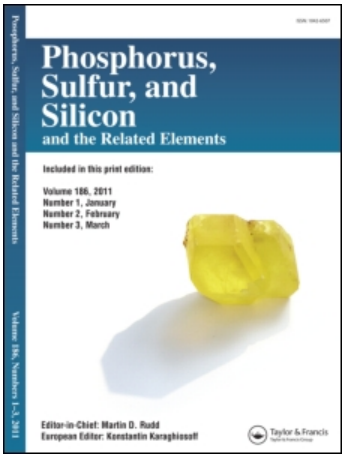

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Comparative Study of Nucleophilic am) Enzymic Reactions of 2-Propyl Methylphosphonate Derivatives

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COMPARATIVE STUDY OF NUCLEOPHILIC AND ENZYMIC REACTIONS OF 2-PROPYL METHYLPHOSPHONATE DERIVATIVES

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Abstract Solvent isotope effects have been measured for the reactions of 4-nitrophenyl 2-propyl methylphosphonate (IMN) with acetylcholinesterase (AChE), chymotrypsin, imidazole base, hydroxide ion, phosphate dianion, and water, and for the reactions of 2-propyl methyl phosphonofluoridate (sarin) with AChE. Kinetics and structural features of the dealkylation of AChE and chymotrypsin adducts were also studied.

INTRODUCTION

The overall objective of this project is a complete understanding of the molecular origin of all phases of the inhibition of serine hydrolase enzymes by organophosphorus (OP) compounds. This investigation, unlike previous ones, holds the premise that some unique interactions exist in numerous OP-serine hydrolase adducts that stabilize these intermediates and that their hydrolysis off the enzymes is hampered by some interference with the normal acid-base catalytic function of these enzymes (1).

The molecular origin of inactivation of serine hydrolase enzymes by phosphonate esters was investigated in this laboratory a few years ago (2-3). To further probe the active-site characteristics of AChE, lately we have undertaken a comparative study of AChE, chymotrypsin, trypsin, and selected nucleophiles with two structural analogs the fluoro and 4-nitrophenyl esters of 2-propyl methylphosphonic acid. The dealkylation of the inactivated enzymes has also been studied by conventional and computational techniques.

RESULTS

Nucleophilic Reactions and Enzyme Inactivation. The second order rate constants for the reactions of electric eel AChE, bovine

chymotrypsin, imidazole base, hydroxide ion, phosphate dianion, and water with IMN and sarin are reported in Tables I and II, respectively. The solvent isotope effect for each reaction has also been measured and is reported in the last column of each table.

Table I. Second-order rate constants at 25°C and solvent isotope effects for enzymic and nucleophilic reactions of 4-nitrophenyl 2-propyl methylphosphonate.

Enzyme or nucleophile	k_2, M^{-1} s^{-1}	$\frac{k_2HOH}{k_2DOD}$(°C)
AChE[a]	8130 ± 380(S) 50(R)[b]	1.29 ± 0.06 (25.0)
Chymotrypsin[c]	14.2 ± 1 (S) 2.1 ± 0.2(R)	1.24 ± 0.20 (25.0)
Imidazole	2×10^{-5}[d]	1.40 ± 0.07 (73.0)
Hydroxide ion	0.27 ± 0.01	0.94 ± 0.02 (25.0)
Phosphate dianion	2.65×10^{-5}[d]	1.11 (54.7)
Water	$(1 \pm 0.4) \times 10^{-7}$	1.70 ± 0.72 (25.0)

[a]Ref. 2b; pH 7.6, 0.05 M phosphate buffer, 5% CH_3OH.
[b]Estimate from Ref. 4.
[c]pH 7.6, 0.05 M phosphate buffer, measured with excess enzyme; time course data fitted to the biexponential rate law; the solvent isotope effect is the same for the enantiomers within experimental error.
[d]Extrapolated from temperature dependence; Ref. 5. Estimated precision ±30%.

Table II. Second-order rate constants and solvent isotope effects at 25°C for enzymic and nucleophilic reactions of 2-propyl methylphosphonofluoridate.

Enzyme or nucleophile	k_2, M^{-1} s^{-1}	$\frac{k_2HOH}{k_2DOD}$
AChE[a]	$(4.61 \pm 0.18) \times 10^5$(S) 50(R)[b]	1.27 ± 0.08
Chymotrypsin[c]	367(S) 50(R)	---
Imidazole	$(5.47 \pm 0.05) \times 10^{-3}$	---
Hydroxide ion[d]	60	

[a]pH 7.6, 0.05 M phosphate buffer, 1% CH_3OH, determined in the presence of 8 mM phenyl acetate, competitively.
[b]Estimate from Ref. 4.
[c]Ref. 6.
[d]Ref. 7.

Dealkylation of 2-propyl methylphosphonyl-AChE. The reaction has been followed at 25°C, pH 6.5, in 0.05 M phosphate buffer in H_2O and D_2O by drawing aliquots, reactivating with TMB4 for 30 min and then assaying with the Ellman method (2) for remaining AChE activity. The first-order rate constant in H_2O is $(7.0 \pm 0.2) \times 10^{-3}$, s^{-1} and the solvent isotope effect is 1.20 ± 0.06. The analogous adduct of chymotrypsin has been studied by ^{31}P NMR in a preliminary experiment. The rate of dealkylation at pH 8.0 in 0.05M phosphate buffer was very similar to the value above for AChE. Molecular modeling of the corresponding trypsin adduct was done with the use of FRODO and the structure was optimized with molecular mechanics program YETI.

DISCUSSION

Nucleophiles. The imidazole-catalyzed hydrolysis of IMN shows a solvent isotope effect of 1.4 ± 0.07, which is more similar to the reactions of phosphate dianion than to the much larger solvent isotope effects of 2.6 - 2.9 for the reactions of other more reactive phosphonate derivatives with imidazole (5). A nucleophilic reaction between IMN and imidazole seems surprising and is inconsistent with our previous experience with analogs (5). The relatively small solvent isotope effects observed for the reactions of IMN with imidazole and water (1.7 ± 0.7) can be rationalized with the assumption of parallel routes: proteolytic and nucleophilic occurring.

The solvent isotope effect for hydroxide attack on IMN is only slightly inverse, 0.94 ± 0.02, and consistent with an early transition state in which there is a significant hydrate shell around the incipient nucleophile ($\phi \sim 0.5$). The overall fractionation factor for hydroxide ion calculated from exchange equilibria (8) is in the range of 0.41 - 0.50. These originate from one covalently bonded hydrogen and probably from hydrogen bonds, one from each of the three water solvate molecules from the first hydration shell.

Probably imidazole partially and phosphate dianion, water and hydroxide ion fully follow a nucleophilic pathway with IMN. This difference in the mode of reaction with imidazole between IMN and more electrophilic analogs is likely to be a consequence of a much smaller change in the charge at P between reactant and transition states for IMN. The reactant state of IMN is likely to be less

positive at P than for soman and other more electrophilic phosphonate esters (9) and our data support the contention that negative charge does not accumulate significantly at P either.

Enzymes. The solvent isotope effects for the inactivation of the two enzymes are similar to what we observed earlier for structural analogs of the two compounds with AChE. In spite of the apparently more restrictive specificity requirements in AChE catalysis and inhibition (10-11) than in serine protease catalysis, major features of the enzymic mechanism are shared by the two groups of enzymes (12). Small phosphonate esters use proteolytic catalysis provided by the enzyme as unnatural acyl substrates do. The mechanism involves a one proton transfer, presumably the imidazole base catalyzed removal of the proton from the catalytic serine, in the rate-limiting step for serine proteases (3). Although it is more complex for AChE, a more sophisticated enzyme, here, too, the one proton transfer with the aid of a similar general acid-base catalytic apparatus to the serine proteases is the key to the catalytic efficiency. In a recent communication (2b), we reported on the concurrence of the formation of the new bond to P and the extensive breakage of the bond to the 4-nitrophenyl leaving group in IMN as indicated by a sizable (1.06 ± 0.03) ^{18}O-(phenol) isotope effect. For both compounds with AChE and for IMN with chymotrypsin, the concurrence of an isotopically silent step with general-base catalysis of the removal of the proton from the active-site Ser only partially rate limiting can account for the small solvent isotope effect.

The stereoselectivity of phosphonylation of the active-site Ser has long been recognized for compounds with the general structure $CH_3ORP(O)X$, where X is a good leaving group (4,6). Our data for chymotrypsin inactivation by IMN fall in the range of rate ratios, k_S/k_R, of 7 to 20 reported for serine proteases (6). The corresponding ratios for AChE inactivation are much higher (10 to 1000) (4).

Removal of the 2-propyl group from 2-propyl methylphosphonyl AChE occurs with an isotope effect of 1.2 ± 0.06 indicative of preprotonation of the isopropyl group before water attack.

Rationale for a Proposed Mechanism of Serine Hydrolase Inhibition by Organophosphorus Compounds (see scheme in Ref. 1). Similarly to the

evolutionarily anticipated acyl transfer, characteristics of the enzymes, i.e., specificity and recognition, dominate the initial steps of phosphonylation (2-3). Later developments in inhibition involving covalent rearrangements and transient species are probably marked by the characteristics of P, and appear to be analogous in the different enzymes. The major differences between acylation and phosphorylation must originate from the differences in valence. Phosphorus chemistry provides a greater diversity of structural arrangements along the reaction coordinate than does carbon chemistry. Consequently, the types of interactions with components of the active site of serine hydrolases must be different between the phosphoryl adducts and acyl intermediates. To the extent that these components are similar in the various enzymes, the characteristics of OP inhibition should also be similar. Until the structural details of AChE become available from X-ray diffractional data, the best assumption seems to be that the key active-site residues are the same or very similar to serine proteases. This notion is partially supported by the data on the primary structure of some AChEs (13).

One major difference between the mechanisms of acylation and phosphorylation of serine hydrolases might be the availability of general acid catalysis of leaving group departure in the former and lack of it in the latter case. This seems to be a consequence of the anchoring of the $C\text{-}O^-$ or P=O fragment in the oxyanion hole which imposes further restraints on the rest of the molecules. In contrast to carbonyl compounds, OP compounds have their leaving groups probably out of place for optimal H-bonding interaction with the general acid catalyst. Evidently only those compounds with good leaving groups react efficiently (1). Fixation of the P=O group, however, may also result in an additional and detrimental H-bond between an electron-rich ligand of the P if it gets within the proper distance from the $HN\epsilon 2$ of the His-57. This third stabilizing H-bond becomes even more counterproductive when it promotes departure of the ligand from the central P whereby a negatively charged adduct is created: this is called aging (10,11). Calculations (14) using molecular mechanics for the relative energies of the active-site components of trypsin adducts with and without positive charge on His-57 show that

at least a -13 kcal/mol stabilization of the aged adduct can be gained if the proton remains on His-57 and interacts with the phosphonate hemiester anion. Molecular modeling shows that the isopropyl group in the "up" position ages in the trypsin adduct.

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