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The Arrangement of Substrate and Organophosphorus-Inhibitor Leaving Groups in Acetylcholinesterase Active Site

By J. JÄRV

*Department of Organic Chemistry, Tartu State University, Kingissepa Str. 16/14,
Tartu, Estonian S.S.R., U.S.S.R.*

and A. AAVIKSAAR

*Department of Biochemistry, Institute of Cybernetics of the Academy of Sciences of the Estonian S.S.R.,
P.O. Box 670, Tallinn-26, Estonian S.S.R., U.S.S.R.*

and N. GODOVIKOV and D. LOBANOV

*Laboratory of Organophosphorus Compounds, Institute of Organo Element Compounds of
the Academy of Sciences of the U.S.S.R., Vavilov Str. 38, Moscow, U.S.S.R.*

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The leaving groups of serine esterase substrates and organophosphorus inhibitors bind to different loci at the enzyme active site. Kinetic proof of this general conclusion is presented in the case of acetylcholinesterase reactions. The proposed model for acetylcholinesterase active site provides a new stereochemical mechanism for the phosphorylated enzyme reactivation.

The reactions of serine esterases with their substrates and organophosphorus inhibitors involve a nucleophilic attack by an activated serine hydroxyl group in the enzyme catalytic centre (Bender, 1971). The bond-breaking step of the process is in both cases preceded by a non-covalent binding state, which provides favourable arrangement of the substrate or inhibitor molecule in the enzyme active site. In the present paper we show that the leaving groups of the substrates and organophosphorus inhibitors in these non-covalent complexes are located at different regions of the enzyme active site.

The first argument follows from the study of non-enzymic reactions. It has been shown that the geometry of the transition state in nucleophilic substitution reaction at carbonyl carbon and tetrahedral phosphorus differs by the spatial arrangement of the attacking and leaving groups. The most probable courses of the reaction are the perpendicular approach of the attacking nucleophile in the case of carbonyl compounds (Bender, 1960; Chiong *et al.*, 1975), and its approach from the side opposite to the leaving-group displacement in the case of organophosphorus compounds (Kirby & Warren, 1967). This has led us to the conclusion that the leaving groups of substrates and organophosphorus inhibitors cannot bind at the same region of the enzyme surface, if the attacking nucleophile in the active site has a fixed position.

Here we present an analysis, confirming this conclusion in the case of acetylcholinesterase (EC 3.1.1.7), which has in its active site a special slit for the binding of the acetic ester leaving groups (Järv *et al.*, 1976a).

However, the suggestions given below should be applicable to all serine esterases.

The assumed arrangement of the substrate molecule in acetylcholinesterase active site is shown in Scheme 1, where ρ_1 and ρ_2 denote the binding areas for the substrate leaving group and the acyl part respectively. The direction of nucleophilic attack is shown by an arrow. It should be noted that, in addition to the interaction of the substituents with the ρ_1 - and ρ_2 -loci, the carbonyl oxygen atom of the substrate may form a hydrogen-bond with some acidic group of the protein (Brestkin & Rozengart, 1965).

Provided that the conformational properties of the acetylcholinesterase active site are the same in reactions with organophosphorus inhibitors and carboxylic ester substrates, we have in Scheme 2 located the compound

on the active surface regions ρ_1 and ρ_2 in such a way as to meet the requirement that the serine hydroxyl-group nucleophilic-attack direction must be on a line that coincides with the leaving-group displacement. Under these circumstances either the group Y or Z, but not X, can be in contact with the substrate leaving-group binding-region ρ_1 . Similar arrangement of the oxygen atoms in the C=O and P=O groups allows the above-mentioned hydrogen-bond formation in the enzyme phosphorylation reaction as well.

The kinetic proof of the proposed arrangement of substrate and organophosphorus-inhibitor molecules

Scheme 1.

Scheme 2.

in the enzyme active site is based on comparison of the data on acetylcholinesterase-catalysed hydrolysis of the substrates $\text{CH}_3\text{C}(\text{O})\text{OR}$ (Järv *et al.*, 1976a) with the data on acetylcholinesterase inhibition by the compounds $(\text{RO})(\text{CH}_3)\text{P}(\text{O})\text{SR}$ (Kabachnik *et al.*, 1970) and $(\text{C}_2\text{H}_5\text{O})_2\text{P}(\text{O})\text{SR}$ (Järv *et al.*, 1976b). In Fig. 1 the logarithms of the second-order rate constants of acetylcholinesterase reaction with $(\text{RO})(\text{CH}_3)\text{P}(\text{O})\text{SC}_n\text{H}_{2n+1}$ and $\text{CH}_3\text{C}(\text{O})\text{OR}$ have been plotted against the hydrophobicity of the substituent $\text{R} = \text{C}_n\text{H}_{2n+1}$. The data show that in both reaction series the relationship between $\log k_{\text{II}}^{\text{R}}$ and the π -constants of Hansch *et al.* (1973) splits into two separate straight lines with a point of intersection at $\pi = 2$ (*n*-butyl radical), which indicates that the group R must bind in a hydrophobic slit with a limited spatial range. The straight lines can be expressed by the equation:

$$\log k_{\text{II}}^{\text{R}} = \log k_{\text{II}}^0 + \phi \pi_{\text{R}} \quad (1)$$

where ϕ gives the sensitivity of a reaction series to substituent hydrophobicity. The ϕ -values for the substrates and for the inhibitors, 1.6 and 1.5 respectively, are in good agreement with each other. It is interesting to note that the length of the substituent, but not its hydrophobicity, is critical for the breaking point of the $\log k_{\text{II}}^{\text{R}}$ versus π_{R} plot. The initial straight lines in Fig. 1 fit the data for the compounds with branched substituents, which have the π -values considerably greater than 2.

For the inhibitors $(\text{C}_2\text{H}_5\text{O})(\text{CH}_3)\text{P}(\text{O})\text{SC}_n\text{H}_{2n+1}$ and $(\text{C}_2\text{H}_5\text{O})_2\text{P}(\text{O})\text{SC}_n\text{H}_{2n+1}$ with normal alkyl substituents in the leaving group, eqn. (1) holds up to $n = 8$ (see Fig. 2) and the straight lines have the slopes $\phi = 0.8$ and 0.6 , which are essentially

Fig. 1. Plot of $\log k_{\text{II}}^{\text{R}}$ against π_{R} for the acetylation of acetylcholinesterase by the substrates $\text{CH}_3\text{C}(\text{O})\text{OR}$ (a) and phosphorylation by the inhibitors $(\text{RO})(\text{CH}_3)\text{P}(\text{O})\text{SC}_n\text{H}_{2n+1}$ (b)

$\text{R} = \text{C}_n\text{H}_{2n+1}$; $\circ$, normal hydrocarbon radicals; $\bullet$, branched-chain hydrocarbon radicals.

lower than the ϕ -value for the substrates $\text{CH}_3\text{C}(\text{O})\text{OC}_n\text{H}_{2n+1}$.

These facts suggest that the substituents R in

Scheme 3.

Fig. 2. Plot of $\log k_{11}^R$ against π_R for the phosphorylation of acetylcholinesterase by the compounds $(C_2H_5O)(CH_3)P(O)SR$ (○) and $(C_2H_5O)_2P(O)SR$ (●) $R = C_nH_{2n+1}$; $n = 2-10$ (○) and $n = 4-8$ (●).

$CH_3C(O)OR$ and $(RO)(CH_3)P(O)SC_4H_9$ interact with the same locus at the active site of the enzyme and the leaving group of an organophosphorus inhibitor probably binds into another hydrophobic region (ρ_3 -region in Scheme 2).

It is known that increasing substituent size in the acyl part of the carboxylic esters makes them poorer substrates for acetylcholinesterase (Mounter, 1951). This can be explained with a limited range of the ρ_2 -locus, which is particularly adapted for the binding of the methyl group of acetic acid. In accordance with the proposed scheme, the restrictedness of the acetylcholinesterase active-site ρ_2 -locus must also manifest itself in the interaction of the enzyme with organophosphorus inhibitors. We suggest that the *O*-isopropyl methylphosphonofluoridate (–)-isomer, which reacts with acetylcholinesterase more rapidly than its (+)-isomer (Boter & van Dijk, 1969), is bound in the enzyme active site with the methyl group located in the ρ_2 -region (see Scheme 3). The knowledge of the absolute configuration of the inhibitor enabled us to determine the spatial location

of the ρ_1 - and ρ_2 -areas in the active site of acetylcholinesterase. Thus in the presented schemes it has been taken into consideration that the highly reactive (–)-isomer of the inhibitor $[(CH_3)_2CHO](CH_3)P(O)F$ has *S*-configuration (Benschop, 1975).

In Schemes 1 and 2, the structures of the acetylated and phosphorylated active site of acetylcholinesterase are also given. By an arrow in Scheme 1, we have pointed out the direction of the nucleophilic attack by an activated water molecule in acetyl-enzyme hydrolysis. As one can see in Scheme 2, the arrangement of catalytically active groups in the active site is unfavourable for the splitting of the organophosphorus ester bond between the inhibitor and enzyme by this mechanism. To perform a nucleophilic attack from the opposite side of the ester bond, the re-activating nucleophile must remain in solution or bind at the ρ_3 -region ($\bar{N}$ -group in Scheme 2).

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