Evidence for P−N Bond Scission in Phosphoroamidate Nerve Agent Adducts of Human Acetylcholinesterase[†]

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ABSTRACT: Acetylcholinesterases (AChEs) form conjugates with certain highly toxic organophosphorus (OP) agents that become gradually resistant to reactivation. This phenomenon termed "aging" is a major factor limiting the effectiveness of therapy in certain cases of OP poisoning. While AChE adducts with phosphonates and phosphates are known to age through scission of the alkoxy C−O bond, the aging path for adducts with phosphoroamidates (P−N agents) like the nerve agent *N*,*N*-dimethylphosphonocyanoamidate (tabun) is not clear. Here we report that conjugates of tabun and of its butyl analogue (butyl-tabun) with the E202Q and F338A human AChEs (HuAChEs) age at similar rates to that of the wild-type enzyme. This is in marked contrast to the large effect of these substitutions on the aging of corresponding adducts with phosphates and phosphonates, suggesting that a different aging mechanism may be involved. Both tabun and butyl-tabun appear to be similarly accommodated in the active center, as suggested by molecular modeling and by kinetic studies of phosphylation and aging with a series of HuAChE mutants (E202Q, F338A, F295A, F297A, and F295L/F297V). Mass spectrometric analysis shows that HuAChE adduct formation with tabun and butyl-tabun occurs through loss of cyanide and that during the aging process both of these adducts show a mass decrease of 28 ± 4 Da. Due to the nature of the alkoxy substituent, such mass decrease can be unequivocally assigned to loss of the dimethylamino group, at least for the butyl-tabun conjugate. This is the first demonstration that AChE adducts with toxic P−N agents can undergo aging through scission of the P−N bond.

Phosphylation[1] of acetylcholinesterases (AChE,[2] EC 3.1.1.7) leads to formation of stable covalent conjugates from which the enzyme usually can be reactivated through reaction with nucleophilic agents such as quaternary oximes or fluoride ions (*2, 3*). In certain cases, reactivability of these conjugates decreases with time due to a unimolecular process termed "aging" (*4*−*6*). While aging of phosphonyl−AChE derivatives, of the organophosphorus (OP) nerve agents soman and sarin, involves the loss of an alkyl group from the phosphyl alkoxy substituent (*7*−*10*), there is no information regarding the nature of the aging process for conjugates with tabun and other phosphoroamidates. Reactivation of these conjugates may be impeded due to conformational change of the protein−inhibitor adducts; making them less accessible to reactivators (*11*). For example, recent study has suggested that the nonreactivability of AChE and BuChE adducts with *N*,*N*′-dialkylphosphorodiamidates such as mipafox, which was thought to involve a rapid aging process, is in fact due to a combination of a nonreactive phosphoryl moiety and a local disturbance around the active site (*12*). In the case of the AChE−tabun conjugate; reaction with reactivators was found to regenerate catalytic activity but also accelerate the development of nonreactivatability (*13*). Thus, the mechanism by which AChE conjugates of P−N agents become resistant to reactivation may be different from those proposed for the corresponding phosphate and phosphonate adducts (*6*−*10, 14*−*19*). Information regarding the potential diversity of aging mechanisms is essential for further elucidation of the role of the AChE active center in these processes, as part of the development of effective treatment for human intoxication by organophosphorus agents, like certain insecticides and nerve agents. Any progress in this direction is of considerable importance since aging is probably the major factor limiting the effectiveness of oxime reactivation therapy in cases of OP poisoning (*20*).

Recent studies of aging of phosphonyl−AChE conjugates assessed the participation of various active-center residues in the dealkylation reaction (*7, 8, 14*−*16, 21*). In particular, replacement of either of the residues Glu-202 or Phe338 in

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[1] The comprehensive term "phosphyl" is adopted from Bourne and Williams (*1*) for all tetravalent phosphorus electrophilic groups.

[2] Abbreviations: AChE, acetylcholinesterase; HuAChE, human acetylcholinesterase; TcAChE, *Torpedo californica* acetylcholinesterase; ATC, acetylthiocholine; BTC, butyrylthiocholine; OP, organophosphorus; soman, 1,2,2-trimethylpropyl methylphosphonofluoridate; DFP, diisopropyl phosphorofluoridate; tabun, ethyl *N*,*N*-dimethylphosphonocyanoamidate; butyl-tabun, *n*-butyl *N*,*N*-dimethylphosphonocyanoamidate.

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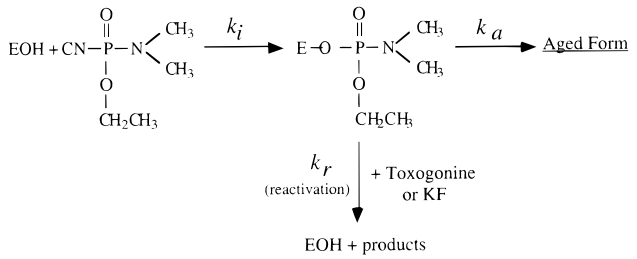
Scheme 1



HuAChE reduced by about 150-fold the rates of aging of the respective soman conjugates (*8*, *15*, *17*). These residues are thought to contribute to the aging process by stabilizing the imidazolium of the catalytic triad His-447, facilitating the formation of an oxonium on the phosphonyl moiety. The proposed mechanism is consistent with the previously proposed scission of the O−C bond and formation of a carbocation (*5*, *22*), with the structures of the aging products as determined by X-ray crystallography (*10*) and electrospray ionization mass spectrometry [ESI-MS (*9*)], and with the observed absolute stereoselectivity of the aging process (*17*, *23*). In marked contrast to these results, we now find that conjugates of the E202Q and F338A enzymes with P−N agents such as tabun age at rates similar to those of the wild-type HuAChE enzyme, suggesting that different products as well as aging mechanisms may be involved (Scheme 1).

Further investigation of this possibility included monitoring the phosphylation rates of certain HuAChE enzymes by P−N agents such as tabun (see Scheme 1) and its butyl analogue (butyl-tabun) and the aging rates of the corresponding conjugates, as well as mass spectrometric measurements of the macromolecular aging products. The results indicate that HuAChE adducts with toxic OP agents such as P−N derivatives and with phosphonates undergo aging by different mechanisms.

MATERIALS AND METHODS

Enzymes, Reagents, and Inhibitors. Expression of recombinant HuAChE and its mutants in a human embryonal kidney-derived cell line (HEK-293) (*24*−*26*) and generation of all the mutants were described previously (*7*, *24*, *27*−*29*). Stable recombinant cell clones expressing high levels of each of the mutants were established according to the procedure described previously (*25*). Enzymes were purified (over 90% purity) either by ligand affinity chromatography (*25*) or by fractionation on monoclonal antibody affinity column (*25*). The monomeric C580S HuAChE enzyme, expressed in *Escherichia coli* with an N-terminal sequence Met-Glu-Gly-Arg (*30*), was a gift from Professor I. Silman. Acetylthiocholine iodide (ATC), toxogonine, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma. Generation of the alkyl *N*,*N*-dimethylphosphoramidocyanidates used in this study were carried out by modification of a previously published procedure (*31*), using dimethylamidophosphoric dichloride and the appropriate alcohol. Phosphoroamidates were purified either by distillation (tabun, 72−74 °C/1.5 mmHg) or by column chromatography (butyl analogue) to >95% purity according to ^{31}P NMR.

Kinetic Studies. HuAChE activity was assayed according to Ellman et al. (*32*) (in the presence of 0.1 mg/mL BSA, 0.3 mM DTNB, 50 mM sodium phosphate buffer, pH 8.0, and various concentrations of ATC), carried out at 27 °C and monitored by a Thermomax microplate reader (Molecular Devices).

Measurements of phosphylation rates, with the mammalian recombinant HuAChE, were carried out with at least four different concentrations of tabun and butyl-tabun (*I*), and enzyme residual activity (*E*) at various times was monitored. The apparent bimolecular phosphonylation rate constants (k_i, see Scheme 1) determined under pseudo-first-order conditions were computed from the plot of slopes of ln *E* vs time at different inhibitor concentrations. Rate constants under second-order conditions were determined from plots of ln $\{E/[I_o - (E_o - E)]\}$ versus time (*8*).

The rates of aging were monitored by measuring the reactivatable fraction of the conjugate in the presence of oxime reactivator (toxogonin or potassium fluoride) under conditions where the rates of reactivation (the pseudo-first-order rates) were greater than the corresponding rates of aging (*8*, *17*). Under the experimental conditions used for reactivation (pH = 8.0; 37 °C), full regeneration of the enzymatic activity was observed.

Mass Spectrometric Analysis. Molecular mass measurements were carried out on a VG Platform mass spectrometer, which consists essentially of an electrospray ion source operating at atmospheric pressure followed by a quadrupole mass analyzer. Samples of phosphonoamidoyl−HuAChE conjugates, prepared by mixing the enzyme (30−40 pmol/μL) with an excess of appropriate phosphonoamidate in deionized water (pH ∼ 6.5), were assayed as described before (*9*, *17*). The multiply charged electrospray ionization mass spectra were converted to the true molecular weight spectra by using the VG MaxEnt algorithm of the MassLynx NT software. The process of dealkylation of phosphonoamidoyl−HuAChE conjugates was monitored over the period of 90 h by mixing aliquots from an appropriate phosphylation mixture (20 μL) with formic acid (1 μL) followed by immediate mass spectrometric analysis in a mass range of 64 000−66 000 Da.

Molecular Modeling. Molecular models of P_S and P_R diastereomeric HuAChE conjugates with tabun and butyl-tabun were generated as described before for conjugates of soman (*17*), using HuAChE coordinates from the recently determined crystal structure of its complex with fasciculin (*33*) and SYBYL modeling software (Tripos Inc.). In these experiments it is essential to use HuAChE coordinates since the measured stereoselectivity of eel AChE is low (6.3-fold; see ref *38*) and therefore minor changes in the active center may reverse stereoselectivity. The models were optimized by molecular mechanics using the MAXMIN force field (and AMBER charge parameters for the enzyme) and zone-refined, including 127 amino acids (15 Å substructure sphere around O^{γ}-Ser203). Optimization of the initial models included restriction of the distances between the phosphonyl oxygen and the amide nitrogen atoms of residues Gly121 and Gly122, which were relieved in the subsequent refinement.

RESULTS AND DISCUSSION

Reactivation of AChE conjugates with P−N agents may be impeded either by a chemical process at the phospho-

Table 1: Rate Constants of Phosphoroamidation (k_i) and Aging (k_a) of Tabun- or Butyl-tabun-Inhibited HuAChE Enzymes

	phosphoroamidation k_i[a] ($\times 10^{-6}$ M^{-1} min^{-1})		aging k_a[b] ($\times 10^4$ min^{-1})			
			tabun		butyl-tabun	
	tabun	butyl-tabun	pH 6	pH 8	pH 6	pH 8
wild type	15 ± 4 [1.0][c]	6.0 ± 0.8 [1.0]	16.0 ± 4.0 [1.0]	1.7 ± 0.5 [1.0]	9.0 ± 3.0 [1.0]	0.6 ± 0.3 [1.0]
E202Q	1.6 ± 0.1 [9.4]	1.3 ± 0.1 [4.6]	3.0 ± 1.0 [5.3]	0.4 ± 0.2 [4.3]	1.5 ± 0.5 [6.0]	0.2 ± 0.1 [3.0]
F338A	48 ± 9 [0.3]	6.0 ± 1.0 [1.0]	ND[d]	0.6 ± 0.2 [3.8]	ND	0.2 ± 0.1 [3.0]
F295A	30 ± 9 [0.5]	25.0 ± 4.0 [0.2]	5.5 ± 2.0 [2.9]	ND	4.5 ± 1.5 [2.0]	ND
F297A	21 ± 3 [0.7]	5.8 ± 0.3 [1.0]	7.5 ± 3.0 [2.1]	ND	5.5 ± 2.0 [1.6]	ND
F295L/F297V	4.0 ± 0.3 [3.8]	1.4 ± 0.1 [4.3]	ND	ND	ND	ND

[a] Measured at pH 8 and 24 °C; values are means ± SD for at least three independent experiments. [b] Measured at indicated pH and 37 °C; values are means ± SD for at least three independent experiments. [c] The number in brackets represents the ratio of the rate constants to that of wild-type HuAChE. [d] Not done.

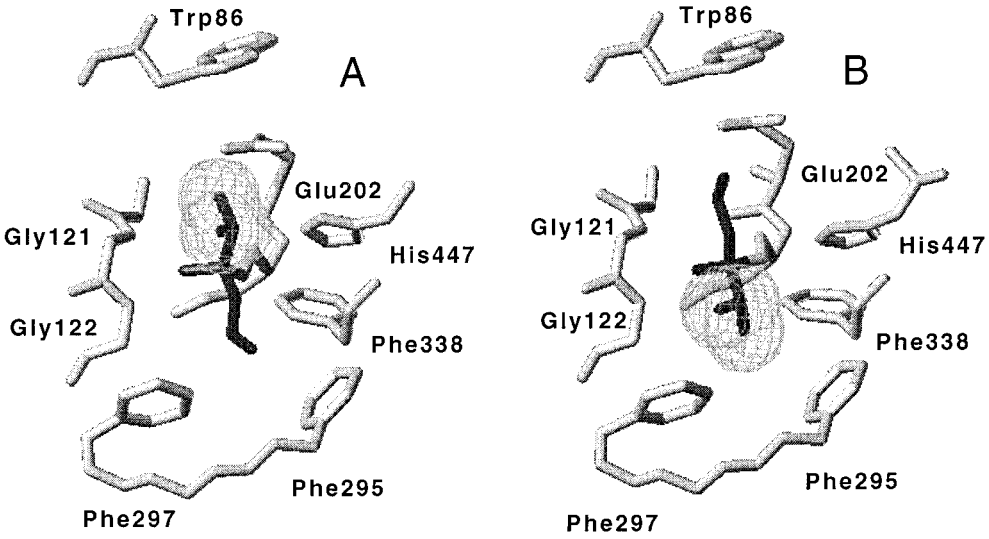
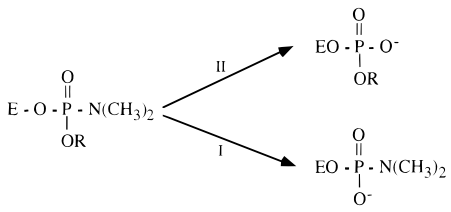


FIGURE 1: Covalent HuAChE conjugates of the P_S and the P_R tabun diastereomers. Only amino acids adjacent to the inhibitor are shown, and hydrogen atoms of the protein are omitted for clarity. The phosphoroamidyl moiety is shown in heavy line and the volume of the dimethylamino group is illustrated by a grid. (A) P_S Tabun−HuAChE conjugate. Note that the nitrogen of the phosphyl dimethylamino substituent is proximal to the carboxylate of Glu202 and within hydrogen-bond distance (2.72 Å) from $N^{\epsilon 1}$-His447; the ethoxy moiety is accommodated within the acyl pocket (Phe295, Phe297) and the phosphoryl oxygen interacts with the oxyanion hole residues Gly121 and Gly122. (B) P_R Tabun−HuAChE conjugate. The dimethylamino group points toward the acyl pocket; the alkoxy oxygen is within H-bond distance (2.95 Å) from $N^{\epsilon 1}$-His447. The P_S diastereomeric conjugate was calculated to be more stable than the corresponding P_R diastereomer by 1.65 kcal/mol.

noamidyl moiety (aging) or by another postinhibitory event like conformational changes of the protein. Different elimination mechanisms leading to somewhat different aging products also can be envisaged (*11*). Indeed, the possibility of an alternative aging mechanism of tabun−AChE conjugates, in which the ethoxy group or even the dimethylamino group is split off from the tabun−enzyme conjugate, has already been suggested (*13*). Unlike the classical aging mechanism, it may involve P−N or P−O, rather than O−C bond scission. Such bond scission should involve a displacement at the phosphoroamidoyl moiety by water molecule, in analogy to the aging processes of diphenylphosphoryl−AChE (*34*) or methylfluorophosphonyl−AChE. To further explore these alternative mechanisms, the kinetics of aging for tabun-HuAChE conjugates were compared to those of the homologous phosphonoamidate bearing butoxy substituent on the phosphorus atom (butyl-tabun).

Both phosphoroamidates are nearly equally efficient in phosphylating various HuAChE mutant enzymes (see Table 1), suggesting that the two inhibitors are similarly accommodated in the active center (see Figure 1). In particular, this is implied by the similar reactivity variations of the two P−N agents toward the different mutants of the HuAChE acyl pocket. Relative to the wild-type HuAChE, both agents exhibit comparable reactivity toward the F297A mutant, 2−4-fold higher reactivity toward the F295A enzyme, and 4-fold lower reactivity toward the F295L/F297V HuAChE. These findings are consistent with the notion that the acyl pocket subsite is juxtaposed with equivalent substituents of the phosphoroamidyl moiety. Another significant observation in this respect is the relatively minor effect of phosphylation rates by tabun and its analogue due to replacement of residue Glu202 (Table 1), as compared to the corresponding effects on the phosphylation rates by phosphates or phosphonates (about 30-fold decrease in E202Q HuAChE) (*8*, *35*). This difference may suggest that during phosphylation by these phosphonoamidates the dimethylamino rather than the alkoxy substituent is preferentially introduced in the vicinity of residue Glu202 of the HuAChE active center (see Figure 1A). We note that, in molecular modeling of the diastereomeric tabun− and butyl-tabun−HuAChE conjugates, the P_S diastereomers were found to be somewhat more stable than the corresponding P_R isomers (Figure 1B).

Scheme 2: Alternative Pathways of the Aging Process in AChE Adducts of Alkyl *N*,*N*-Dimethylphosphoramidates[a]



[a] Tabun, R = C_2H_5; butyl-tabun, R = *n*-C_4H_9.

The rate constants of aging of tabun adducts with the E202Q and F338A HuAChEs are similar to those of the corresponding butyl-tabun adducts (see Table 1). These results suggest that the nature of the phosphoroamidyl alkoxy substituent studied has a limited effect, if any, on the aging process and that the aging mechanism may involve displacement of the dimethylamino group by water (see Scheme 2, pathway II). In accordance with the effect of pH on the rates of aging (Table 1), the loss of the dimethylamino moiety from the P−N adducts is most probably facilitated through proton transfer from residue His447 to the phosphoroamidoyl nitrogen, in analogy to the dealkylation processes in methylphosphono-AChEs (*8*). However, in the case of phosphoroamidates the rate-limiting steps are probably the development of a trigonal bipyramidal intermediate, its reorganization (pseudorotation) to position the dimethylammonium group in an apical position (*36*), and its elimination. This could explain the surprisingly minimal effects due to replacement of residues Glu202 or Phe338 on the facility of aging of the studied P−N agents.

Replacement of either residue Phe295 or Phe297 by alanine had small and equivalent effects on the aging of the respective tabun and butyl-tabun conjugates (see Table 1). As in the case of the corresponding phosphoroamidation reactions, this observation is consistent with the idea that the acyl pocket accommodates equivalent substituents of the phosphoroamidyl moiety.

Although aging of HuAChE conjugates of the two phosphoroamidates seems to proceed by pathway II (Scheme 2), contributions of the alternative pathway I cannot be ruled out. Such a contribution is related to the stereoselectivity of HuAChE phosphylation since in both pathways elimination seems to include the phosphoramidyl substituent vicinal to residues Glu202 and His447. These points are exemplified by the recently resolved structures of ethyl methylphosphono-TcAChE and of its aging product (*37*) where the ethyl group, to be cleaved off during aging, is juxtaposed with residues Glu199 and His440 (corresponding to Glu202 and His447 in HuAChE). Stereoselectivity of both agents toward HuAChE is presently unknown. Yet it is probably low, as suggested by the previously observed very limited (6.3-fold) stereoselectivity of AChE from electric eel toward the (−)-tabun enantiomer (*38*).

The comparable reactivities of tabun and butyl-tabun toward the different HuAChE enzymes combined with the similar aging rate constants of the corresponding phosphoroamidyl-HuAChE conjugates and the results from molecular modeling are all consistent with an aging process proceeding according to pathway II in Scheme 2. To provide direct evidence for this conclusion, mass spectrometric characterization of both the P−N phosphyl conjugates and of their respective aging products was carried out. Recently we have shown that the aging products of certain methylphosphono−HuAChEs can be characterized by electrospray ionization mass spectrometry (ESI-MS), with mass resolution that allows one to distinguish between elimination of alkyl groups differing by a single methylene (*9*, *17*). While in the case of the aged tabun conjugate the mass changes resulting from elimination of the ethyl group or of the dimethyl amino moiety are nearly equivalent, such limitation does not apply to the corresponding butyl-tabun conjugate (see Table 2). Mass spectrometric monitoring of the progressive changes in the molecular masses of HuAChE adducts with tabun and butyl-tabun was carried out over a time period required to complete the aging process. For these measurements, recombinant HuAChE expressed in bacteria was used since with such an enzyme we could avoid the extensive molecular heterogeneity due to glycosylation (*9*). Examination of Figure 2 shows that the molecular masses of the phosphoroamidyl−HuAChEs appear immediately after addition of the phosphylating agents, with mass increase in excellent agreement with those expected from the respective phosphoroamidyl fragments following displacement of cyanide [136 Da for $C_2H_5OP(O)N(CH_3)_2$; 164 Da for $C_4H_9OP(O)N(CH_3)_2$]. The molecular species of the adducts are progressively replaced by those of the aged products, which in both cases exhibit a mass decrease of 28 ± 4 Da compared with that of the original adduct. For the butyl-tabun conjugate these observations unequivocally demonstrate that the aging process involves replacement of the dimethylamino substituent according to a pathway involving P−N bond scission (pathway II, Scheme 2) rather than elimination of either butyl or butoxy moieties (see Table 2).

The molecular masses of aging products of the HuAChE conjugates with P−N agents such as tabun and butyl-tabun demonstrate that, as for other phosphylated AChEs, the aging process involves elimination at the phosphyl moiety. Al-

Table 2: Calculated and ESMS Measured Molecular Masses of the P−N Phosphylated AChE Adducts and Their Aging Products

	formation of phosphoroamidyl−HuAChEs		"aging" of phosphoroamidyl−HuAChEs					
			molecular mass (Da)			Δ mass[c] (Da)		
	molecular mass (Da)		calculated[b]			calculated[b]		
	calculated[a]	measured	I	II	measured	I	II	measured
tabun	64 836	64 836 ± 4	64 807	64 808	64 808 ± 4	29	28	28 ± 4
Bu-tabun	64 864	64 864 ± 4	64 807	64 836	64 836 ± 4	57	28	28 ± 4

[a] Calculated molecular mass based on the measured mass of the free enzyme (64 700 Da) and theoretical mass increase due to the corresponding phosphoroamidyl fragment, assuming that cyanide is the leaving group. [b] Mass calculated according to pathway I (Scheme 2), where aging occurs through loss of the alkoxy substituent, or pathway II, where aging involves loss of dimethylamine. [c] Difference between mass values of phosphoroamidyl−HuAChE and their corresponding aged forms.

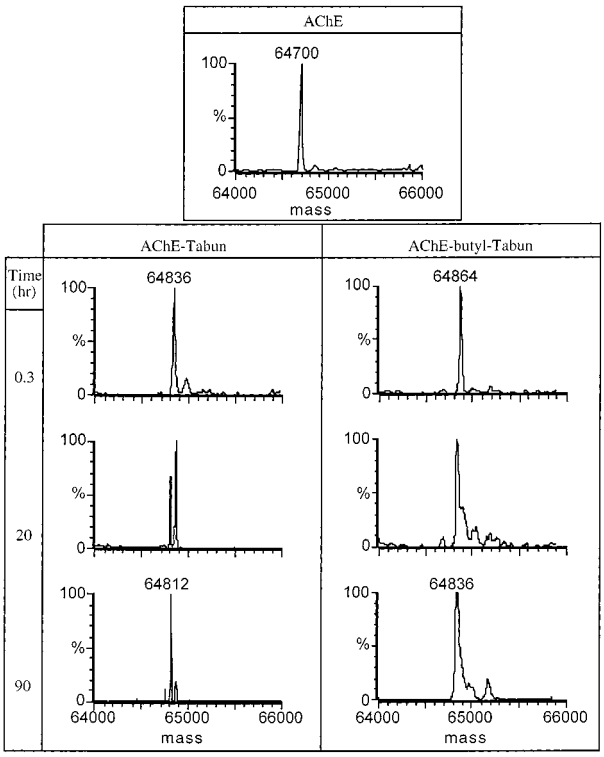


FIGURE 2: Molecular mass changes during phosphylation of HuAChE by tabun and butyl-tabun and during the aging of the corresponding conjugates monitored by positive-ion ESI-MS mass spectrometry. Masses of the molecular species were obtained for each spectrum after processing by the VG MaxEnt software over a mass range of 64 000−66 000 Da (*9*, *17*). Note that due to this method of data transformation the shapes of the peaks are not related to the mass resolution of the spectrum. (Upper panel) Analysis of the free C580S HuAChE from a bacterial source yielded experimental molecular mass 64 700 Da. Mass spectra of the reaction mixtures with tabun (left panels) and with butyl-tabun (right panels) were determined at the indicated time intervals after reaction onset.

though in this case elimination seems to occur through scission of the P−N bond, the nature of the aging products should be equivalent to those resulting from phosphoryl-AChEs. Therefore, regardless of the aging mechanism, the nonreactivatability of the aged adducts may be attributed mainly to the stabilization imparted by a salt bridge between the negative charge on the phosphoryl oxygen and the His447 imidazolium. Structures of the aged phosphoroamidyl−HuAChEs are probably similar to those recently observed for the corresponding adducts with phosphonates (soman and sarin), phosphates (diisopropyl phosphorofluoridate, DFP) (*10*), and phosphonothiolates (VX) (*37*).

Due to the relative resistance to reactivation (*39*) of AChE conjugates with certain P−N nerve agents such as tabun, their aging can become a major obstacle to an effective treatment of intoxication by these agents. Recently certain AChE mutants, for which aging of the phosphonate adducts is severely retarded, have been proposed as potential OP scavengers in combination with appropriate oxime reactivators (*40*−*42*). In view of the different aging mechanism, such enzymes may not be effective in cases of tabun and related phosphonoamidates. On the other hand, elucidation of this mechanism may be of considerable value in guiding further enzyme engineering of ChEs to enhance their tabun scavenging properties.

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