Carbocation-Mediated Processes in Biocatalysts. Contribution of Aromatic Moieties

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The cation π interaction is currently receiving considerable attention as a new type of binding force that is important in the function of biological systems[1] such as neurotransmitters interactions with their receptors,[2] and enzyme recognition of substrates and inhibitors.[3] In particular, the involvement of residue Trp86 at the active center of human acetylcholinesterase (HuAChE)[4] and corresponding tryptophanes in other AChE species[5] in accommodating the quaternary ammonium moieties of acetylcholine and of several covalent and noncovalent inhibitors is well documented. The involvement of aromatic residues, in stabilization of partially charged transition states for certain enzymatic processes mediated by carbocations or sulfonium ions, has also been suggested as a means to provide highly polar interaction loci which are compatible with the hydrophobic environments of the enzyme active centers.[6] However, a direct experimental evidence for such involvement is not yet available.

Recently we have reported that replacement of residue Trp86 in HuAChE by alanine resulted in a dramatic decrease (over 10^3-fold) in the dealkylation rate of 1,2,2-trimethylpropyl methylphosphonofluoridate (soman) inhibited enzyme,[7] a process known also as "aging"[8] (Scheme 1). The participation of HuAChE in the aging process was hypothesized[7] to involve the stabilization of partial positive charges of the C_β methyl substituents, imparted through hyperconjugation with the evolving carbocation on the C_α of the 1,2,2-trimethylpropyl moiety, by cation π interaction with the indole ring of the active center residue Trp86 (Figure 1). If this idea is correct, then (a) the extent of methylation at the alkoxy C_β of the phosphono conjugate should be of a crucial importance to the facility of aging and (b) in the absence of aromatic residue at position 86,

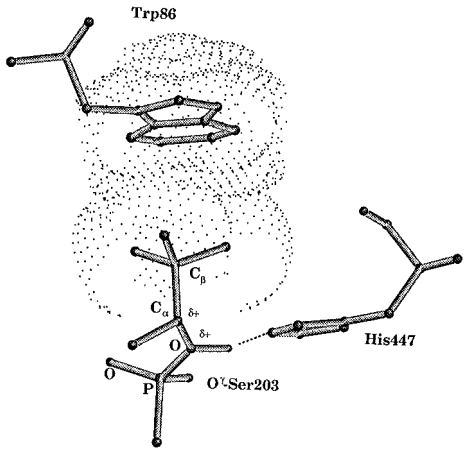
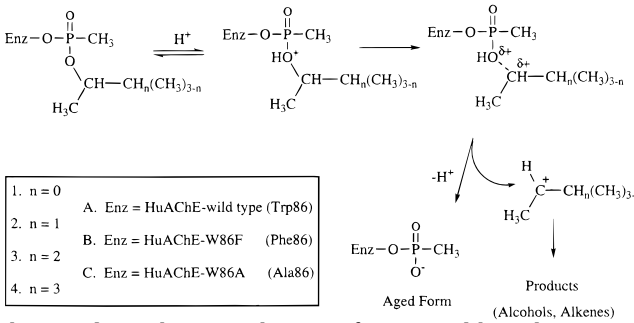


Figure 1. Orientation of the protonated methylphosphonyl moiety in **1A** relative to the residues presumably involved in the catalysis of aging. The catalytic triad residue His447 is probably involved in proton transfer to the phosphonyl moiety, maintaining a sufficient steady-state concentration of the oxonium reactive species for the dealkylation to take place.[7] The methylphosphonyl C_β methyl substituents and the indole group of residue Trp86 exhibit a tight matching of their van der Waals surfaces and are favorably juxtaposed for the development of the stabilizing cation π interaction, during the charge separation (O−C_α bond-breaking) step. The positive charge on C_α is dispersed through partial overlap of the empty p orbital of C_α and the σ C_β−CH_3 bonds (hyperconjugation).

Scheme 1. Carbocation Mechanism of the Aging Process[8] in Alkyl Methylphosphono HuAChE Conjugates



the correlation between the rate of aging and branching at C_β of the alkoxy substituent should resemble that of limiting solvolysis reactions of analogous tosylates or phosphonates.[8b,d]

Results (Table 1) of the kinetics of aging for 12 methylphosphono HuAChE conjugates (Scheme 1), differing in the number of methyl groups at C_β and in the nature of the residue at position 86 of the enzyme,[9] are in accordance with the above predictions. The data support the notion that HuAChE is involved in the mechanism of aging through cation π interactions. To our knowledge this is the first actual demonstration of biocatalysis through cation π stabilization of a carbocationic transition state.

For the conjugates of the wild type enzyme, a progressive decrease in rates of aging was observed (from 0.675−0.003

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(1) Kumpf, R. A.; Dougherty, D. A. *Science* **1993**, *261*, 1708.
(2) Schwartz, T. W. *Curr. Opin. Biotechnol.* **1994**, *5*, 434.
(3) Ortiz, A. R.; Pisabarro, M. T.; Gallego, J.; Gago, F. *Biochemistry* **1992**, *31*, 2887.
(4) (a) Ordentlich, A.; Barak, D.; Kronman, C.; Flashner, Y.; Leitner, M.; Segall, Y.; Ariel, N.; Cohen, S.; Velan, B.; Shafferman, A. *J. Biol. Chem.* **1993**, *268*, 17083. (b) Ordentlich, A.; Barak, D.; Kronman, C.; Ariel, N.; Segall, Y.; Velan, B.; Shafferman, A. *J. Biol. Chem.* **1995**, *270*, 2082.
(5) (a) Weise, C.; Kreienkamp, H. J.; Raba, R.; Pedak, A.; Aaviksaar, A.; Hucho, F. *EMBO J.* **1990**, *9*, 3885. (b) Radic, Z.; Quinn, D. M.; Vellom, D. C.; Camp, S.; Taylor, P. *J. Biol. Chem.* **1995**, *270*, 20391. (c) Harel, M.; Quinn, D. M.; Nair, H. K.; Silman, I.; Sussman, J. L. *J. Am. Chem. Soc.* **1996**, *118*, 2340.
(6) Dougherty, D. A. *Science* **1996**, *271*, 163.
(7) Shafferman, A.; Ordentlich, A.; Barak, D.; Stein, D.; Ariel, N.; Velan, B. *Biochem. J.* **1996**, *318*, 833.
(8) (a) "Aging" is the loss of reactivability of phosphyl ChE adducts by reaction with nucleophilic agents such as quaternary oximes and fluoride ions. This process prevents treatment and exacerbates the effects of human intoxication by organophosphorus agents. (b) Benschop, H. P.; Keijer, J. H.; Kienhuis, H. In *Structure and Reactions of DFP Sensitive Enzymes*; Heilbronn, E., Ed.; Forsvarets Forskninsanstalt: Stockholm, 1967; pp 193−199. (c) Michel, H. O.; Hackley, B. E.; Jr.; Berkowitz, L.; List, G.; Hackley, E. B.; Gilliam, W.; Paukan, M. *Arch. Biochem. Biophys.* **1967**, *121*, 29. (d) Cadogan, J. I. G.; Eastlick, D.; Hampson, F.; Mackie, R. K. *J. Chem. Soc. B* **1969**, 144.

(9) The **A**−**C** series of conjugates were obtained from phosphonylation of the wild type of the W86F and the W86A enzymes, respectively, as described in ref 4b. Generation of the mutant enzymes was described before.[4] Preparation of alkyl methylphosphonofluoridates, used to obtain conjugates **A**−**C** followed an accepted synthetic procedure using methylphosphonodifluoride (Monard, C.; Quinchon, J. *Bull. Soc. Chim. Fr.* **1961**, 1084) and the appropriate alcohol. The rates of aging were monitored by measuring the reactivatable fraction of the conjugate in the presence of the oxime reactivator HI-6 and under conditions where the rates of reactivation (the pseudo first order rates) were greater than the corresponding rates of aging. Under the experimental conditions used (pH = 7.0; T = 37 °C), a substantial regeneration of the enzymatic activity was observed even for **1A**, for which the aging is fairly rapid. Although the conjugates **A**−**C** (except for **4A**−**C**) are presumably each a mixture of two diastereomers,[9] in most cases monoexponential aging kinetics have been observed. For **2B** and **3B**, where the bisexponential kinetic behavior becomes evident, the rate constant corresponding to the faster reaction has been cited.

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Table 1. Rate Constants of Aging (k_a) of HuAChE Mutants Conjugated with -OP(O)(CH_3)OCH(CH_3)CH_n(CH_3)$_{3-n}$ at pH 7.0, 37 °C

phosphonyl enzyme	10^3 k_a (min^{-1})			
	1 ($n = 0$)	**2** ($n = 1$)	**3** ($n = 2$)	**4** ($n = 3$)
A (wild type)	675 ± 150	75 ± 15	30 ± 6	3.6 ± 0.6
B (W86F)	46 ± 6	5.9 ± 1	2.0 ± 0.2	0.21 ± 0.02
C (W86A)	0.6 ± 0.1	0.20 ± 0.06	0.08 ± 0.02	0.06 ± 0.01

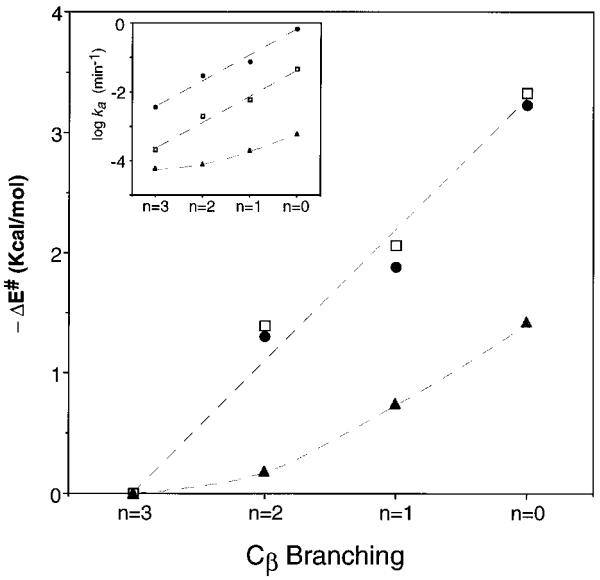

Figure 2. Differences in activation energy of aging, between the alkyl methylphosphono HuAChE conjugates and the corresponding 2-propyl methylphosphono HuAChE species **A** (●), **B** (□), and **C** (▲), as a function of the number (n) of methyl substituents at C_β. Values of $\Delta E^{\ddagger}$ were calculated from Arrhenius equation ($R = 1.99 \times 10^{-3}$ kcal mol^{-1} K^{-1}; $T = 310$ K) assuming a constant frequency factor. Inset: correlation of rate constants of aging and C_β branching.

min^{-1}) concomitant with a decrease in branching of the alkyl group of the methylphosphonyl moiety (Scheme 1 and Table 1). Examination of the effects due to amino acid replacements at position 86 of HuAChE on the rates of aging of the corresponding methylphosphono conjugates (compare **A** and **B** in Table 1) shows that substitution Trp → Phe results in a moderate and uniform decrease of the rates (13−16-fold), irrespective of the nature of the phosphonyl moiety. A similar decrease in affinities toward positively charged ligands (edrophonium, decamethonium)[4b] was also observed in the W86F enzyme as compared to that of the wild type enzyme. This parallelism indicates that Phe86 may indeed participate in the aging process through carbocation stabilization. In addition, the constant ratio of the aging rates for the conjugates of the wild type and the W86F enzymes (**A** and **B**) is consistent with the notion that tryptophane and phenylalanine play equivalent roles in the aging process and that their relative contributions are determined only by the size of their respective π electron systems.[6] On the other hand, for the various alkyl substituents in the methylphosphono conjugates, comparison of the ratio k_a^{A}/k_a^{C} for **1**−**4** shows a decline from 1120 to 60, respectively. This decline demonstrates that the extent of involvement of residue Trp86 in **A**, relative to Ala86 in **C**, in the aging process shows a much stronger dependence upon the number of methyl groups at C_β of the phosphono alkoxy substituent.

Aging of **1C** is 10-fold faster than that of **4C** [compare 1,2,2-trimethylpropyl methylphosphono W86A ($n = 0$) and 2-propyl methylphosphono W86A ($n = 3$) in Table 1], corresponding to a relatively small difference in the activation energies of the two reactions ($\Delta E^{\ddagger}_{\mathbf{4C\text{-}1C}} = 1.42$ kcal mol^{-1}; Figure 2). A similar rate dependence on the C_β substitution has been observed for the analogous limiting solvolysis reactions of the corresponding alkyl brosylates.[10] This similarity suggests that the W86A enzyme does not participate in facilitation of the charge separation step and that the decrease in activation energy is mainly due to hyperconjugative effects.[11] In contrast, for the two enzymes bearing aromatic residues at position 86, the decrease in activation energy of dealkylation reaction of **1A** and **1B**, relative to **4A** and **4B**, respectively, ($\Delta E^{\ddagger}_{\mathbf{4A-1A}} \approx \Delta E^{\ddagger}_{\mathbf{4B-1B}} >$ 3.2 kcal mol^{-1}; Figure 2) is much larger. This enzymatic acceleration appears to involve interaction with aromatic system since the values of $\Delta E^{\ddagger}_{\mathbf{4A-1A}}$ and $\Delta E^{\ddagger}_{\mathbf{4B-1B}}$ are practically equivalent irrespective of the aromatic residue at position 86. Note that such similarities in $\Delta E^{\ddagger}$ values exist also for the pairs $\Delta E^{\ddagger}_{\mathbf{3A-1A}}$ and $\Delta E^{\ddagger}_{\mathbf{3B-1B}}$ or $\Delta E^{\ddagger}_{\mathbf{2A-1A}}$ and $\Delta E^{\ddagger}_{\mathbf{2B-1B}}$. On the other hand, the greater contributions of tryptophan in type **A** conjugates as compared to those of phenylalanine in type **B** conjugates to the overall rate of aging (Table 1 and inset of Figure 2) is consistent with the relative capacities for cation π interactions of these two aromatic moieties.[6] Presentation of the kinetic data through the respective values of $\Delta E^{\ddagger}$ (Figure 2) for the entire **A** and **B** series (relative to **4A** and **4B**, respectively) demonstrates the extent of enzyme participation in the aging process as well as a clear correlation between the branching of the alkyl substituent. This correlation is consistent with the mechanistic hypothesis which relates the contribution of cation π interactions to stabilization of transition state with the polarizabilities of the interacting partners.[6] Transition state polarizability of the aging process should be enhanced by dispersion of the evolving positive charge and therefore by branching at the C_β of the alkylphosphono group. Ultimately, this enhancement results in a uniquely high rate of aging of HuAChE conjugated with the 1,2,2-trimethylpropyl methylphosphono moiety, with a half-life of 1 min (pH = 7, 37 °C, Table 1). The correlation between branching at C_β of the alkyl phosphono substituent and the acceleration of the aging process can be also rationalized in terms of steric congestion at the active center.[8d] However, this explanation is inconsistent with the nearly identical $\Delta E^{\ddagger}$ values for the **A** and **B** series of conjugates bearing aromatic moieties of considerably different volume and shape.

Carbocationic transition states in enzymes may be stabilized through polar interactions with acidic residues,[12] and yet, nature appears to use aromatic moieties as "hydrophobic negative charges" for polar interactions in apolar environments.[6] Carbocation formation during the enzymatic cyclization of squalene by aromatic residues is an example of the possible utilization of such interactions in biocatalysis.[13] Yet, recognition of cation π interactions as one of the noncovalent forces contributing to catalytic activity is mostly speculative, due to the absence of structural data for any of the relevant enzymes.[6] For the system studied here, we can rely on the 3D structure of the closely related enzyme (*Torpedo californica* AChE) for which there is also information on some covalent and noncolvant adducts.[5c] This structural background together with the experimental findings regarding aging of methylphosphono HuAChE conjugates strongly support the molecular mechanism of biocatalysis operating through cation π interactions.

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(10) The rate constants of limiting formolysis for 2-methylpropyl, 2,3-dimethylpropyl, and 2,3,3-trimethylpropyl *p*-bromobenzenesulfonates, relative to the 2-propyl derivative are 2.5, 14, and 14, respectively, see: Winstein, S.; Marshall, H. *J. Am. Chem. Soc.* **1952**, *74*, 1120.

(11) The protein environment may still have some effect since the geometry of the transition state of the **4C** aging reaction appears to be somewhat different from that of the analogous solvolysis of 2,3,3-trimethylpropyl brosylate.

(12) Warshel, A. In *Computer Modeling of Chemical Reactions in Enzymes and Solutions*; Wiley-Interscience Publishers: New York, 1991; Chapter 6.

(13) Shi, Z.; Buntel, C. J.; Griffin, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7370.