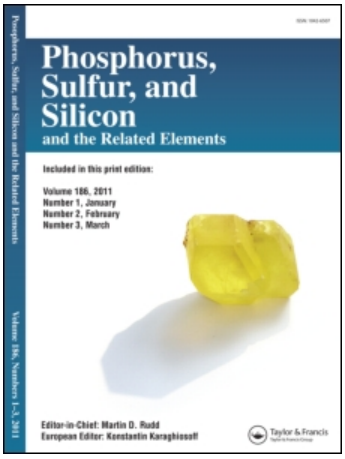

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Ligand and Active-Site Dependent P-O Versus C-O Bond Cleavage in Organophosphorus Adducts of Serine Hydrolases

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The stereoselectivity of phosphonylation of serine hydrolases by the ROR'P(O)X group of compounds is governed by the electronic properties of X and the size of RO. The electronic properties of ligands attached to P are decisive in whether C-O or P-O bond cleavage occurs in phosphonate diesters of serine hydrolases. Phenolate ions leave readily with P-O cleavage from chymotrypsin and the cholinesterases. The architecture and electrostatic character of the active site governs the fate of a covalently attached phosphonyl fragment. Strong negative electrostatic and hydrophobic forces in the cholinesterases preferentially promote C-O bond cleavage with occasional methyl migration whereas this route of dealkylation is nearly absent in phosphonate esters of serine proteases.

Keywords: acetylcholinesterase inhibition; butyrylcholinesterase inhibition; serine protease inhibition; inhibition by soman; irreversible inhibition

INTRODUCTION

The inhibition of serine hydrolases by organophosphorus compounds is generally considered in three distinct phases: phosphonylation, dephosphonylation and aging or dealkylation or dearylation [1]. Phosphonylation is avidly catalyzed by the enzymes and occurs with great efficiency if the leaving group is good. The adducts formed are considered transition state analogs for acylation of the enzymes by substrates. There is, however; greater charge separation and polarization in the phosphonylated adducts than in transients in acylation [2] which confers unique stabilizing interactions between active-site residues and phosphonate fragment resulting in irreversible inhibition. It has been our hypothesis that hydrolysis of the phosphonate fragment from the enzymes is hampered by interference with the normal acid base-catalytic function of the catalytic triad (Asp/Glu. His. Ser) of the enzymes [1]. This hypothesis has been tested by studying the enantioselectivity and dynamics of phosphonylation and the nature of interactions leading to dephosphonylation versus secondary reactions leading to the formation of anionic monoester adducts.

Recently, the properties of the transition states for these secondary reactions with C-O [3] and P-O [4] cleavage have been characterized by pH dependence, the use of isotope effects, thermodynamic parameters and computational techniques [5]. Catalytic residues that induce C-O bond cleavage have been identified especially in the cholinesterases (ChE) [3].

The molecular features that promote P-O bond breaking in chymotrypsin covalently modified with 4-nitrophenyl methyl- and propylphosphonate have been studied in depth [4].

METHODS

Inhibition of ChEs with 2-(3,3-Dimethylbutyl) methylphosphonofluoridate [3]

Inhibition of wild-type ChEs and their mutants by2-(3,3-dimethylbutyl) methylphosphonofluoridate (soman), was initiated by adding one of the two P(S)-diastereomers of somar (10-250 nM) and measuring enzyme activity by the Ellman assay at various time intervals. ***pH Dependence of Dealkylation in Soman-inhibited ChEs*** A three-step protocol for monitoring the aging reaction was followed: 1) dealkylation was carried out in a small vial in the required medium in a thermostated reactor at 4.0 ± 0.2 °C for rapid aging and at 25.0 ± 0.2 °C in most cases; 2) aliquots were drawn in a regular time-course and the reaction was quenched by dilution into a prethermostated reactivation mixture at 38.0 ± 0.2 °C; and 3) after an hour the fully reactivated enzyme was assayed for activity. Spontaneous recovery of enzyme activity and the stability of the native enzymes were monitored in controls. An average of 2-4 enzyme activity-time pairs for 4 half lives were fit to the first-order rate law at each experimental pH value. The weighted average of first-order rate constant - pH data obtained were fit to one, (sigmoid), two or three-pK (bell-shaped) models.

Inhibition of Serine Proteases by bis-4-Nitrophenyl methylphosphonate (NMN) and bis-4-Nitrophenyl propylphosphonate (NPN) [4]

The release of 4-nitrophenol was monitored at 400 nm in kinetic studies. The rate constants for inactivation of serine proteases by NMN and NPN was measured by stopped flow techniques under single turnover with a few exceptions when conventional spectrophotometric techniques were used. Adducts of the enzymes were generated with 20% free enzyme in excess. The release of the second stoichiometric equivalent of 4-nitrophenol was monitored as a function of time. The data were fit to a first-order rate equation.

RESULTS AND DISCUSSION

Enantioselectivity of Phosphonylation in Serine Hydrolases

The eanatioselectivity of phosphonylation with compounds of the general structure ROR'P(O)X of ChE, is for the enantiomer with P(S) configuration: The enantioselectivity is the greatest when X=F among F, Cl, and 4-nitrophenol. The effect of R in the side chain is also the greatest when X=F and R is branched with resemblance to the choline side chain of the natural substrate: the best example is soman. With increasing size of R, however, the mode of binding and the preference for the P(S) enantiomer may change [6]. For comparison, chymotrypsin also has preference for the P(S) enantiomers of the same inhibitors but with much smaller enantioselectivity than ChEs. In contrast to chymotrypsin, the preference is for P(R) in trypsin and subtilisin if R is sizable. In general the architecture and electrostatic character at the active site of an enzyme are decisive factors.

The Fate of Covalently Attached Phosphonyl Fragments in Serine Hydrolases

Cholinesterases [3] For example, bimolecular rate constants for the inactivation of

recombinant (r) human (Hu) butyrylcholinesterase (BChE) with P(S)C(S)- and P(S)C(R)-soman are (92 ± 7) x10^6 M^{-1} min^{-1} and (13.7 ± 0.8) x 10^6 M^{-1} min^{-1} at pH 7.4, μ=0.1 M and 25°C. Mutations of E197(*199*) to D or Q and W82(*84*) to A result in reductions in the rate constants for inactivation with P(S)C(S)-soman 4.3-, 11.8-, and 263- fold and with P(S)C(R)-soman by 6.5-, 47.3-, and 685-fold, respectively. The pH-dependence of dealkylation (aging) in electric eel (Ee), fetal bovine serum (FBS), r mouse (Mo) AChE and rHu BChE and mutants of the latter two inactivated with P(S)C(S)- and P(S)C(R)-soman were compared. Best-fit parameters for the asymmetric bell-curves for the adducts of wild-type, Ee, FBS and Mo AChE are $pK_1 = pK_2 = 4.0$-4.9 and $pK_3 = 5.2$-6.6. These pKs are consistent with the involvement of two carboxylic acids, possibly E199/202 and either E327/334 or E443/450, and H440/447H$^+$ in the dealkylation of AChE. E202Q MoAChE inactivated with the soman diastereomers yielded $pK_3 = 5.5$-5.8. Nearly symmetric pH curves for soman-inhibited wild-type and E197D Hu BChE gave $pK_2 = 3.7$-4.6 and $pK_3 = 7.3$-8.0, but much lower, $pK_3 \sim 5$, for the corresponding adduct of the E197Q mutant. Dealkylation in soman-inhibited BChE is consistent with the participation of a carboxylic acid side chain and H438(*440*)H$^+$. Maximal rate constants for dealkylation are 1-6 min^{-1} for AChE and 2 min^{-1} for BChE at 25°C. The W82 to A mutation in BChE results in a 2500-6000-fold reduction in the rate constant for dealkylation. The reduction in the rate constants for dealkylation in the E197 mutants are highly pH dependent. The solvent isotope effects at the pH maxima are 1.3-1.4 indicating unlikely preprotonation or proton in 'flight' at the enzymic transition states.

A Self-consistent Mechanism of Dealkylation in Soman-inhibited ChEs The extensive pH profiles and solvent isotope effects for the dealkylation reaction in soman-inhibited ChEs support the push-pull mechanism [3]. The essence of the mechanism is that the impetus for methyl migration stems from the electrostatic and steric push from the anionic binding site including E199 and W84 in the ground state. Concerted with methyl migration from Cβ to Cα, the C-O bond breaks without sharp charge polarization at the transition state and provides the soft interactions which are the hallmark of enzyme catalysis. H440H$^+$ and the electropositive oxyanion hole provide the pulling effect to the C-O bond breaking and the ensuing development of the negative charge on the phosphonate monoester anion. It appears that the enzyme stabilizes the transition state for dealkylation by ~ 14 kcal/mol with respect to an appropriate nonenzymic reaction by avoiding the formation of at least one intermediate. In the tertiary carbenium ion the center of positive charge on the alkyl fragment is on Cβ which is only 4 Å from the N in the indole ring of W84, whereas Cα is ~ 7 Å from the same point, thus electrostatic stabilization by aromatic π electrons of the positive charge on Cβ is more substantial than on Cα. The first intermediate formed is the tertiary carbenium which then rapidly rearranges into neutral products. The catalytic function of E199 is apparently two fold: electrostatic in stabilizing the developing positive charge at the transition state for the formation of the tertiary carbenium ion and general base catalysis.

The earlier (oxonium ion) mechanism [7] often cited is not consistent with the fact that there were essentially no products isolated that originate from the secondary cation. Rapid preprotonation followed by rate-determining formation of a secondary carbenium ion would be associated with an inverse solvent isotope effect, whereas the observed values are between 1.1 and 1.4 for the maximal rate constant for aging in soman-inhibited ChEs. The pH-dependence of the reaction, is consistent with pK values of the catalytic H in the phosphonate diester; two pK units higher in Hu BChE than in AChE. Differential calorimetric masurements for aged-soman-inhibited Hu BChE [8] and earlier strcutural

investigations of serine proteases support a serine methylphosphonate anion-histidinium ion-pair product of dealkylation below pH ~8-9, which preclude oxygen preprotonation.

Irreversible Inhibition of Serine Proteases [4] Chymotrypsin and subtilisin BPN' can be inhibited by NMN and NPN very efficiently with second-order rate constants, k_i/K_i, between 544 and 4300 M^{-1} s^{-1} at 25.0 ± 0.1 °C at the pH maxima. The second-order rate constants for the inhibition of trypsin are 26.3 ± 1.4 M^{-1} s^{-1} with NMN and 891 ± 14 M^{-1} s^{-1} with NPN at pH 8.3 and 25.0 ± 0.1 °C. A second stoichiometric equivalent 4-nitrophenol is also lost from 4-nitrophenyl alkylphosphonyl adducts of chymotrypsin but not from trypsin and subtilisin BPN'. Elimination of 4-nitrophenol from the propylphosphonyl adduct is at a rate only about twice the rate of hydrolysis of a comparable phosphonate diester, whereas 4-nitrophenol is eliminated 270-times faster from the methylphosphonyl adduct of chymotrypsin. The activation enthalpies, in kcal/mol, for 4-nitrophenol elimination from 4-nitrophenyl alkylphosphonyl-chymotrypsin are 15.0 ± 1.3 for the propyl derivative, 16.4 ± 0.5 for the methyl derivative in H_2O and 18.0 ± 0.5 in D_2O. The activation entropies, in cal mol^{-1} K^{-1}, are -29.7 ± 2.4 for the propyl derivative, -14.8 ± 0.5 for the methyl derivative in H_2O and -10.3 ± 0.3 in D_2O. Partial solvent isotope effects for the elimination of 4-nitrophenol from 4-nitrophenyl methylphosphonyl-chymotrypsin give best fits to two-site proton models: These give primary isotope effects between 1.9 and 2.0 ($\phi_1^{\ddagger}$ = 0.52 ± 0.14 or 0.49 ± 0.07) for a proton in flight, possibly from the water attacking at phosphorus to the catalytic His, and an α-secondary effect of 1.3 ($\phi_2^{\ddagger}$ = 0.75 ± 0.20) or a term for solvent contribution of 1.25 (Φ = 0.80 ± 0.10). The secondary β-deuterium isotope effect on the elimination of the second 4-nitrophenol from the adduct of chymotrypsin with NMN-l_3 (l=h or d) is 0.94 ± 0.2 possibly due to hyperconjugation. The mechanisms of secondary reactions from phosphonylated serine protease enzymes are markedly different from cholinesterases.

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