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Double effect of organic amines (activation and inhibition) on the phosphotriesterase

Victoria S. Sergeeva, Elena N. Efremenko *, Gregory M. Kazankov, Sergei D. Varfolomeyev

Chemical Enzymology Division of Chemical Department, M.V. Lomonosov Moscow State University, Moscow 119899, Russia

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Abstract

The kinetic behavior of the phosphotriesterase in the presence of organic amines (diethylamine, diisopropylamine, triethylamine, pyridine and others) capable of activating the enzyme at the concentration up to 0.10 M and inhibiting it at higher concentrations is described. The form acting as activator in a solution is the nonprotonated form of amines and the value of activation effect depends on pK_a of amine and its structure. The activation by amines at the above concentrations has a noncompetitive character, whereas the inhibition at higher concentrations has competitive one. The mechanism of phosphotriesterase action in the presence of amines is discussed.

Keywords: Phosphotriesterase; Active site; Molecular mechanism; Activation; Paraoxon

1. Introduction

Phosphotriesterase (EC 3.1.8.1) initially isolated from various microbial bacterial cells [1–4] catalyzes hydrolysis of a broad spectrum of organophosphorus compounds containing P–O, P–F and P–CN bonds [5–12]. The hydrolysis occurs via S_N2-like mechanism [13]. The phosphotriesterase is a metalloenzyme, and its molecule contains two Zn^{2+} or Co^{2+} ions per subunit. The substitution of Zn^{2+} in the active site by Co^{2+}, Cd^{2+}, Ni^{2+} or Mn^{2+} retains the enzyme catalytic activity [14–16]. EPR-study of the Mn^{2+}/Mn^{2+}-substituted enzyme has been shown that two metal ions in the phosphotriesterase active site are antiferromagnetically coupled through a common ligand [15]. The NMR-spectra of the ^{113}Cd-substituted enzyme, site-directed mutagenesis, and studies of the three-dimensional structures of apo- and holoenzymes by the X-ray analysis have shown that the first metal ion of the phosphotriesterase is ligated by His^{55}, His^{57} and Asp^{301} and the second one is connected with His^{230} and His^{201} [11,14,17–20].

Besides the above ligands, a coordination sphere of the second metal ion contains two water molecules. The metal ions are linked by

* Corresponding author. Fax: +7-95-9395417.

E-mail address: efremenko@enzyme.chem.msu.ru (E.N. Efremenko).

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the carbamylated lysine residue (Lys^{169}) and a water molecule as bridging ligands. The coordination sphere of the first metal ion has a trigonal bipyramidal geometry and the other has octahedral configuration [18].

The influence of various effectors such as chelating ligands and amino acids on the phosphotriesterase activity has been described [6,21]. The phosphotriesterase inactivation in the presence of 1,10-phenantroline, 2,2′-bipyridine, EDTA, 2,6-pyridine dicarboxylate has been investigated. The amino acids cysteine and histidine inactivate the enzyme. β-Mercaptoethanol, dithiothreitol and dithioerythritol competitively inhibit the phosphotriesterase.

In this study, the phosphotriesterase catalytic activity in the presence of various amines was investigated. The amines have been shown to be able to both activate and inhibit the phosphotriesterase.

2. Materials and methods

The Co^{2+}-dependent phosphotriesterase isolated from gene-engineered strain *Escherichia coli* DH5α was used in the study. The enzyme was purified as described previously [6]. The paraoxon aqueous solution (10 mM) was used as a substrate. To study the kinetic behavior of the phosphotriesterase in the presence of effectors, the following organic amines were chosen: diethylamine, diisopropylamine, triethylamine, morpholine, *t*-butylamine, dimethylbenzylamine, pyridine, piperidine, ammonium hydroxide and imidazole. All chemicals were purchased from Sigma and Fisher Scientific. The reaction progress was followed by the Hitachi-150-20 spectrophotometer registration of the reaction product — the *p*-nitrophenolate anion ($\varepsilon_{405} = 17{,}000\ M^{-1}\ cm^{-1}$) accumulation, in 200 mM CHES (2-(cyclohexylamino) ethansulfonic acid) buffer, pH 9.0 at 25°C. As a reference solution, the same one but without enzyme was used.

The Michaelis–Menten scheme was applied to describe the paraoxon hydrolysis catalyzed by the phosphotriesterase. Initial rates of the enzymatic reaction at various initial substrate concentrations were analyzed using the Lineweaver–Burk routine ($1/v_0$ vs. $1/[S]$). The investigated amines accelerated the paraoxon hydrolysis and dependencies of k_{cat} on the amine concentration represent a difference in the rate constants of the enzymatic and spontaneous hydrolysis of paraoxon in the presence of amine. The ion strength values of the solutions in all experiments were equal.

3. Results and discussion

Addition of amines into the reaction media containing the phosphotriesterase and the substrate provide different effects on the catalytic activity of the enzyme. Amines can be classified into two groups according to their influence on phosphotriesterase behavior: (1) inhibitors and (2) activators of the enzyme at 0.05–0.1 M but inhibitors at the concentrations above 0.1 M. The results obtained are summarized in Table 1 and Figs. 1 and 2.

3.1. Amines inhibiting the phosphotriesterase

The dependence of the initial rate of enzymatic reaction vs. inhibitor concentration at various substrate concentrations (as $1/v_0$ vs. [I]) showed that amines of the first group (pyridine,

Table 1
Inhibition constants of phosphotriesterase by amines

Amine	pK_a[a]	K_i, M
Pyridine	5.23	0.014
Imidazole	6.95	0.402
Morpholine	8.33	0.416
Diethylamine	10.49	0.178
Diisopropylamine	10.96	0.112
Piperidine	11.12	0.011

[a] Handbook of Chemistry and Physics; 64-edition, CRC Press, 1983.

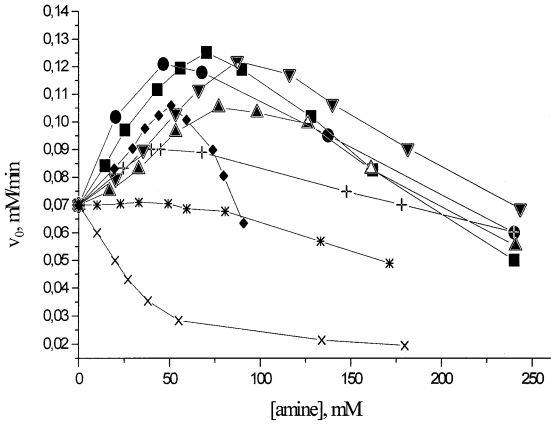


Fig. 1. Influence of various amines on the initial rate of the paraoxon hydrolysis reaction catalyzed by the phosphotriesterase. ■ — Diisopropylamine (pK_a 10.96), ● — diethylamine (pK_a 10.49), ▲ — triethylamine (pK_a 11.01), ▼ — *t*-butylamine (pK_a10.83), ♦ — piperidine (pK_a 11.23), + — ammonium hydroxide (pK_a 9.25), × — pyridine (pK_a 5.23), * — morpholine (pK_a 8.33). Reaction was assayed in CHES-buffer, pH 9.0 at 25°C, [E] = 2.5×10^{-7} M, [paraoxon] = 1.2 mM.

morpholine, dimethylbenzylamine and imidazole) are competitive inhibitors (Table 1).

3.2. Amines activating the phosphotriesterase

As it follows from Fig. 2, the k_{cat} changes from 11.8 to 18.8 s^{-1} are observed at optimal

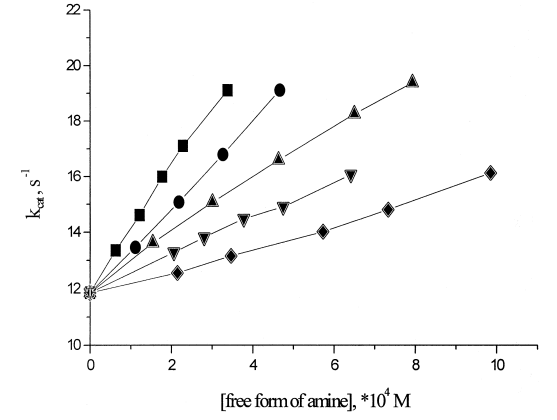


Fig. 2. Activation of the phosphotriesterase by amines. The activation by amine is proportional to its pK_a value and also is connected with structural features of amine molecule, i.e. the effect of activation is less for tertiary amines and for amines with the bulky radical. ■ — Piperidine (pK_a 11.23), ● — diisopropylamine (pK_a 10.96), ▲ — diethylamine (pK_a 10.49), ▼ — triethylamine (pK_a 11.01), ♦ — *t*-butylamine (pK_a10.83). pH 9.0 at 25°C, [E] = 2.5×10^{-7} M.

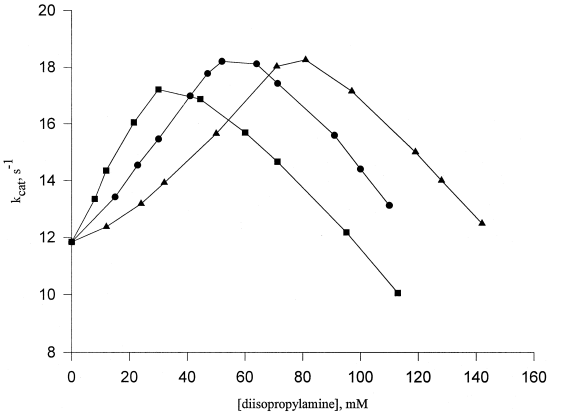


Fig. 3. Enzymatic hydrolysis of paraoxon in the presence of diisopropylamine at various pH. The activation is directly proportional to concentration of the free form of amine (■ — pH = 9.4, ● — pH = 9.0, ▲ — pH = 8.6). 25°C, pH 9.0, [E] = 2.5×10^{-7} M, [paraoxon] = 1.2 mM.

concentrations of diisopropylamine and diethylamine. The effect of activation by other amines is about 40–60%. To explain the activating effect of amines, it might be supposed that the amine acts as a nucleophile in the reaction instead of water. In this case, the corresponding amide should be formed in the reaction medium. However, the ^{31}P NMR study showed the absence of the amide formation in the reaction medium for diisopropylamine (data not shown).

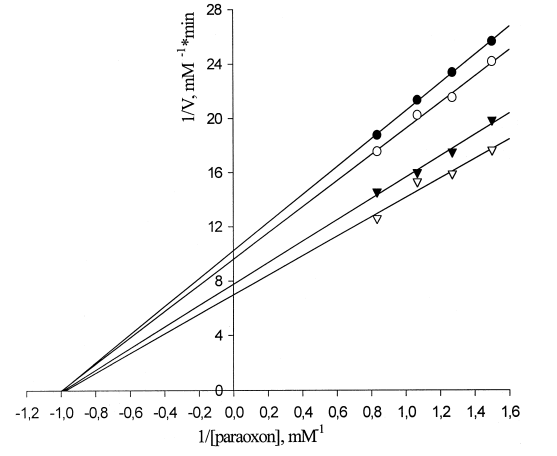


Fig. 4. Noncompetitive activation of the phosphotriesterase by triethylamine (● — in the absence of triethylamine, ○, ▼, ▽ — concentration of triethylamine 0.021, 0.072, and 0.096 M, respectively). 25°C, pH 9.0, [E] = 2.5×10^{-7} M.

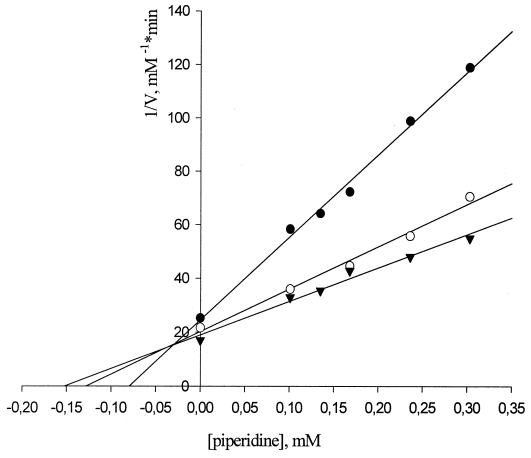


Fig. 5. Competitive inhibition of the phosphotriesterase catalyzed paraoxon hydrolysis reaction by piperidine (concentration of paraoxon ● — 0.9 mM, ○ — 1.2 mM, ▼ — 1.5 mM, respectively). 25°C, pH 9.0, $[E] = 2.5 \times 10^{-7}$ M.

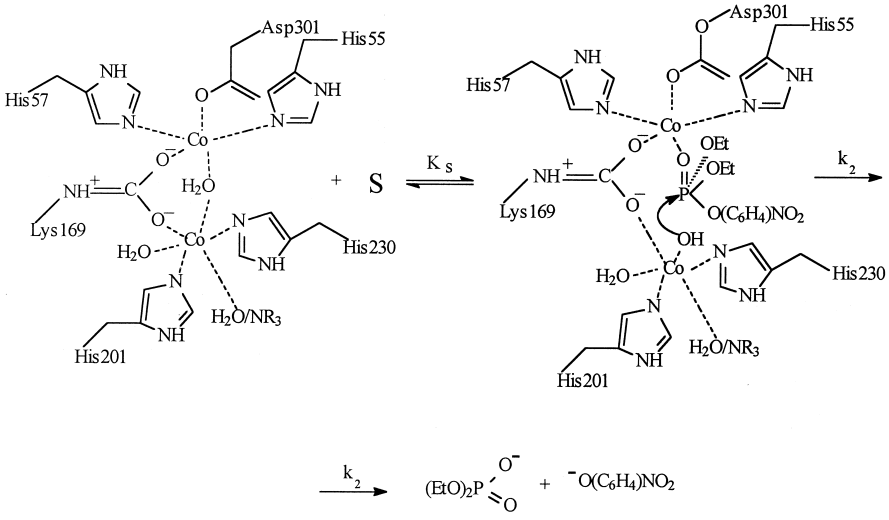
Moreover, a tertiary amine (triethylamine), for which the formation of amides is impossible, enhances the enzymatic paraoxon hydrolysis. This fact forced to propose a different activation mechanism.

The experiments with amines were carried out at such pH conditions supposing amines to exist in both protonated and free forms, it was thus necessary to find out which form was in fact active. Analysis of the kinetic parameters of the enzymatic reaction carried out in the presence of amines at various pH shows that the activation is dependent on the concentration of the free form of amine (Fig. 3).

The activation of the phosphotriesterase by amines was found to be proportional to its pK_a value (thus, the activation decreases in the series: piperidine > diisopropylamine > diethylamine) and to be also affected by bulkiness of the amine molecule (the effect is smaller for tertiary amines and for amines with bulky radicals).

The next step was to define the types of enzyme activation and inhibition by higher concentrations of amines. The initial rate of enzymatic hydrolysis of paraoxon was investigated as a function of concentration of amine (activating and inhibiting) and substrate. The results shown in Fig. 4 allow to conclude that the activation of the phosphotriesterase by amines has a "noncompetitive" character (experimental data were analyzed using $[S]/v_0$ vs. [S] and $1/v_0$ vs. 1/[S] plots). In other words, the substrate and the activator do bind with the enzyme active site independently from each other and this increases the reaction rate [22].

The inhibition of phosphotriesterase by higher amine concentrations was studied for piperidine,



Scheme 1.

diethylamine and diisopropylamine and was found to be competitive (Fig. 5). The experimental values of K_i determined for these amines are given in Table 1.

3.3. The mechanism of amine action on the phosphotriesterase

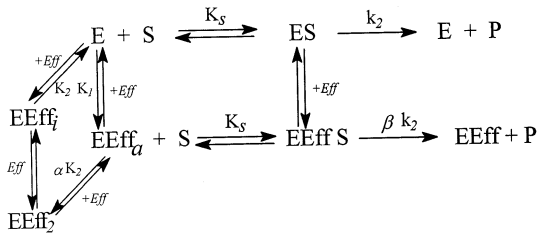
We propose the following mechanism of amine action on the phosphotriesterase (Scheme 1) based on the data from the literature [17–20,23] and obtained in the current work.

According to this scheme, the substrate replaces the bridging water molecule and coordinates with the first Co^{2+} ion in the enzyme active site. The loss of the enzymatic activity at pH higher than 10.0 is explained by the presence of water in the coordination sphere of Co^{2+} and its existence in two forms (H_2O/OH^-). A substrate can replace the water molecule in the coordination sphere of Co^{2+} but cannot replace a hydroxide ion.

The second Co^{2+} ion increases the acidity of water owing to coordination. The coordinated hydroxide ion is formed at low pH. Amine can interact with both Co^{2+} ions in two parallel processes. If an amine molecule interacts with the first metal ion, the competition between the amine and a substrate for the coordination center is observed. If an amine molecule binds with the second Co^{2+} ion replacing the water molecule, the nucleophilicity of attacking nucleophile increases because of the amine is stronger electron pair donor than water in the Co^{2+} coordination sphere. The results obtained in this study are in the complete agreement with the phosphotriesterase three-dimensional structure reported by Benning et al. [17,18].

The kinetic behavior of the phosphotriesterase in the presence of effectors can be described by Scheme 2 assuming the existence of two independent coordination centers in the system.

In conclusion, it should be noted that the study presented and results obtained could help to detail the molecular mechanism of enzyme catalysis of such compounds as phosphotriesters on one hand. On the other hand, the observation of amines as low molecular weight activators of such enzyme as the phosphotriesterase containing two metal ions in its active site gives hope that this example may not be unique, but typical for other metalloenzymes. This supposition has to be confirmed or rejected in the future investigations of corresponding enzymes.



Scheme 2. Eff — amine molecule, $EEff_i$ complex of an amine with substrate binding Co^{2+}-ion, $EEff_a$ — complex of an amine with a hydroxide-forming Co^{2+}-ion, $EEff_2$ — nonactive complex of the phosphotriesterase with two amine molecules.

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