Alkynyl phosphates are potent inhibitors of serine enzyme

Dina Segal[a], Channa Shalitin[a], Yechiel Shalitin[a,*], David R. Fischer[b], Peter J. Stang[b,*]

[a]*Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel*
[b]*Department of Chemistry, University of Utah, Salt Lake City, UT 84112, USA*

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Abstract Propynyl, hexynyl and *t*-butylethynyl diethyl phosphates were found to be very powerful covalent inhibitors of serine enzymes. Esterases were inhibited with second-order rate constants of 10^7–10^8 M^{-1} min^{-1}. Most proteases were inhibited with a rate constant of 10^4–10^5 M^{-1} min^{-1}. By inhibiting chymotrypsin with (3-^{14}C)-1-propynyl diethyl phosphate, it was established that inhibition was caused by binding of the phosphate group to the enzyme active site.

Key words: Alkynyl phosphate; Covalent inhibition; Serine enzyme

1. Introduction

Enzyme inhibitors find wide applications in a variety of areas. Anticholinesterase phosphate and carbamate derivatives are extensively used as pesticides [1,2]. Inhibitors of esterases and proteases are applied as chemotherapeutic drugs. In some cases the unregulated activity of an enzyme can lead to accumulation of undesirable products or to decomposition of vital metabolites, which has pathological effects. Inhibitors of the enzyme involved can serve as drugs which alleviate the pathological results. For example, glaucoma and Alzheimer's disease are treated with acetylcholinesterase (AChE) inhibitors which reduce the activity of the enzyme so that acetylcholine concentration is increased and reduces the pathological effects [3,4].

Studies of inhibitor interactions with enzymes are of importance for the design of drugs for therapeutic application.

A few years ago we showed that the alkynyl esters propynyl benzoate and propynyl methoxybenzoate reacted with a variety of proteases and esterases forming a relatively stable acyl-enzyme intermediate so that the enzymes were practically inhibited [5].

As many 'active' phosphates are generally good inhibitors of proteases and esterases, it was of interest to study the interaction of the new types of compounds alkynyl phosphates [6,7] with a variety of enzymes.

1-Propynyl diethyl phosphate (PDEP, I), 1-hexynyl diethyl phosphate (HDEP, II) and 1-*t*-butylethynyl diethyl phosphate (*t*-BEDEP, III) were found to be very potent inhibitors of serine enzymes. Chymotrypsin, for instance, was inhibited with a rate constant of 10^5 M^{-1} min^{-1}, while AChE was inhibited with a rate constant of 4×10^7 M^{-1} min^{-1}. These rate constants are higher than those found for other powerful inhibitors of the above-mentioned enzymes.

$$CH_3C\equiv C-O-P(O)(OC_2H_5)_2 \quad \text{I}$$

$$CH_3(CH_2)_3C\equiv C-O-P(O)(OC_2H_5)_2 \quad \text{II}$$

$$(CH_3)_3CC\equiv C-O-P(O)(OC_2H_5)_2 \quad \text{III}$$

Alkynyl phosphates can react with serine enzymes via different pathways:

(1) A phosphorylation of the serine group at the active site (Scheme 1).

$$EOH + RC{\equiv}COP(O)(OC_2H_5)_2 \xrightarrow{H_2O} EOP(O)(OC_2H_5)_2 + RCH_2COOH$$

EOH designates the serine enzyme.

Scheme 1.

(2) Previous studies suggested that in some cases alkynyl esters behave as suicide substrates. Thus, pig liver esterase started to hydrolyze propynyl benzoate quite rapidly, but the rate slowed down during the course of the reaction and eventually stopped when a portion of the substrate was still not affected by the enzyme [5]. Raushel and Stang and coworkers [8,9] studied the reaction of phosphotriesterase with alkynyl phosphate esters and demonstrated that the ester was a good substrate of the enzyme but the product formed inhibited the enzyme. It was concluded that the 'latent' ketene in the alkynyl group is liberated by hydrolysis and inhibits the enzyme. Thus the inhibition is of a mechanism-based type (Scheme 2).

$$EXH + RC{\equiv}CO\text{-}P(O)(OC_2H_5)_2 \rightarrow EXH + HO\text{-}P(O)(OC_2H_5)_2 + RCH{=}C{=}O$$

$$EXH + RCH{=}C{=}O \rightarrow EX\text{-}C(O)CH_2R$$

X denotes a nucleophilic group on the enzyme.

Scheme 2.

(3) Theoretically, a nucleophile can add to the alkynyl triple bond forming a covalent adduct which is enzymatically inactive (Scheme 3).

$$EXH + RC{\equiv}COP(O)(C_2H_5)_2 \xrightarrow{H_2O} EX\text{-}\underset{R}{C}{=}CHOP(O)(OC_2H_5)_2$$

Scheme 3.

It was of interest to establish which of the above mechanisms was operative in our study of enzymic inhibition. To this

*Corresponding authors. Y. Shalitin, Fax: (972) 4-8225153 or P.J. Stang, Fax: (801) 581-8433.

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CT, chymotrypsin; *t*-BEDEP, 1-*t*-butylethynyl diethyl phosphate; HDEP, 1-hexynyl diethyl phosphate; PDEP, 1-propynyl diethyl phosphate; DFP, diisopropyl fluoro phosphate; TCA, trichloroacetic acid; PAM, pyridine aldoxime methiodide; Z-Ala-pNP, benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester; BAEE, benzoylarginine ethyl ester; ATEE, acetyltyrosine ethyl ester; PMSF, phenylmethanesulfonyl fluoride; cpm, counts per minute

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end we treated α-chymotrypsin (CT) with PDEP labeled with ^{14}C at the propynyl moiety and found that practically no radioactivity was bound to the enzyme, indicating that the phosphate and not the alkynyl group was responsible for the covalent inhibition.

2. Materials and methods

Enzymes were purchased from Worthington and Sigma. Substrates were obtained from Sigma. The inhibitors PDEP, HDEP and *t*-BEDEP were prepared according to Stang et al. [6,7]. 3-^{14}C-labeled PDEP [8] was prepared with a specific activity of 0.17 mCi/mmol.

2.1. Enzymic reactions

The activities of chymotrypsin (CT), subtilisin and proteinase K were determined with acetyltyrosine ethyl ester (ATEE) and those of trypsin, plasmin, kallikrein and thrombin were assayed with benzoylarginine ethyl ester (BAEE). The activity of elastase was estimated by using benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester (Z-Ala-pNP). Pig liver esterase was assayed with ethyl butyrate and cholesterol esterase and lipase were assayed using *p*-nitrophenyl decanoate as a substrate. Enzymic activity was generally followed in the pH stat at pH 8, 25°C in the presence of 0.1 M NaCl. Hydrolysis of the chromogenic *p*-nitrophenyl ester substrates (0.1 mM) was monitored spectrophotometrically at 400 nm. The activities of AChE and butyrylcholinesterase (BChE) were determined by the method of Ellman et al. [10] using acetylthiocholine iodide and butyrylthiocholine iodide, respectively, as substrates.

For conducting the inhibition experiments a stock solution (1–10 mM) of the alkynyl phosphate dissolved in acetonitrile was prepared and a measured amount of the solution was added to an enzyme solution (0.05–1 mg/ml) at pH 8 (0.1 M phosphate buffer) 25°C. The final inhibition mixture contained 5% (v/v) acetonitrile. At timed intervals an aliquot of the reaction mixture was removed and added to a substrate solution and the enzymic activity followed. From the residual activity as a function of time the rate constant of inhibition was calculated. In order to estimate the rate constant for very fast reactions, we used the method of competitive covalent inhibition of the enzyme in the presence of a substrate [11].

2.2. Determination of active site concentration by titration

To several vials containing the same amount of enzyme in 0.1 M phosphate buffer, pH 8 and 25°C, we added increasing amounts of alkynyl phosphate. The residual activity was assessed at different times until it remained unchanged. The loss of enzymic activity was proportional to the amount of alkynyl phosphate added, and the minimal concentration of the ester that abolished activity was equivalent to the active enzyme concentration.

2.3. Radioactive labeling of chymotrypsin

In order to elucidate the mechanism by which alkynyl phosphates inhibit enzymes we treated CT with (3-^{14}C)-1-propynyl diethyl phosphate.

One milligram of CT (40 nmol) was dissolved in 1.0 ml of phosphate buffer, pH 8. ^{14}C-labeled PDEP was dissolved in acetonitrile to a concentration of 12 mM (determined by radioactivity counting). Five microliters of this solution was added to the enzyme solution (50% molar excess of PDEP). After 5 min (a period of time sufficient for complete inhibition) 2 ml of cold 25% trichloroacetic acid (TCA) was added to the reaction mixture and it was placed on ice for 30 min. A precipitate of the protein formed and was then filtered through a GF/C glass-fiber filter. The filtrate was saved, and the precipitate on the filter was thoroughly washed with 10 ml of 8% cold TCA. The glass-fiber filter containing the precipitate was dried under a lamp, inserted in 3 ml Opti-Fluor liquid scintillation cocktail (Packard) and the radioactivity determined in a Packard scintillation counter. One milliliter of the filtrate was mixed with 2 ml of scintillation liquid and its radioactivity counted. Also the radioactivity of the washings was determined. The experiment was run in triplicate. In another experiment chymotrypsinogen, which cannot react with active-site-directed inhibitors, was treated similarly to CT. A parallel control experiment was performed in which no protein was present.

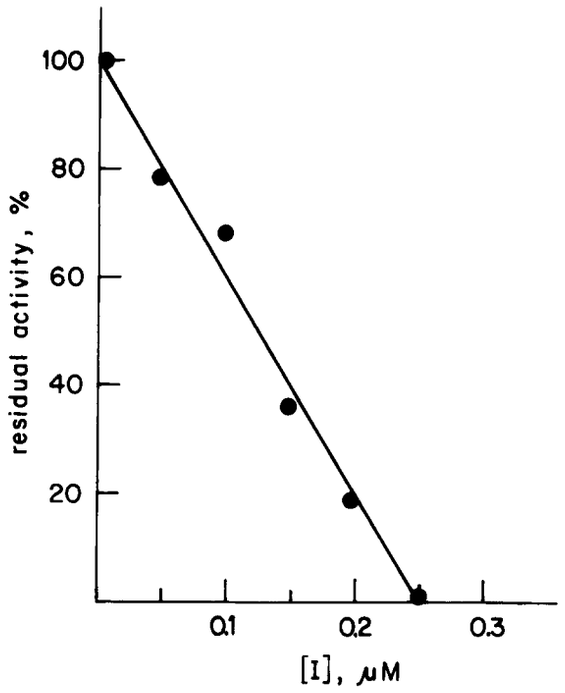

Fig. 1. To 5 mg of horse serum BChE in 1.0 ml of 0.1 M phosphate buffer pH 8 and 25°, *t*-BEDEP was added to the indicated concentrations and the residual enzymic activity was measured.

3. Results and discussion

3.1. Inhibition kinetics

When alkynyl phosphates were added to solutions of enzymes of various classes it was found that only serine esterases and proteases were efficiently inhibited. Lysozyme, pepsin, alcohol dehydrogenase and lactate dehydrogenase were not affected. Introduction of alkynyl phosphates in excess molar concentration to enzyme solution resulted in time-dependent loss of activity until it was completely abolished. Analysis of the course of the inhibition reaction yielded the second-order specificity constant $k_2 = k_i/K_I$ for the reaction described by scheme 4.

$$E + I \overset{K_I}{\leftrightarrow} EI \overset{k_i}{\rightarrow} EI^x$$

Scheme 4.

where E denotes enzyme, I the alkynyl phosphate inhibitor, EI the reversible enzyme-inhibitor complex with a dissociation constant of K_I, and k_i is the rate constant of formation of EI^x, the covalently inhibited enzyme, from EI.

The second-order inhibition rate constants for alkynyl phosphates are listed in Table 1.

In many cases the rate constants listed in Table 1 exceed those found with conventional simple covalent inhibitors. Thus, the second-order rate constant of the alkynyl phosphates with CT is 5.7×10^4–1.8×10^5 M^{-1} min^{-1} which is several fold greater than the values with other powerful inhibitors of the enzyme. Phenylmethanesulfonyl fluoride (PMSF) and disopropyl fluorophosphate (DFP) inhibit the enzyme with rate constants of 18 000 and 20 000 M^{-1} min^{-1}, respectively [12]. Diphenylcarbamoyl chloride reacts with CT with a rate constant of 37 000 M^{-1} min^{-1} [13], while *p*-nitrophenyl

Table 1
Second-order rate constants of the inhibition of enzymes by alkynyl phosphates (in M^{-1} min^{-1})

Enzyme	PDEP	HDEP	*t*-BEDEP	DFP [10]	PARAOXON [10]
α-Chymotrypsin (bovine)	5.7×10^4	1.8×10^5	8×10^4	2×10^4	9×10^2
Trypsin (bovine)		3×10^4			
Elastase (porcine)	3.2×10^4	2.5×10^5			
Subtilisin BPN'	1.8×10^4	0.9×10^4			
Subtilisin Carlsberg	1.3×10^4	1.4×10^4	0.7×10^4		
Proteinase K (tritiracium album)	0.8×10^4	2.7×10^4	0.5×10^4		
Thrombin (bovine)	1.7×10^3	1.4×10^3			
Kallikrein (porcine)	0.7×10^3	3.4×10^3			
Lipase (*Candida rugosa*)	3.4×10^6	5.5×10^6	3×10^6		
Cholesterol esterase (*Pseudonomas*)	0.9×10^6	7.3×10^7	1.6×10^7		
Acetylcholinesterase (electric eel)	1.5×10^7	4.8×10^7	5×10^7	4.6×10^4	4.5×10^5
Acetylcholinesterase (human erythrocytes)		1.1×10^7			
Butyrylcholinesterase (horse serum)	4.6×10^7	4×10^7	10×10^7	1.3×10^7	1.5×10^6
Esterase (porcine)	0.7×10^7	7.2×10^7	1.8×10^7	3.2×10^5	7×10^6

diethyl phosphate (paraoxon) reacts with a rate constant of 9×10^2 M^{-1} min^{-1} [14]. Noteworthy is the very high rate constants of the inhibition of esterases, the last five enzymes in Table 1, which are in the range of 10^6–10^8 M^{-1} min^{-1}. These rates are even greater than those reported for most powerful phosphate inhibitors. DFP, for example, inhibits AChE, BChE and esterase with rate constants of 4.6×10^4, $1.\times10^7$ and 3.2×10^5 M^{-1} min^{-1}, respectively [14], and the rate constants of inhibition by paraoxon are 4.5×10^5, $1.\times10^6$ and 7×10^6 M^{-1} min^{-1}, respectively [14], whereas the alkynyl phosphates display rate constants up to 10–100 times greater. Probably the active site of the esterases can better accommodate the alkynyl phosphates than the proteases do, leading to a most potent inhibition.

3.3. Titration of the active site concentration of enzymes

As commercial preparations of certain enzymes are generally not pure and in some cases contain only a small portion of active enzyme, we utilized the powerful inhibitory capacity of the alkynyl phosphates to titrate the active-site concentration of enzymes. A representative experiment is depicted in Fig. 1. The addition of sub-equivalent amounts of 1-*t*-butyl ethynyl diethyl phosphate to a commercial low-activity BChE solution resulted in loss of activity which was directly proportional to the amount of inhibitor added. The minimal concentration of alkynyl phosphate required to completely eliminate enzymic activity is the active-site concentration. The result of the titration indicated that the BChE contained only 0.42% active enzyme.

3.4. Reaction with radioactively labeled alkynyl phosphate

The inhibition by alkynyl phosphate can theoretically proceed via several mechanisms (see Section 1). In order to distinguish between the different possibilities we used the radioactively labeled ^{14}C-PDEP to inhibit chymotrypsin. One milligram of the inhibited CT was precipitated with TCA, filtered and thoroughly washed. In a typical experiment the counted radioactivity of the precipitate was 900 cpm, whereas the filtrates and washings had 22 093 cpm, so 3.9% of the radioactivity label was found on the filter. In a parallel experiment that contained all the components of the previous one but was devoid of CT, 0.9% of the radioactivity remained on the filter, indicating that the protein precipitate contained only 3% of the radioactivity applied. In a control experiment we added alkynyl phosphate to chymotrypsinogen under identical conditions used in the CT inhibition. Chymotrypsinogen does not react with 'active' phosphates. Nevertheless the filter contained 3% of the label applied. These results indicate that only a negligible amount of the radioactive label was non-specifically adsorbed to the protein, and practically the alkynyl moiety did not bind to the protein, negating mechanisms 2 and 3 described in Section 1. The phosphate group of the alkynyl phosphate is therefore responsible for the high inhibitory potency of the compound.

3.4. Reactivation of inhibited cholinesterases

Phosphorylated cholinesterases can be reactivated by specifically designed nucleophiles such as pyridine aldoxime methiodide (PAM) [15]. We added 5 mM PAM to AChE inhibited by HDEP and noticed a rapid restoration of enzymic activity which was complete in a few minutes. Again, supporting the conclusion that phosphorylation by alkynyl phosphate causes the inhibition of the enzymes studied.

We have demonstrated that alkynyl diethyl phosphates are highly potent inhibitors of serine esterases and proteases. The triple bond adjacent to the phosphate moeity renders the alkynyloxy group an excellent leaving group when reacting with serine enzymes, thus leading to a rapid phosphorylation of the enzyme. The structure of the alkynyl moiety, which in many cases satisfies the specificity requirements of esterases, turn the alkynyl phosphates studied as most powerful covalent inhibitors of serine esterases. The present alkynyl phosphates lack the structural elements required for specific recognition of proteases' active site, nonetheless they are good inhibitors. It is expected that modification of alkynyl phosphates by attaching to them appropriate groups complementary to particular protease binding site will lead to even more efficient inhibitors.

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