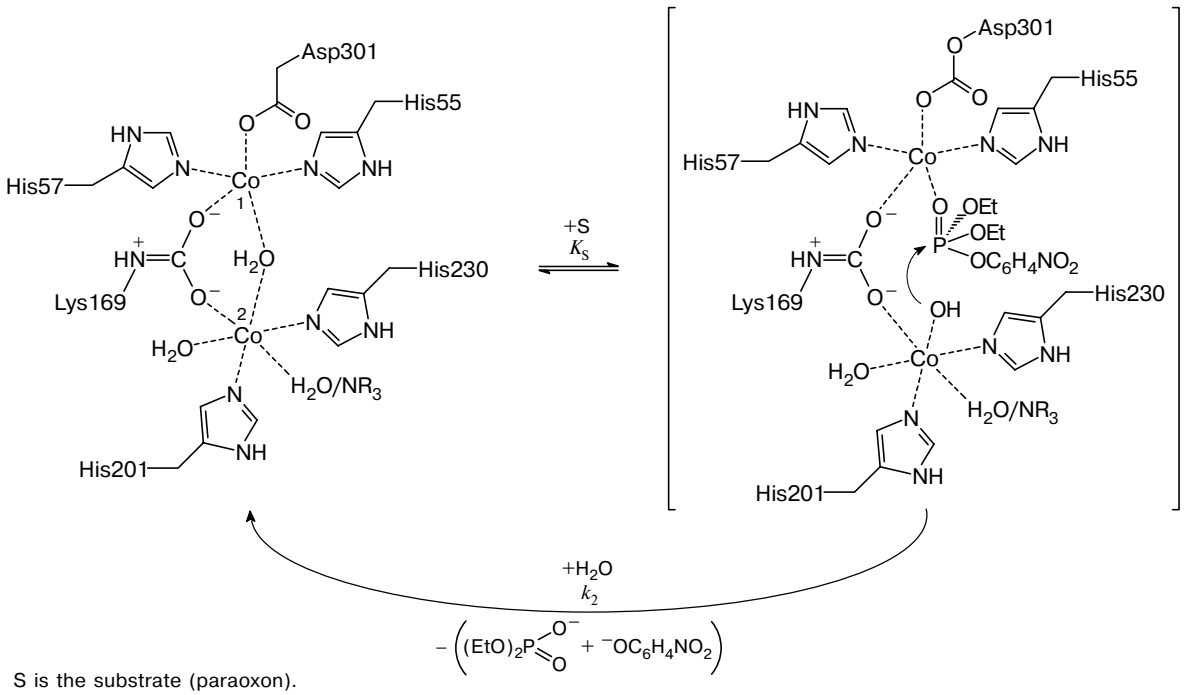
According to the proposed mechanism (Scheme 1),[35] the substrate interacts with the $Co^{2+}(1)$ ion by displacing the bridging water molecule. In our opinion, it is this process that is responsible for a sharp decrease in the activity of OPH at pH > 10. The substrate can displace the water molecule from the coordination sphere of the $Co^{2+}$ ions, but cannot replace the hydroxide ion at higher pH.

Apparently, the amine, which is present in the reaction medium, can be coordinated to both metal ions in the enzyme active site. In the case of interaction with the substrate-binding site, competitive inhibition of the enzyme is observed.

The second $Co^{2+}(2)$ ion decreases $pK_a$ of the water molecule through coordination, thus promoting the formation of the hydroxide ion at pH close to the neutral value.

Binding of the amine with the $Co^{2+}(2)$ ion leads to the replacement of a weaker electron donor (the water molecule) in the coordination sphere of the $Co^{2+}$ ion by a stronger electron donor (amine). As a result, the electron density on the $Co^{2+}$ ion increases, which, in turn, leads to an increase in the nucleophilicity of the attacking $OH^-/H_2O$ ligand. If the water molecule acts as the attacking species in the reaction, the presence of the amine in the coordination sphere of the $Co^{2+}$ ion is favorable for its deprotonation, *i.e.*, for the formation of a strong nucleophile, *viz.*, the hydroxide ion. Apparently, this effect is most pronounced when the amine

**Scheme 1**




S is the substrate (paraoxon).

replaces the water molecule located in the *trans* position with respect to the attacking aqua/hydroxo ligand. It is known that a ligand in the *trans* position with respect to the leaving ligand has a more pronounced effect on the rate of its elimination from the complex compared with the groups located in *cis* positions.[36] The *trans* effect of the ligand decreases in the following series $NR_3 > OH^- > H_2O$.[37] Coordination of the amine molecule in the *trans* position leads to an increase in the nucleophilicity of the leaving aqua ligand. In addition, the latter becomes more labile and undergoes substitution more readily.

To summarize, the following general conclusion about the role of the metal ions in the active site of OPH can be made: one of the metal ions increases the electrophilicity of the phosphorus center through coordination with the non-ester oxygen atom of the substrate, whereas the second metal ion acts as a promoter of the attacking nucleophile.

### Influence of inhibitors on organophosphate hydrolase

Undoubtedly, various metal-chelating agents occupy the first place among the now available inhibitors of OPH. Investigations of the influence of *o*-phenanthroline, EDTA, and pyridine-2,6-dicarboxylate on OPH demonstrated[10,38] that the enzyme inactivation has the first order with respect to the concentration of the chelating ligand. The second-order rate constants determined at pH 9.0 are 5.3, $1.6 \cdot 10^{-3}$, and $2.2 \cdot 10^{-3}$ $mol^{-1}$ L $s^{-1}$ for *o*-phenanthroline, EDTA, and pyridine-2,6-dicarboxylate, respectively.

It was also established that only cysteine and histidine residues (of 20 amino acids) form coordination bonds with the metal ion in the enzyme active site and inactivate OPH with the second-order rate constants of 0.19 and $4.1 \cdot 10^{-2}$ $mol^{-1}$ L $s^{-1}$, respectively.[10]

Compounds containing the sulfhydryl group comprise another known class of OPH inhibitors. β-Mercaptoethanol, dithiothreitol, and dithioerythritol perform competitive inhibition of OPH; the constants $K_i$, determined for these compounds[10] are more than an order of magnitude larger than $K_m$ for **1**.

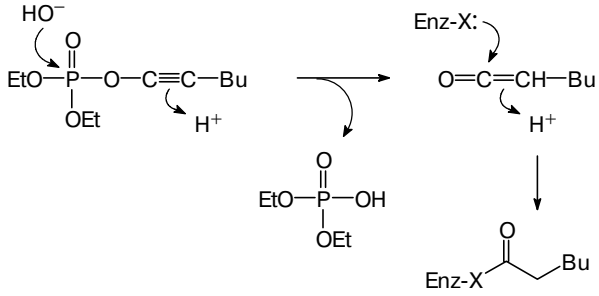
Analysis of the data presented in Fig. 3 allows the conclusion that amines possessing rather low $pK_a$ (Table 4) can also exert inhibiting effects on OPH. It was found that the inhibition was competitive and that the observed value of the constant $K_m$ increased as the amine concentration in the reaction medium increased.[35]

**Table 4.** Inhibition constants of organophosphate hydrolase with amines

| Amine | $K_i$/mol $L^{-1}$ |
|---|---|
| Pyridine | 0.014±0.004 |
| Imidazole ($pK_a$ 6.95) | 0.40±0.03 |
| Morpholine | 0.42±0.05 |
| Diethylamine | 0.18±0.01 |
| Diisopropylamine | 0.11±0.02 |
| Piperidine | 0.011±0.003 |

The introduction of alkynyl phosphates into the reaction medium also resulted in the enzyme inactivation.[39] Hydrolysis of the P—O bond in these esters affords highly reactive ketene intermediates, which, in turn, react with imidazole of the histidine residue (Scheme 2) giving rise to inactive covalent adducts.

**Scheme 2**




Since the use of organic solvents is indispensable in the studies of hydrolysis of organophosphorus neurotoxins which are poorly soluble in water (except for paraoxon), problems associated with the examination of the influence of various solvents on the kinetic properties of OPH attract the attention of researchers.

The inactivating effect of some organic solvents on OPH has been demonstrated.[40—43] Analysis of the data published in the literature allows the conclusion that all polar solvents studied (dioxane, MeCN, EtOH, MeOH, DMF, DMSO, and AcOEt) are competitive inhibitors of OPH, which suggests that it is the formation of the enzyme-substrate complex that is affected. It was found (Table 5) that the inhibiting action of the solvent on OPH correlates with its polarity.[43] Since the binding of uncharged orthophosphates in the active site has a hydrophobic character, a decrease in the polarity of the reaction medium leads, apparently, to a decrease in the

**Table 5.** Inhibition constants ($K_i$) for organophosphate hydrolase in paraoxon hydrolysis in polar solvents with different dielectric permeabilities (ε)

| Solvent | Characteristics of the solvent | | $K_i$/mol $L^{-1}$ |
|---|---|---|---|
| | ε | logP*[44] | |
| AcOEt | 6.0 | 0.68 | 0.030±0.002 |
| EtOH | 24.3 | −0.24 | 0.038±0.04 |
| MeCN | 37.4 | −0.33 | 0.26±0.02 |
| DMF | 37.6 | −1.00 | 0.38±0.03 |
| DMSO | 46.7 | −1.30 | 0.42±0.04 |

* logP is the characteristic of the hydrophobicity of the solvent and P is the partition coefficient for the solvent in a standard two-phase octanol—water system.

rate of the substrate transfer to the hydrophobic microenvironment of the enzyme active site. Hence, polar solvents with high dielectric permeabilities are solvents of choice for enzymatic hydrolysis of OP compounds.

## Conclusion

The available data on the structure and the mechanism of action of OPH allow one to carry out new investigations aimed at performing desired changes in the properties of this enzyme. Thus, attempts are made to replace individual amino acid residues using site-directed mutagenesis with the aim to change the substrate specificity of OPH, in particular, to enhance the affinity of the enzyme for such substrates as chemical warfare agents.[34] It should also be noted that the enzyme exhibits the optimum activity at alkaline pH, whereas most of the reaction products of enzymatic hydrolysis cause substantial acidification of the reaction medium, which leads to enzyme inactivation. In this connection, it is desirable to change the structure of the OPH molecule in such a way as to shift the pH optimum of the enzyme to the neutral or weakly acidic region or to achieve substantial stabilization of the enzyme at low pH. It is generally agreed that the enzyme immobilization is an efficient route for the enhancement of the OPH stability.[2,41,42]

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