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ORGANOPHOSPHORUS COMPOUNDS AS ACTIVE SITE-DIRECTED INHIBITORS OF ELASTASE

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SUMMARY

Four ethyl p-nitrophenyl alkylphosphonates were studied for the inhibition of elastase. A pH-dependence study using the assay substrate BOC-Ala-ONp or the phosphonate inhibitors showed the participation of an ionizing group with an apparent pK_a of 6.9 and a decrease of reaction or inhibition at higher pH. Out of the four compounds investigated ethyl p-nitrophenyl pentylphosphonate was found to be the best inhibitor of elastase as judged from the value of k_2/K_I. This value, which is the measure of inhibitory capacity, is the highest reported so far for the inhibition of elastase.

INTRODUCTION

Elastase is a pancreatic (1), proteolytic (2) enzyme which possesses remarkable similarities in its chemical, physical, and biological properties to two other pancreatic proteolytic enzymes, trypsin and chymotrypsin. These three enzymes differ only in their substrate specificity: chymotrypsin recognizes large hydrophobic side chains; trypsin positively-charged side chains of lysine and arginine; and elastase small aliphatic residues especially alanine derivatives. Several groups of workers have determined the complete three-dimensional structures of all three enzymes (3-6). There is a remarkable degree of similarity in their catalytically active sites. They are almost identical, all containing an active site serine, which is acylated in the catalytic process, and the side chains of histidine and aspartic acid in very similar geometrical arrangements. Furthermore, these three enzymes are characterized by the presence of binding pockets adjacent to the catalytic sites. The binding pocket of chymotrypsin is hydrophobic and open, the trypsin binding pocket is similar to that of chymotrypsin but contains a

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negatively charged aspartate ion in the bottom, and the binding pocket of elastase is narrow and shallower because of the presence of a bulky valine residue near the front of the cavity (7).

A number of investigations have suggested that elastase may be involved in pathological processes associated with elastic tissue damage in pulmonary emphysema (8).

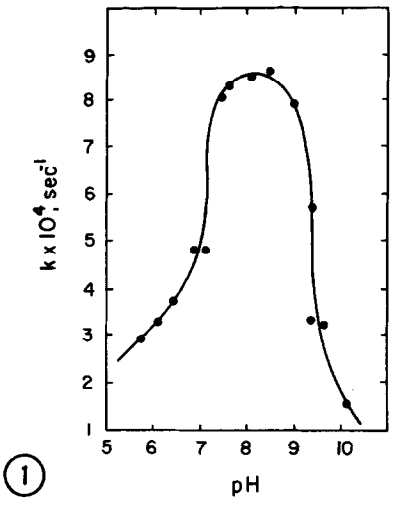
Inhibitors provide a valuable tool both for the characterization of purified enzymes as well as for deducing their role in vivo. Synthetic inhibitors with a variety of functional groups have been shown to react stoichiometrically with elastase. Organophosphorus compounds have served as tools in the investigation of primary structure of the active center since their radioactive derivatives could be used as tags. Additionally, these stoichiometric inhibitors can provide a means of studying the catalytic mechanism in a unique way because, in reality, they are substrates and, therefore, in reacting with the enzyme, make use of the same specificity factors involved in the attack of the enzyme upon substrates.

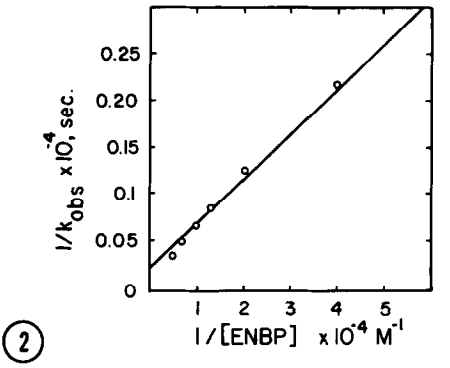
In this communication, we wish to report an account of the inhibition of elastase with four ethyl p-nitrophenyl alkylphosphonates (alkyl = n-propyl, n-butyl, n-pentyl and n-hexyl), the phosphorus analogs of specific substrates of elastase.

MATERIALS AND METHODS

Porcine elastase was a Worthington Biochemical Corp. purified product used without further purification; its substrate BOC-Ala-ONp, was purchased from Vega-Fox Biochemicals. Synthesis of ethyl p-nitrophenyl butylphosphonate (ENBP): Diethyl butyl-phosphonate was prepared by the application of Michaelis-Arbuzov reaction (9) in which equimolar quantities of triethylphosphite and n-butyl bromide were heated under reflux at 150° to give diethyl butylphosphonate and ethyl bromide. Ethyl butylphosphonochloridate was synthesized by the reaction of diethyl butylphosphonate and phosphorus pentachloride in the cold. Ethyl p-nitrophenyl butylphosphonate was prepared by reacting ethyl butylphosphonochloridate with sodium p-nitrophenoxide in toluene according to the method of Fukuto and Metcalf (10). The other three inhibitors which involve a change in the alkyl groups to n-propyl, n-pentyl and n-hexyl were synthesized according to the above procedure.

Stock solutions of substrate and inhibitor were prepared in acetonitrile (Spectro grade, Eastman Kodak Co.). Buffer solutions were made from





1. pH dependence of the inhibition of elastase by ENBP, 25°, E = 2×10^{-6} M; I = 1×10^{-4} M; substrate = BOC-Ala-ONp = 5×10^{-5} M.
2. Determination of the inhibition constant of elastase by ENBP at 25°, pH 7. E = 2×10^{-6} M, I = 0.25 - 2×10^{-4} M, substrate = BOC-Ala-ONp = 5×10^{-5} M.

reagent grade chemicals of ionic strength, I - 0.1.

Elastase inhibition. A 25 to 100 fold molar excess of inhibitor over enzyme was used for the inhibition studies. Inhibition was carried out at 25° in buffers of pH 5-6, acetate; 6-8, phosphate; 8-10, Tris. Progress of inhibition was followed by assaying against BOC-Ala-ONp as substrate using a Cary 14 PM recording spectrophotometer equipped with thermostatted cell compartment at 347 nm.

RESULTS AND DISCUSSION

The effect of pH upon the reaction of elastase with ethyl p-nitrophenyl n-butylphosphonate (ENBP) was studied in the pH range 5.8 - 10. The pH dependence of the butyl compound was studied, since inhibition by the pentyl compound might make it too fast to measure at the optimal pH. The bell-shaped pH-rate profile for this inhibition is shown in Fig. 1. Maximum inhibition was observed between pH 7.5 to 9. The pH profile shows a dependence on the presence of an ionizable group of pK_a 6.9, which can be identified as of an imidazole moiety of an histidine residue. Geneste and Bender (11) reported a similar rate profile with an apparent pK_a of 6.85 in the hydrolysis of the sub-

strate, N-furyl-acryloyl-L-alanine methyl ester, by elastase. The decrease in rate of inhibition at higher pH is quite interesting. This might be due to either a conformational change of the active site of the enzyme or to denaturation of the enzyme.

Kinetics of Inhibition

The rate of inhibition of elastase by ENBP was investigated as a function of inhibitor concentration at pH 7 and 25°. Fig. 2 reveals the presence of a reversible complex between enzyme and inhibitor preceding covalent bond formation. The reaction pathway may be expressed as follows:

$$E + I \rightleftharpoons E \cdot I \longrightarrow E - I \qquad (1)$$

$$K_I = [E][I] / [E \cdot I] \qquad (2)$$

Where E · I represents a noncovalent complex of enzyme with inhibitor, and E - I is the final product with the inhibitor irreversibly bound to the enzyme. Here K_I is the dissociation constant of the E · I complex and k_2 is the limiting rate of inhibition. Since the initial inhibitor concentration is considerably higher than the total enzyme concentration, the pseudo-first-order rate constant of elastase inhibition, k_{obs}, may be given by the equation

$$1/k_{obs} = \frac{1}{k_2} + \frac{K_I}{k_2[ENBP]} \qquad (3)$$

In Fig. 2, $1/k_{obs}$ is plotted vs. $1/[ENBP]$ and the value of $1/K_I = -1/[ENBP]$ when $1/k_{obs} = 0$. The values of K_I and k_2 were computed to be 2×10^{-4} M and 4×10^{-3} sec^{-1}, respectively. The inhibition parameter, k_2/K_I, analogous to the catalytic parameter, k_{cat}/K_m, was computed to be 20. The k_2/K_I values for three other alkylphosphonate esters made by changing the alkyl group to n-propyl, n-pentyl and n-hexyl were computed to be 8.5, 90 and 4 respectively. It is evident that out of the four alkylphosphon-

ate esters studied for the inhibition of elastase, the most effective and specific inhibitor is ethyl p-nitrophenyl n-pentylphosphonate. It inhibits elastase 100% within 20 minutes and the k_2/K_I value (which is the best measure of inhibitory capacity) is 90. This is higher than any value reported in the literature (12-13), using various inhibitors of elastase.

In recent years several investigators have initiated studies of elastase inhibitors in animal models of emphysema (14-15). There is a good possibility that the course of the disease could be arrested by the use of an appropriate inhibitor. Since ethyl p-nitrophenyl n-pentylphosphonate is a potent inhibitor for elastase, this might be used as a powerful synthetic inhibitor for the treatment of emphysema for which at present there is no suitable treatment.

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