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STRUCTURE AND DYNAMICS OF SERINE HYDROLASE-ORGANOPHOSPHATE ADDUCTS

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The structural profile for the interactions between serine proteases and organophosphorus (OP) compounds can be deduced from recent NMR and X-ray crystallographic data. Using the rationale proposed for serine proteases, dynamic data on the inhibition of acetylcholinesterase by OP compounds is also consistent with structural constraints and an impairment of the proton switch mechanism during phosphylation.

KEY WORDS: Organophosphorus compounds, acetylcholinesterase, serine proteases, phosphylation, inhibition.

INTRODUCTION

The toxic effects of organophosphorus (OP) compounds originate from their nearly irreversible inhibition of the catalytic action of serine hydrolases: acetylcholinerase (AChE) and the serine proteases.[1,2] With the mediation of specific interactions between the compounds and the hydrolases, the enzyme's catalytic apparatus is mobilized to effect rapid phosphylation, which is a similar process to acylation in the analogous reactions with natural substrates of the enzymes (equation 1).

$$R_1R_2POX + EOH \longrightarrow R_1R_2POE + X^- \quad (1)$$

The enzyme-OP adducts, however, are greatly stabilized by interactions between the active-site residues of the enzyme and the ligands around the phosphorus atom, while the substrates from transient intermediates. The consequence is that, unlike with the substrates, the phosphyl group does not readily hydrolyze off the enzyme, the reason for which is poorly understood at best.

The resistance of many phosphylated serine hydrolases to reactivation by water or other nucleophiles has been attributed to an inaccessibility of the phosphorus in the covalent adducts. Spacial restrictions,[3] such as tightness of the active-site or the bulk of the substituents,[2,4] are believed to be refractory to dephosphylation. An increasing availability of structural details, by X-ray and NMR-techniques, for serine protease catalysis and inhibition, could shed some light on the types of forces involved in stabilization of the phosphylated serine hydrolases. In the wake of current discoveries[5] on the molecular dynamics of catalysis by serine proteases and of their inactivation by diisopropyl phosphorofluoridate one can perceive a more holistic explanation of the formation of the surprisingly stable phosphyl-serine hydrolase adducts. The view is taken here, that some of the hitherto elusive aspects of AChE catalysis and inhibition can be much clarified by a correlation of the energies of dynamics of AChE inhibition by OPs, with the structural information from serine proteases.

DYNAMICS OF ORGANOPHOSPHORUS INHIBITION

OP inhibitors of serine hydrolases demonstrate a variety of chemical behaviour toward the members of this group of enzymes[2,3] and toward chemical models, which have some of the active-site properties of these enzymes.[4,6] Phosphono- and phosphine-chloridates and fluoridates react very rapidly[6] with most of these enzymes, but sometimes the rate is not greatly enhanced over that which would be observed with a suitable nucleophile to model the active-site of these enzymes. A group of OP compounds shows substantial selectivity for a particular serine hydrolase, a characteristic which could be related to the active-site specificity of the particular enzyme.[7] The most outstanding magnitudes for rate acceleration of phosphylation of AChE with respect to water can be calculated for OP compounds with fluoride and thiol leaving groups e.g. 10^{11} for pinacolyl methylphosphono-fluoridate[8a] and 3×10^{11} estimated for $(CH_3)_2CH(CH_2)_3OP(O)CH_3SC_4H_9^t$ to be compared with 3×10^{17} for acylation by acetylcholine.[9] Likewise, rate acceleration of phosphylation of chymotrypsin relative to water can be calculated as follows: diisopropyl fluorophosphate reacts 10^9 times faster[8b] and bis 4-nitrophenyl propylphosphonate reacts 5×10^8 times faster[8a] with chymotrypsin than with water. The enzyme facilitates the hydrolysis of its natural substrates by a factor of 10^{11}–10^{12}.[8a] The acceleration factors translate into 60–70% (11/17 or 9/12) mobilization of the catalytic power of each enzyme by the respective OP comound as compared with good substrates.

The exceptional efficiency of catalysis by AChE in the hydrolysis of its natural substrate is accompanied by a strong stabilization of the transition state (TS) for acylation, formation of a transient acetylenzyme, and then a facile deacetylation. In Figure 1, a quantitative comparison of free energies for enzymic and corresponding hydrolytic reactions is presented. Calculations were done for the molar standard state from kinetic data in the indicated references. The free energy of equilibrium for the formation of enzymic intermediates and the ethyl derivatives for reference is arbitrarily set to -5 kcal/mol, which is consistent with data for both AChE acetylation[12] and diethylphosphorylation.[13] Acetylation goes through a barrier of $\Delta G^{\ddagger}$ 6.3 kcal/mol,[10] 24 kcal/mol less than that for the hydrolysis of acetylcholine.[9] Deacetylation of acetyl-AChE takes place through a barrier of $\Delta G^{\ddagger}$ 11.8 kcal/mol,[12] 18 kcal/mol less than that for the hydrolysis of ethyl acetate. For an example in contrast:[13] phoshorylation of AChE by diethylphosphorylcholine involves barriers of $\Delta G^{\ddagger}$ 22 kcal/mol for the generation of the phosphorylated AChE adduct and $\Delta G^{\ddagger}$ 23 kcal/mol for dephosphorylation.[2b] These values are to be compared[14] with a $\Delta G^{\ddagger}$ 30 kcal/mol barrier for the neutral hydrolysis of diethylphosphorylcholine and a $\Delta G^{\ddagger}$ 32 kcal/mol barrier for the hydrolysis of triethylphosphate.[14] Phosphates with better leaving groups react through lower activation barriers. It is noteworthy that with diethylphosphorylthiocholine the value of $\Delta G^{\ddagger}$ for phosphorylation is only 11.2 kcal/mol, quite close to the value of 6.1 kcal/mol calculated from Rosenberry's data for the acetylation of AChE by acetylthiocholine.[10] The difference in acivation free energy between the nonenzymic hydrolysis of choline and thiocholine derivatives is < 1 kcal/mol for acetate and 1 kcal/mol for diethylphosphate. For AChE acetylation the TS free energy is 0.2 kcal/mol lower if the leaving group is thiocholine instead of choline, but for phosphorylation it is 11 kcal/mol lower for the same change. Enzymic acceleration of phosphylation by AChE is most pronounced when thiol and fluoride leaving groups are involved.

Indeed, AChE sometimes appears to respond sensitively to the differences between

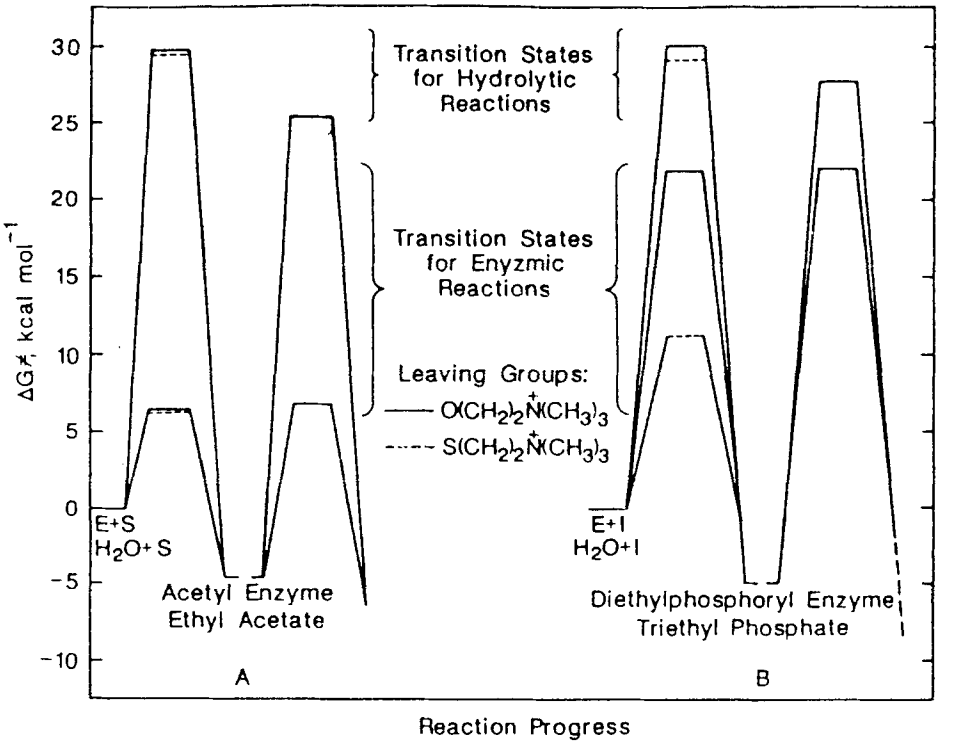
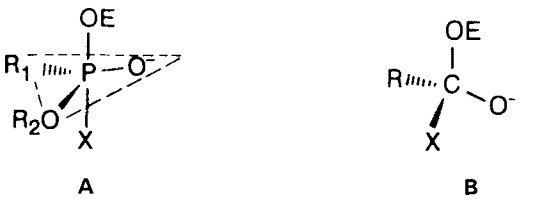


FIGURE 1 Free energy profiles for the comparison of the AChe catalyzed and neutral hydrolyses of A. acetylcholine and acetylthiocholine[10,12] B. diethylphosphorylcholine and diethylphosphorylthiocholine,[13,14] The acetyl and diethylphosphoryl AChE adducts are modeled by the corresponding ethyl compounds as serine analogs. Thus, the barriers for enzymic deacetylation and dephosphorylation steps are compared to those for the neutral hydrolysis of the ethyl analogs. The best estimate for the free energy for all equilibria is -5 kcal/mol.[12,13]

the TS structures for acyclation and phosphylation. The differences in TS structures are inherent in the differences in valence and consequently in size and geometry. How this can result in disorientation of key ligands on the phosphorus with respect to key catalytic units at the active-site may be discernible. In the pentavalent phosphyl-enzyme TS structure **A,** the oxygen bearing the negative charge.



has to take an equitorial position,[15] that is, it will be 90° from the newly formed serine–O–P bond, with a bond length of 1.54–1.7 Å for each Ser–195–O–P and P–O.[16] This might be compared with a nearly tetrahedral angle (109°) and the bond

length of 1.42 Å for the Ser–195–O–C and C–O^{-} [17] in TS **B,** for the natural substrate. Even if TS geometries are closer to the reactant state geometries respectively, the quantitative differences in angles and bond lengths remain significant. The consequences must be that the instruments of catalysis at the active-site of serine hydrolases can not fully be called into action. The most important faculties, at least for serine proteases, that may not be fully used are the two hydrogen (H)-bonds in the oxyanion hole.[18] The center of charge accumulation in the pentavalent phosphyl-enzyme structure should be spatially removed with respect to its position in the tetrahedral acyl-enzyme structure. This shift may cause a weakening of the fixation of the pentavalent structure by the two H-bonds in the oxyanion hole,[18] resulting in a smaller stabilization of the TS for the phosphylation reaction than that of the acylation reaction.

STRUCTURAL FEATURES OF SERINE PROTEASE INHIBITION BY OP COMPOUNDS

A structural investigation of the phosphylated AChE has not yet been possible. However, similarity between the active-site properties of AChE and those of the serine proteases has been well established.[3,12] Results of extensive examinations of diisopropylphosphoryl-serine proteases by X-ray crystallography[19] and by NMR spectroscopy[5] offer a quite plausible explanation for the kinetically observed unusual stability of the phosphorylated-serine hydrolases. The X ray data[19] indicate two stabilizing H-bonds to the P = O oxygen from the Gly-192 and Ser-195 NH groups in the oxyanion hole[18,19] and, most likely, a third important H-bond from His-57 to one isopropyloxy moiety attached to the central phosphorus[19] Because of the long recognized role of the two H-bonds in the oxyanion hole in stabilizing the quasi-tetrahedral TS of the reaction of acyl derivatives,[18] the analogy of the phosphylated enzymes to the TS for acylation has long been contemplated.[19] For a more recent analysis of their behavior it was concluded that the inhibitors are not TS analogs, as had previously been suggested, but rather the phosphyl-enzyme is an analog of the TS or tetrahedral intermediate for deacylation.[11]

A combination of two structural factors has a profound effect on the dynamics of serine protease catalysis.[5] The concurrence of the formation of the tetrahedral intermediate and the proton transfer from Ser-195 to the $N_{\varepsilon}2$ of His-57 are necessary for a slight movement, a tilt less than 30°, of the His-57 ring away from the Asp-102.[18] This results in the splitting of the Asp-His H-bond and the approach of the leaving group by the $N_{\varepsilon}2H$ of the His-57.[5] By this movement of the His-57, the $N_{\varepsilon}2H$ is correctly poised for general acid catalysis of the departure of a peptide (and, supposedly, other poor) leaving group(s). It is not clear what the orientation of the $N_{\varepsilon}2H$ of His-57 becomes without the formation of the evolutionarily anticipated ion-pair in which the negative charge is localized in tetrahedral geometry and anchored by two H-bonds in the oxyanion hole. But even if the anticipated movement of the His-57 can be effected by the formation of a pentavalent phosphyl-enzyme species, the leaving group of this species will also be in the wrong position for the His-57 to assist in its departure. Good leaving groups are expected to be in the axial position, 180° from the Ser–195–O–P bond **(A)**, in a trigonal bipyramidal structure to be compared to the tetrahedral angle at 109° for the substrate reaction **(B)**. Distortion from the trigonal bipyramid toward a square pyramidal arrangement might be expected if the eletronic

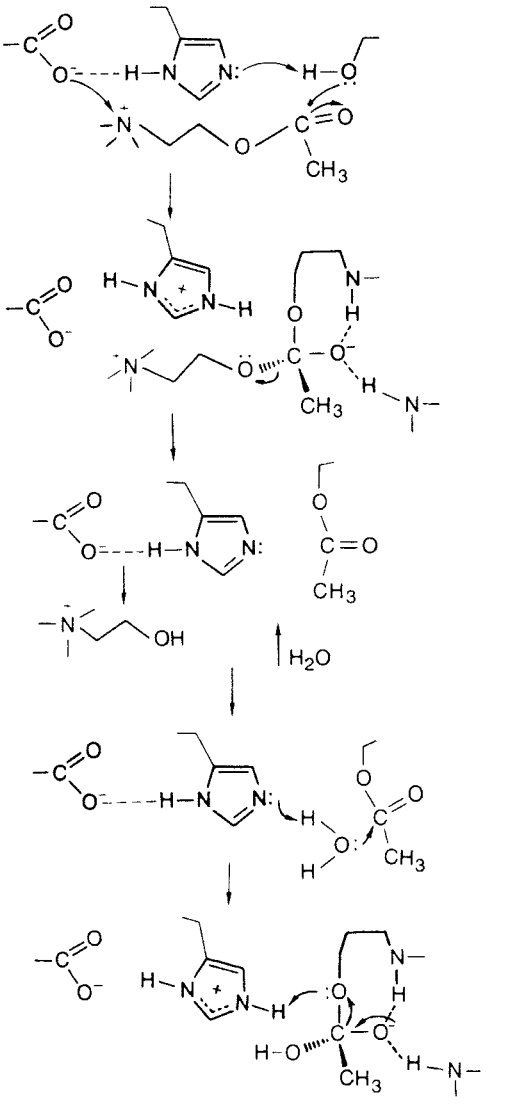
character of the ligands around phosphorus are more alike and therefore the innate leaving tendencies of the ligands are also similar. Under these circumstances the His-57 probably could not promote departure of the leaving group from the pentavalent phosphorus adduct in some positions, but perhaps would favor departure in other locations. General acid catalysis is needed more if the electrons are localized on the atom adjacent to phosphorus. Intuitively, it may be received in a small range of locations that fall close to the 109° angle expected between the Ser–195–O–P bond and the leaving group as in a distorted square pyramidal arrangement. Alkyl phosphates may fulfill these conditions the best.

Departure of the leaving group occurs efficiently for the most reactive OPs, since good leaving groups are involved. Alternatively, catalysis by some other residue or water, possibly needed for the departure of F^-, could be involved. Thus, phosphylation of serine hydrolases can take place with general base catalysis of the attack of Ser-195,[7] presumably, through the formation of a pentacovalent species with either concerted or time shifted departure of the leaving group. Catalytic participation of the His-57 in the later step is likely to be hampered or non existent. The phosphyl-enzyme thus formed, however, resembles the tetrahedral intermediate in the deacylation of serine hydrolases in charge distribution and geometry. If so, the forces that stabilize the tetrahedral adduct can act on the phosphylated enzyme. As suggested by the X-ray structure of diisopropyl trypsin,[19] two stable H-bonds are formed between the free O on the phosphorus and the NHs of the Ser-195 and Gly-192 in the oxyanion hole. This fixation must confer a fairly stringent spacial arrangement upon the rest of the molecule, locating one ligand on the phosphorus within the sphere of the $N_\varepsilon 2H$ of His-57 for H-bonding. If the His-57 ring had not previously (during proton transfer from Ser-195) moved, it certainly could at this late stage, when a tetrahedral arrangement is attained in the stable phosphyl-adduct. This phase shift, compared to the sequence in acyclation, for the adaption of the correct conformation required for the acceptance of a proton by His-57 has a serious consequence. A counterproductive additional H-bond can form between an electron rich (alkoxy) ligand of the phosphorus if it gets within the proper distance from the $N_\varepsilon 2H$ of the His-57. The third stabilizing H-bond betrayes the evolutionary mission of catalysis when it promotes departure of the alkyl ligand attached to the H-bonded O, thereby generating a negatively charged adduct:[19] a process called "aging". The negatively charged adduct adheres even more strongly to the active-site region of the enzyme by the same type of electrostatic (H-bond) forces. Three hydrogen bonds in the enzyme-OP adducts can account for 6–15 kcal/mol stabilization. Although reliable values for the formation of phosphylated serine hydrolases are not yet available, the equilibrium formation of diethylphosphoryl-AChE from some diethylphosphoryl compounds has been reported to be exothermic by < -10 kcal/mol.[13]

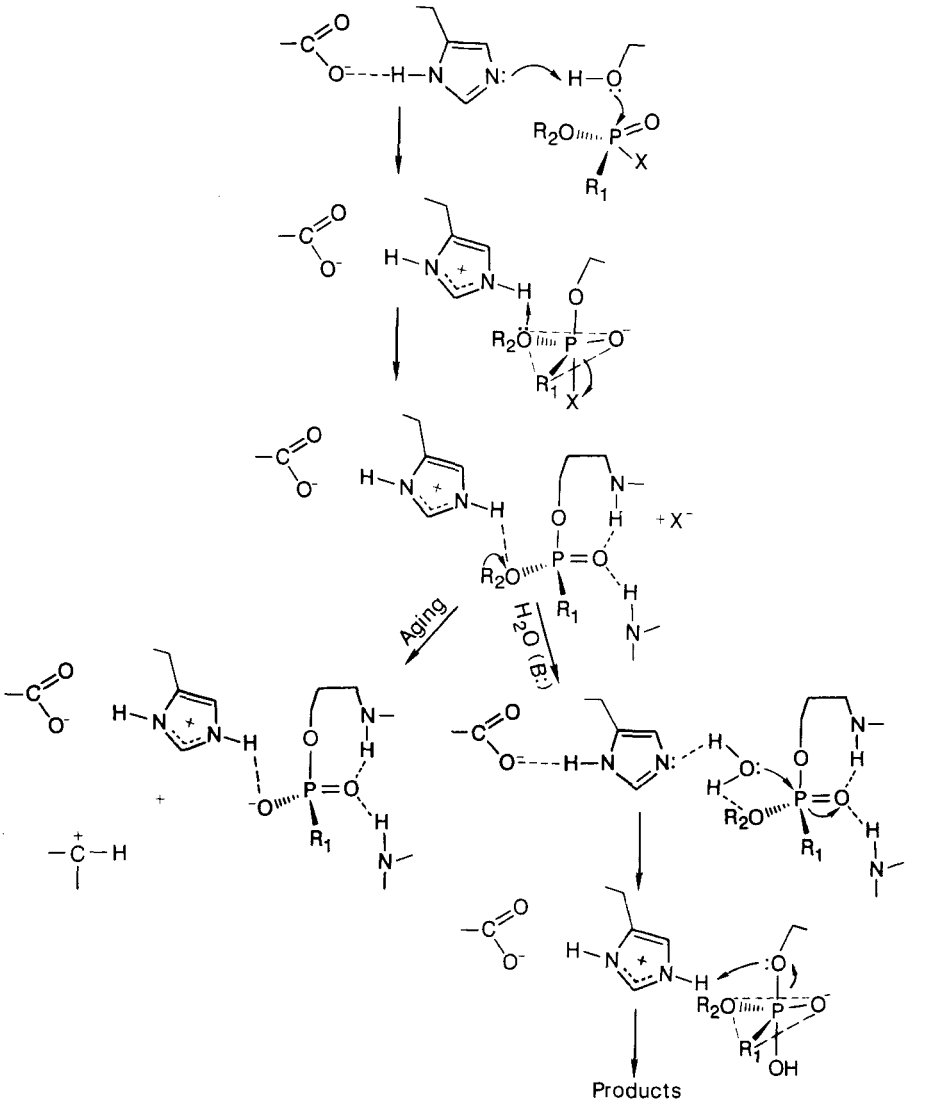
For serine proteases, the physical event behind inactivation is the freezing of His-57 in the wrong protonation state and at the wrong location for general base catalysis of an attack by water at the phosphorus atom. Such a restriction would interfere with the proton-switch mechanism. The phosphylated-serine protease adducts are not flowless analogs of an early TS for deacylation, because the His-57 is in the wrong conformation for general base assistance of water attack for deacylation. If, however, the rate limiting step involves a fairly advanced transfer of the proton from the attacking water molecule to His-57, the structure and conformation of the TS for such a case is conceivably similar to those of the phosphylated serine hydrolase adducts. For most phosphyl groups, hydrolysis from the Ser-195 of the proteases is remarkably slow with half lives in days.

A WORKING HYPOTHESIS FOR ACETYLCHOLINESTERASE MECHANISMS

The involvement of the active-site His of AChE, in essentially the same manner as with the serine proteases (Scheme 1), is consistent with the kinetic data for catalysis and inhibition by OPs. Do other residues intercede in tandem to provide conformational stability and/or storage of protons for the His? The answer , unfortunately, is not known. It is conceivable that the carboxylate believed to provide charge stabilization for the choline moiety of the natural substrate also has the propensity to keep the



SCHEME 1a



SCHEME 1b

His in the catalytically competent protonation form in the resting enzyme, much like the Asp-102 does in trypsin. According to recent estimates[20] the distance between the Ser-O and the Asp of the anionic-site may only be 4.7 Å. The presence of a tryptophan residue at the active-site, so instrumental in substrate binding at the active-site of serine proteases, also seems to be conserved.[20] Scheme 1 describes a proposition for the catalytic events for AChE hydrolysis and for OP inhibition in the manner described for the serine proteases. The anionic-center provides the anchoring of His by H-bonding for attack. Substrate binding, with the positive charge of the choline replacing the H-bonding interaction at the anionic-site by the ionic interaction, can

occur with simultaneous His base catalyzed attack of the Ser. Upon protonation, the His moves into the correct location for stabilizing the quasi-tetrahydral TS and then donates the proton back to the leaving group for departure. When the proton is lost, the His resumes the resting conformation, which involves resumption of the H-bond to the anionic-center while the leaving group is liberated to diffuse out of the active-site. This sequence is interrupted in the course of phosphylation if instead of the leaving group another ligand on the phosphorus engages in H-bonding to the His. Slow restoration of the His from the wrong conformation in the phosphyl-AChE is somehow possible though, because some catalytic acceleration of dephosphylation is observed[21] even in the worst case. It may be that water can slowly replace the H-bond to the alkoxy ligand at phosphorus. Conformational changes during "aging" and dephosphorylation have been reported[20] that might be associated with the required movement of the His back to the unprotonated form which is needed for catalysis of the water attack. The restoration of His to the resting conformation via stabilization by the anionic-center is consistent with suggestions of the participation of a carboxylic acid in the partitioning of the phosphyl-AChE between "aging" and dephosphylation.[21,23]

CONSISTENCY OF THE MODEL

One outcome of this model, for example, is that OP agents with poor leaving groups have little chance to phosphylate, whereas some of the natural substrates (peptides or even acetylcholine) with poor leaving groups enjoy outstanding catalytic rate enhancements by serine hydrolases, because they can mobilize the active-site His for general acid catalysis of the leaving group departure. This prediction is borne out by the AChE example, where acetylcholine reacts much faster than its diethylphosphoryl analog. In contrast, the thiocholine analogs have good leaving groups and therefore depart more promptly at more similar rates and without general acid catalysis in either case. Carbamoylcholine inhibitors of AChE, that have thiol and selenol choline leaving groups react similarly (or slower) than the acetylcholine analogs.[23] This might be expected on grounds of similarity in the structure of the TSS for acyl transfer and carbamyl transfer. In these quasi-tetrahedral structures the departure of choline can be promoted via general acid catalysis by the active-site His which bears the proton during catalysis (Scheme 1A). A rapid departure of the leaving group appears to be a critical requisite for efficient enzymic catalysis. It may be one of the first processes to be optimized in the course of evolution of enzymic catalytic power toward perfection.[24] AChE could be the paradigme of a perfect enzyme[25] because in the catalysis of acetylcholine hydrolis, all covalent rearrangements go through lower energy barriers (Figure 1) than the diffusion controlled encounter of enzyme and its natural substrate.[12] This is a remarkable accomplishment best reflected by the acceleration of a sluggish "background" (neutral) hydrolysis of acetylcholine by a factor of 10.[17]

It can also be forecast that the higher the electron density on the atom bonded to phosphorus, the stronger the H-bond to His and, therefore, the less likely the dephosphylation. Experimental data shows that the dephosphorylation rates for diphenyl, diethyl and dimethylphosphoryl-AChE are in the order of 100:1:5.[13b] For branched alcohols on phosphorus, S_N1 type "aging" reactions have been observed, whereas for phenyl ligands, base catalyzed "aging" has been reported[21,22] These can also be related

to the strength of the respective H-bonds formed between His and these ligands. When the alkoxy group is H-bonded strongly, the rupture of the weakened O-C bond, especially when geminal or vicinal to a branched chain, occurs more readily, as with AChE or trypsin. The rate ratios of dephosphylation to aging are 1:50–10000 for this case.[13b,22] When the H-bonding is weaker or nonexistant and the C–O bond is strong, as with the phenols, the dephosphylation rate to the aging rate is 1:0.0001–1.[13b,21] Departure of phenols with P-O cleavage may take place only after restoration of the active-site His to the unprotonated form to receive a proton from the water attacking at phosphorus. The two processes, dephosphylation and "aging", often occur at comparable rates in such cases. This suggests that the liberation of the poorer leaving group, the active-site Ser, is assisted by some enzymic catalysis. It may well be that the proton transferred from the attacking water to the His catalyst is used in the facilitation of the departure of the poorer leaving group, the active-site Ser, whereas the departure of the better aryloxy leaving group from the phosphorus is unassisted.

Studies of the dynamics of dephosphylation and the structure of stable serine protease-OP adducts to test this working hypothesis have begun in the author's laboratory.

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References

1. Koelle, G.B. (Ed.), *Cholinesterase and Anticholinesterase Agents*, Springer-Verlag, Berlin, (1963).
2. a. Aldridge, W.N. and Reiner, E. *Enzyme Inhibitors as Substrates. Interactions of Esterases With Esters of Organophosphorus and Carbamic Acids*, American Elsevier Publishing Company Inc., New York, 1972. b. Aldridge, W.N. *Croatica Chemica Acta*, 47, 215–235, (1975).
3. Jarv, J.L. *J. Bioorg. Chem.*, 259–278, (1984).
4. Kabachnik, M.I. Brestkin, A.P. Godovilov, N.N. Michelson, M.I. Rozengart, E.V. and Rozengart, V.I. *Pharmacol., Rev.*, 22, 355–388, (1970).
5. Bachovchin, W.W. *Biochemistry*, 25, 7751–7759, (1986) and references therein.
6. a. Andersen, R.A., Aaraas, I., Gaare G. and Fonnum, F. *Gen. Pharmac.*, 8, 331–334, (1977). b. Wins P. and Wilson I.B., *Biochim. Biophys. Acta*, 334, 137–145, (1974).
7. Kovach, I.M., Larson, M. and Schowen, R.L. *J. Am. Chem. Soc.*, 108, 5490–5495, (1986).
8. a. Kovach, I.M. and Schowen, R.L. b. H.U. Demuth *et al* in *Peptides and Proteins: Recent Advances*, R.L. Schowen and A. Barth (Eds,), Pergamon, Oxford, 1987.
9. Schowen, R.L. in *Transition States in Biochemical Processes*, R.D. Gandour and R.L. Schowen (Eds.), Plenum, New York, (1978) p. 86.
10. a. Rosenberry, T.L. *Croatia Chemica Acta*, 47, 235–275, (1975). b. Rosenberry T.L. *Adv. Enzymol. Relat. Areas Mol. Biol.*, 43, 103–218, (1975).
11. Ashani, Y. and Green, B.S. *Chemical Approaches to Understanding Enzyme Catalysis: Biomimetic Chemistry and Transition-State Analogs*, Proceedings of the 26th Oholo Conference, Zichron Yaacov, Israel, 22–25 March 1981, Green B.S., Ashani Y. and Chipman D. (Eds.), Studies in Organic Chemistry, V 1o, Elsevier, Amsterdam, (1981), pp 1–10.
12. a. Froede, H.C. and Wilson, J.B. *J. Biol. Chem.*, 259, 11010, (1984). b. Quinn, D.M. *Chem. Rev..*, 87, 955, (1987). c. Lockridge, O., Bartels, C.F. Vaughan, T.A. Wong, C.K. Norton, S.E. and Johnson, L.L. *J. Biol. Chem.*, 262, 549–557, (1987).
13. a. Maglothin, J.H. and Wilson, J.B. *Biochem.*, 13, 3520–3527, (1974). b. Maglothin, J.H. Wins, P. and Wilson, J.B. *Biochim. Biophys. Acta*, 405, 370–387, (1975).
14. The ΔG values were estimated from data in Cox, J.R. and Ramsey, O. B., *Chem. Rev.* 64, 317, (1964),

for diethylfluorophosphate with corrections for the respective leaving groups from the hydrolysis data of OPs in Larson, L., *Swensk Kem. Tids. Kr.* 70, 157–162, (1958). The estimate is probably correct within ± 1 kcal mol.
15. Frank, D.S. and Usher, D.A. *J. Am. Chem. Soc.*, 89, 6360–6361 (1967).
16. Pauling, L. *The Nature of the Chemical Bond*, 3rd ed.; Cornell University Press, Ithaca NY, 1960, p. 332.
17. Hogg, J.L., Rodgers, J., Kovach, I.M. and Schowen, R.L. *J. Am. Chem. Soc.*, 102, 79–85, (1981).
18. a. Polgar, L. and Halasz, P. *Biochem. J.*, 207, 1–10, (1982). b. Fersht, A. *Enzyme Structure and Mechanism.*, 2nd ed;. San Francisco, 1985, Chapter 1, and pp. 395–407.
19. a. Stroud, R.M. Kay, L. and Dickerson, R.E, *J. Mol. Biol.*, 83, 185–208 (1974). b. Chambers, R.L. and Stroud, R.M. *Acta Chrystallogr., Sect. B.*, 33, 1824–1837 (1977) and references therein. c. Kraut, *J. Ann. Rev. Biochem.*, 46, 331–351, (1977). Kossiakoff, A.A. and Spencer, S.A. *Biochemistry*, 20, 6462–6474, (1981).
20. Berman, H.A. and Decker, M.M. a. *J. Biol Chem.*, 261, 10646–10651, (1986). b. *Biochim. Biophys. Acta*, 872, 125–133, (1986) and references therein.
21. Hovanec, J.W. and Lieske, C.N. *Biochemistry* 6, 1051–1056, (1972).
22. Keijer, J.H., Wolring, C.Z. and De Jong, L.P.A. *Biochim. Biophys. Acta* 334, 146–155, (1974).
23. Lindgren, B., Lindgren, G, Artursson, F. Puu, G. Fredriksson J. and Andersson, M.O. *J. Enz. Inhib.* 1, 1 (1985).
24. The notion that optimization of the leaving group departure might be a prerequisite to other improvements on enzymic catalytic efficiency, such as in the initial steps of the reaction, concurres with the experience with artificial enzyme mimics. See; Breslow, R. Trainer, G. and Ueno, A. *J. Am. Chem. Soc.*, 109, 3145–3146 (1987).
25. Albery, W.J. and Knowles, J.R. *Biochemistry*, 15, 5631–5640, (1976).