Reaction Products of Acetylcholinesterase and VX Reveal a Mobile Histidine in the Catalytic Triad

Charles B. Millard,* Gertraud Koellner,† Arie Ordentlich,‡ Avigdor Shafferman,‡ Israel Silman, and Joel L. Sussman

Departments of Structural Biology and Neurobiology The Weizmann Institute of Science Rehovot 76100 Israel, Israel Institute for Biological Research Ness Ziona 70450 Israel

Received July 30, 1999

The presence of a precisely aligned active-site triad (Ser-His-Asp/Glu) in the three-dimensional structures of widely different hydrolytic enzymes has generated intense interest in the chemical *modus operandi* of this catalytic motif.[1] One hypothesis, which has not received wide acceptance, proposes that the imidazole of the catalytic His is mobile during enzyme function.[2] We solved the structures of the phosphonylation and dealkylation ("aging") reaction products of acetylcholinesterase (AChE; EC 3.1.1.7) and an organophosphorus (OP) inhibitor, *O*-ethyl-*S*-[2-[bis(1-methylethyl)amino]ethyl] methylphosphonothioate (VX) by X-ray crystallography. The structures clearly demonstrate reversible movement of the catalytic His. Moreover, the conformational change apparently involves a hydrogen (H−) bond with a glutamate (E199) which had been implicated previously in OP and substrate reactions.

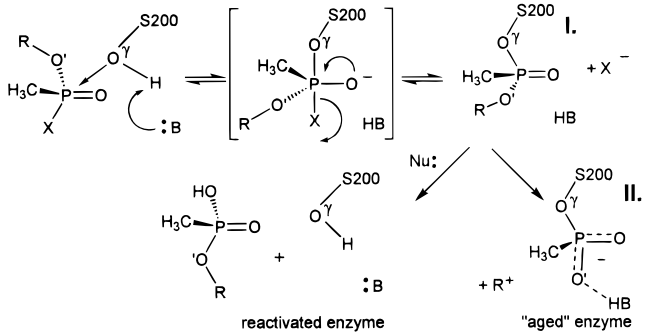
Most serine hydrolases, including AChE, use a catalytic triad and a dipolar oxyanion hole in tandem to catalyze substrate hydrolysis via an acylation−deacylation mechanism.[3] This two-step mechanism is also a weakness, however, because it renders the enzyme susceptible to stoichiometric inhibition by "hemi-substrates" which react to form stable analogues of natural reaction intermediates. Following phosphonylation of the active-site Ser Oγ, some OP-enzyme adducts undergo further post-inhibitory reactions, including dealkylation, which result in truly irreversible enzyme inhibition (collectively called "aging") (Scheme 1).

Structures of the reaction products of *Torpedo californica* (*Tc*) AChE with DFP, sarin or soman[4] after aging reveal that the OP undergoes dealkylation to yield a stable anionic phosphonyl adduct.[5] As reported previously for aged OP-serine proteases,[6] the catalytic imidazole (H440) of *Tc*AChE is positioned to form H-bonds with its normal carboxylic acid partner (E327), and with one oxygen of the negatively charged phosphonyl moiety. Such structures are limited, however, because they reveal only the final product (**II**) of the OP reaction. To overcome this limitation, we employed VX. Although phosphonylation of AChE with VX is rapid ($>10^7$ M^{-1} min^{-1}), the ethyl group of VX dealkylates slowly, thus allowing us to solve the structures of both (**I**) and (**II**) by conventional X-ray crystallography to 2.2 and 2.4 Å resolution, respectively.[7]

The most striking feature of the pro-aged VX−AChE structure (**I**) was disruption of the catalytic triad due to movement of H440. The H440 N∂ was 4.5 Å away from its resting state partner, E327 Oϵ, and within H-bond distance (2.7 Å) of E199 Oϵ (Figure 1). The observed uncoupling of the catalytic triad offers a new explanation for the slow, often negligible spontaneous reactivation of the VX−AChE adduct. Movement of the imidazole was reversible, however, because the catalytic triad could be restored by either: (1) dephosphonylation with a nucleophile (reactivation) or (2) dealkylation of the VX *O*-ethyl group (aging). To confirm that active enzyme could be regenerated from the alternate conformation, VX−*Tc*AChE crystals were dissolved in phosphate buffer (pH 7.5), incubated for 20 h with 10 mM pralidoxime, and AChE activity then measured.[8] Oxime-reactivated VX−*Tc*AChE was indistinguishable from native enzyme with respect to substrate kinetics, corroborating that the H440 movement caused by phosphonylation was reversed upon dephosphonylation.

Alternatively, if crystalline VX−*Tc*AChE was allowed to age completely (**II**), the H440 imidazole moved to a position close to that found in native *Tc*AChE. The aged adduct was essentially identical to those solved previously for sarin or soman,[5] and the catalytic H440−E327 pair had reverted to its native conformation (Figure 1).

Scheme 1. OP Reaction with Serine Hydrolases[a]



[a] The OP undergoes nucleophilic attack by a reactive Ser (represented here by S200 Oγ of *Tc*AChE) assisted by a general base (probably H440 Nϵ2 for *Tc*AChE) in the enzyme active site; the leaving group, X, is expelled, and a stable tetrahedral adduct (**I**) is formed. The adduct can undergo further reactions ("aging"); R is an alkyl group that departs during aging with some OPs, perhaps via a carbonium ion (R+). Prior to aging, a properly positioned nucleophile (Nu:), such as an hydroxyl ion or oxime, can catalyze slow dephosphonylation of **I** to regenerate free enzyme (reactivation).

Functional Significance for the Glu327−His440−Glu199 Array in AChE. Site-specific replacement of E199 with Q reduces the rate constant for aging approximately 100-fold.[9] Furthermore, the pH dependence of OP−AChE aging follows an asymmetric bell curve with a maximum rate at pH 6, and three apparent pK_a's which implicate two carboxylic acids (4.0−4.9) and an imidazole (5.2−6.6).[10] Mechanistic hypotheses of the aging reaction, therefore, have centered upon the role of E199.[11]

The VX−*Tc*AChE structures, obtained near the optimal pH for aging, provide the first evidence that H440 is mobile in

* Author for correspondence. E-mail: limill@sgjs8.weizmann.ac.il. Present address: U.S. Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, MD 21702-5011.

† Present address: Free University of Berlin, Takustrasse 6, D-14195, Berlin, Germany.

‡ Israel Institute for Biological Research.

(1) Reviewed in Dodson, G.; Wlodawer, A. *Trends Biochem. Sci.* **1998**, *23*, 347−352.

(2) (a) Satterthwait, A. C.; Jencks, W. P. *J. Am. Chem. Soc.* **1974**, *96*, 7018−7031. (b) Bachovchin, W. W. *Biochemistry* **1986**, *25*, 7751−7759.

(3) Kraut, J. *Annu. Rev. Biochem.* **1977**, *46*, 331−358.

(4) DFP, di*iso*propylphosphorofluoridate; sarin, *O*-*iso*propylmethylphosphonofluoridate; soman, *O*-pinacolyl methylphosphonofluoridate.

(5) Millard, C. B.; Kryger, G.; Ordentlich, A.; Greenblatt, H. M.; Harel, M.; Raves, M. L.; Segall, Y.; Barak, D.; Shafferman, A.; Silman, I.; Sussman, J. *Biochemistry* **1999**, *38*, 7032−7039.

(6) Kossiakoff, A. A.; Spencer, S. A. *Nature* **1980**, *288*, 414−416.

(7) Coordinates and details of data collection and refinement are available from the Protein Databank under accession codes 1vxr (pro-aged adduct) and 1vxo (aged adduct). Briefly, crystals of space group $P3_121$ were prepared in PEG-200, pH 6.0, and data were collected at 100 K with synchrotron radiation at Elletra, Trieste (1vxr) or Broohaven National Laboratory, NY (1vxo). All crystals were isomorphous with native *Tc*AChE (2ace); see ref 5 and Sussman, J. L.; Harel, M.; Frolow, F.; Oefner, C.; Goldman, A.; Toker, L.; and Silman, I. *Science* **1991**, *253*, 872-879. The final *R*-factors were 18.9% (1vxr) and 19.4% (1vxo), and the free-*R*-factors were 23.0% (1vxr) and 23.4% (1vxo).

(8) The substrate was acetythiocholine and enzyme activity was measured by the method of Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88−95.

(9) (a) Ordentlich, A.; Kronman, C.; Barak, D.; Stein, D.; Ariel, N.; Marcus, D.; Velan, B.; Shafferman, A. *FEBS Lett.* **1993**, *334*, 215−220. (b) Saxena, A.; Doctor, B. P.; Maxwell, D. M.; Lenz, D. E.; Radic, Z.; Taylor, P. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 343−349.

10.1021/ja992704i CCC: $18.00
Published on Web 10/06/1999

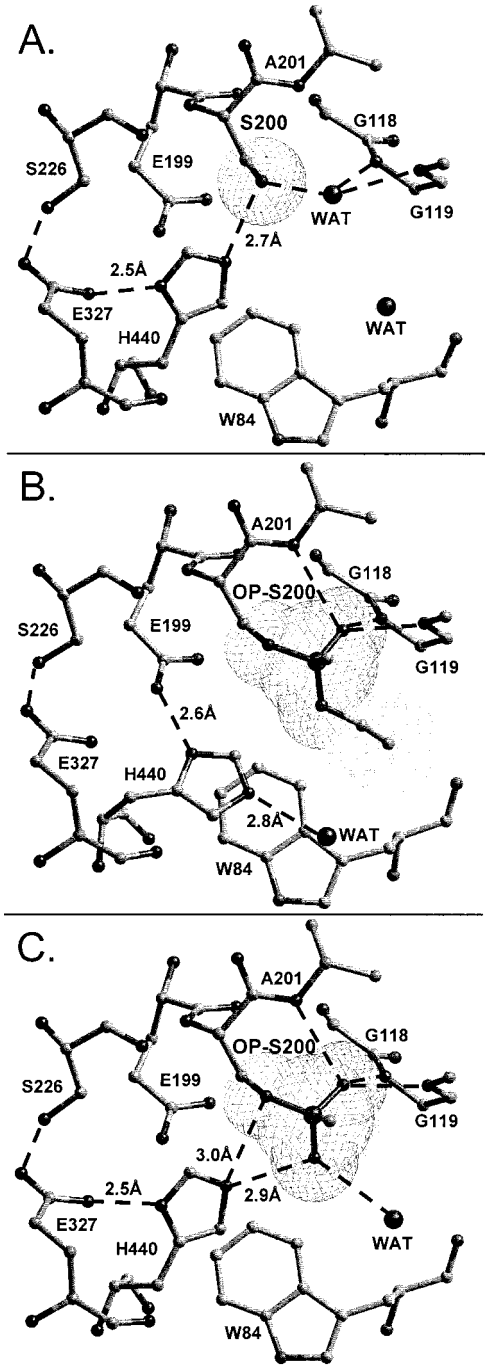


Figure 1. Structural kinetic of VX inhibition of *Tc*AChE by X-ray crystallography at pH 6.0. The active site of *Tc*AChE is depicted with possible H-bonds involving the catalytic triad and the OP moiety (broken lines). Panel A (native structure) shows the active site, including the catalytic triad (S200−H440−E327) and the oxyanion hole (−NH of G118, G119, and A201). Note that two water molecules (WAT) are displaced by the OP. Panel B (pro-aged structure): Phosphonylation triggers a conformational change for H440 that disrupts the H-bond to E327; this may be caused by steric crowding in the pentavalent phosphorus transition state, or by re-distribution of charge on the H440 imidazolium during phosphonylation. Note that E199 and a water (WAT) apparently stabilize the alternate conformation of H440. Subsequently, the H440 imidazole catalyzes either dealkylation (aging), or spontaneous reactivation (Scheme 1). Panel C (aged structure): For reaction of AChE with VX and most phosphonates, aging predominates, and dealkylation results in movement of H440 to the negatively charged pocket formed by E327 Oϵ, S200 Oγ, and one anionic oxygen of the dealkylated OP.

phosphonate adducts of AChE and strongly suggest a cooperative role for E199. It appears that the side chain of H440 rotates around its χ1- and χ2-angles to move between the carboxylic acids of E327 and E199 (Figure 1). A tripartite array, E327−H440−E199, probably persists through much of the span of this motion; this array may be a structural device for orienting the imidazole according to the steric and electrostatic features of the ligand bound to S200. Thus, we propose that the two VX−*Tc*AChE structures (pro-aged and aged) reveal the H440 imidazole trapped in the extreme positions of a continuous, reversible path.

The AChE E199Q mutation was found to reduce k_{cat}/K_m for hydrolysis of optimal ester substrates by 10- to 100-fold,[9] and recent Brownian dynamics simulations have attributed this effect partly to electrostatic stabilization of H440 by E199 during acylation.[12] In addition, Quinn and colleagues proposed a "cryptic catalytic mechanism" that relies upon E199 during hydrolysis of certain relatively slow-reacting carboxyl ester substrates.[13] Although we do not know yet if the mobility of H440 extends to substrate reactions, the widely conserved presence of the E327−H440−E199 array in divergent cholinesterases is consistent with a role in carboxyl ester catalysis.

Comparison with Serine Proteases. The possibility of a mobile catalytic histidine in selected serine protease reactions has been demonstrated and discussed previously.[2b,14] The crystal structure of diethoxyphosphorylated chymotrypsin (analogous to **I** in Scheme 1) showed no movement of the catalytic His,[15] but reaction of α-lytic protease with a more selective phosphonate substrate analogue showed a large movement of H57.[14] Nevertheless, the prevailing view remains that the His-Asp/Glu pair is H-bonded throughout catalysis, and most structures of serine hydrolases bound to transition state analogue inhibitors support this conclusion.

Structural differences in the active-site suggest to us that *Tc*AChE H440 has greater mobility than does the catalytic His found in many serine proteases: (1) flexibility of the longer Glu side chains in the E327−H440−E199 array of AChE should facilitate a larger span of motion for the imidazole than is possible with the Asp residue in the catalytic triad of serine proteases; (2) the handedness of the serine protease triad is opposite to that of AChE, which places the Asp analogous to *Tc*AChE E199 on the opposite side of the OP and too far away to interact with the catalytic His;[11a] and (3) interatomic distances in the crystal structures imply that many serine proteases possess a stabilizing H-bond between the catalytic His Cϵ1 and a nearby main-chain carbonyl oxygen,[16] but this distance is consistently too long for an optimal H-bond in *Tc*AChE.

The VX−*Tc*AChE structures demonstrate that two buried acids in an active-site quartet (Ser-His(-Glu)-Glu) modulate the catalytic imidazole position during reaction with a rapid OP "hemi-substrate." Structural flexibility may underlie the biological persistence of the catalytic triad motif, and offers a new hypothesis for how AChE hydrolyzes such a wide range of substrates, including bulky carboxyl esters, amides, carbamates and, to a limited extent, OPs, in a sterically congested and buried active site.

Acknowledgment. The work was supported by the U.S. Army (agreements DAMD17-97-2-7022 and 17-96-C-6088) and Minerva Foundation, Munich.

JA992704I

(10) Saxena, A.; Viragh, C.; Frazier, D. S.; Kovach, I. M.; Maxwell, D. M.; Lockridge, O.; Doctor, B. P. *Biochemistry* **1998**, *37*, 15086−15096.
(11) (a) Bencsura, A.; Enyedy, I. Y.; Kovach, I. M. *J. Am. Chem. Soc.* **1996**, *118*, 8531−8541. (b) Shafferman, A.; Ordentlich, A.; Barak, D.; Stein, D.; Ariel, N.; Velan, B. *Biochem. J.* **1996**, *318*, 833−840.
(12) Wlodek, S. T.; Antosiewicz, J.; Briggs, J. M. *J. Am. Chem. Soc.* **1997**, *119*, 8159−8165.
(13) Selwood, T.; Feaster, S. R.; States, M. J.; Pryor, A. N.; Quinn, D. M. *J. Am. Chem. Soc.* **1993**, *115*, 10477−10482.
(14) Bone, R.; Sampson, N. S.; Bartlett, P. A.; Agard, D. A. *Biochemistry* **1991**, *30*, 2263−2272.
(15) Harel, M.; Su, C.-T.; Frolow, F.; Ashani, Y.; Silman, I.; Sussman, J. L. *J. Mol. Biol.* **1991**, *221*, 909−918.
(16) Derewenda, Z. S.; Derewenda, U.; Kobos, P. M. *J. Mol. Biol.* **1994**, *241*, 83−93.