Direct determination of the chemical composition of acetylcholinesterase phosphonylation products utilizing electrospray-ionization mass spectrometry

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Abstract While non-reactivability of cholinesterases from their phosphyl conjugates (aging) is attributed to an unimolecular process involving loss of alkyl group from the phosphyl moiety, no conclusive evidence is available that this is the only reaction path and involvement of other post-inhibitory processes cannot be ruled out. To address this issue, molecular masses of the bacterially expressed recombinant human acetylcholinesterase and of its conjugates with a homologous series of alkyl methylphosphonofluoridates, were measured by electrospray-ionization mass spectrometry (ESI-MS). The measured mass of the free enzyme was 64 700 Da (calculated 64 695 Da) and those of the methylphosphono-HuAChE adducts, bearing isopropyl, isobutyl, 1,2-dimethylpropyl and 1,2,2-trimethylpropyl substituents, were 64 820, 64 840, 64 852 and 64 860 Da, respectively. These values reflect both the addition of the phosphonyl moiety and the gradual mass increase due to branching of the alkoxy substituent. The composition of these adducts change with time to yield a common product with molecular mass of 64 780 Da which is consistent with dealkylation of the phosphonyl moieties. Furthermore, in the case of 1,2-dimethylpropyl methylphosphono-HuAChE, the change in the molecular mass and the kinetics of non-reactivability appear to occur in parallel indicating that dealkylation is indeed the predominant molecular transformation leading to 'aging' of phosphonyl-AChE adducts.

Key words: Acetylcholinesterase; Aging; ESI-MS; Organophosphonate inhibitor

1. Introduction

The catalytic power of acetylcholinesterase (AChE, EC 3.1.1.7), which is among the most efficient enzymes with turnover number of $>10^4$ s^{-1} (for recent reviews, see [1,2]), is believed to be a consequence of the unique architecture of its active center [2–15]. This architecture can also account for the marked stereospecificity toward certain alkyl methylphosphonofluoridates and methylphosphonothioates [16–18]. The phosphonylation is thought to proceed through an *in-line* displacement reaction at the phosphorus to form a stoichiometric (1 : 1) and stable conjugate through inversion of configuration around the phosphorus [19–21]. The post-inhibitory behavior of phosphonyl-AChE adducts has been examined mainly through the facility of reactivation of the enzyme by reaction with nucleophilic agents, such as quaternary oximes or fluoride ions [22,23]. Non-reactivability of the adducts is usually associated with a unimolecular process which involves a loss of an alkyl group from the phosphonyl alkoxy substituent and is termed 'aging' [24–28].

In certain cases, resistance to reactivation of phosphonyl-AChE conjugates, like that of (P+)-cycloheptyl methylphosphono-AChE [21], may not be due to the classical 'aging' mechanism. The non-reactivability in these cases can originate from the inability of the protein to regain the conformationally active form following inhibition, even though displacement of the phosphyl moiety from the active site serine has actually occurred [29]. In addition, non-reactivability may in principle also occur through a path for the decomposition of phosphonyl-AChE adducts which is different from the classical 'aging' [30,31]. One of the reasons for the difficulty to estimate the importance of those mechanisms to the reactivability of phosphonyl-AChE adducts, was that the variations in composition of these enzyme adducts could not be determined.

Structures of several serine hydrolase phosphyl conjugates were determined by X-ray crystallography [32–35]. Such data are not available for cholinesterases and the nature of the corresponding phosphyl adducts can be inferred mainly from analogies to other hydrolases. To date, structures of aged cholinesterase conjugates could be suggested only on the basis of capture of low molecular mass decomposition products (corresponding alkyl alcohol or alkene) [25] and more recently by NMR studies [26]. Although these results are consistent with the commonly accepted structure of the aged phosphyl-AChE conjugate, an unequivocal evidence regarding its composition is not yet available.

Here we report that the chemical composition of certain phosphonyl adducts of HuAChE can be directly measured by electrospray-ionization mass spectrometry (ESI-MS). In this method, the molecular mass of HuAChE and its phosphonyl adducts has been measured accurately allowing also to monitor the post-inhibitory processes on a molecular level. In the absence of X-ray structures for the different phosphyl-cholinesterase conjugates, these mass spectrometric results represent a direct evidence for their chemical identity.

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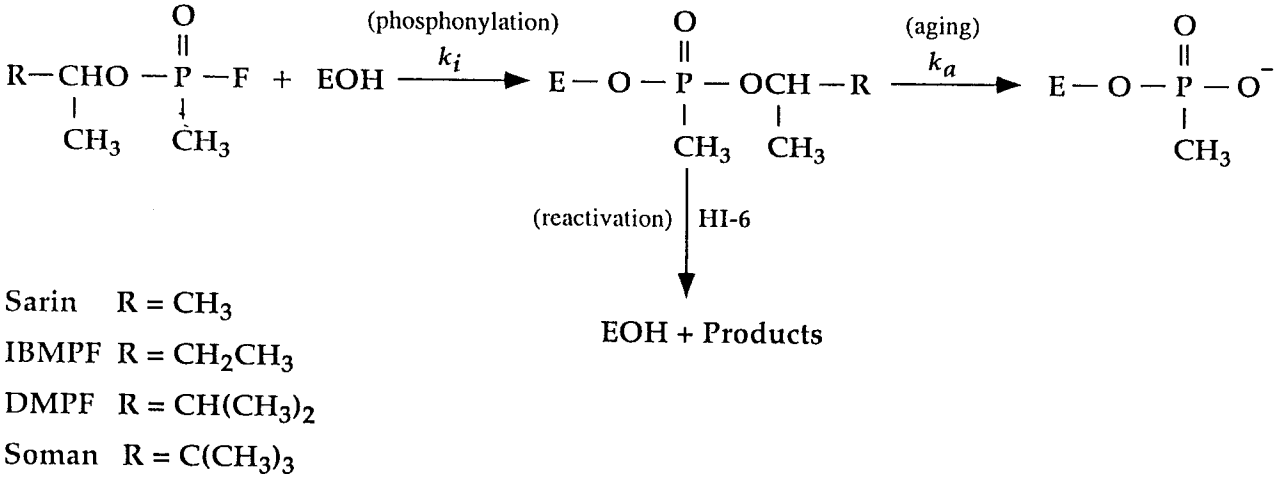

Scheme 1. Generation and 'aging' of phosphonylated AChE.

2. Materials and methods

2.1. Enzymes reagents and inhibitors

Wild-type (WT) recombinant HuAChE and its monomeric mutant C580A were expressed in 293 cells and purified as described before [5,8]. The monomeric C580S bacterially expressed enzyme (HuAChE-bac) was expressed in *E. coli* [36] with an N-terminus sequence Met-Glu-Gly-Arg-. Acetylthiocholine iodide (ATC) and 5 : 5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma. 1-(2-hydroxyimino-methylpyridinium)-1-(4-carboxyimino-pyridinium) dimethylether dichloride (HI-6) was a gift from Dr. G. Amitai. Preparation of the racemic mixtures of 2-propyl, 2-butyl and 1,2-dimethylpropyl methylphosphonofluoridates, used in this study, followed an accepted synthetic procedure using methylphosphonodifluoride [37] and the appropriate alcohol. The purified stereoisomers (C+P+)- and (C+P−)-1,2,2-trimethylpropyl methylphosphono-fluoridates were isolated as described before [38].

2.2. Kinetic studies

HuAChE activity was assayed according to Ellman et al. [39] (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 phosphonylation rates, with the mammalian recombinant HuAChE, were carried out in at least four different concentrations of the alkyl methylphosphonofluoridates (I) and enzyme residual activity (E) at various times was monitored (see Scheme 1). The apparent bimolecular phosphonylation rate constants (k_i) 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 $\ln\{E/[I_o-(E_o-E)]\}$ vs. time [28].

The stabilities of the HuAChE phosphono conjugates were evaluated under conditions where the rate of phosphonylation is much higher than the rate of aging ($k_i[I_o] \gg k_a$, see Scheme 1) and with >98% inhibition of the initial enzyme activity. The excess inhibitor was rapidly removed either by column filtration (Sephadex, G-15) or by 1000-fold dilution, prior to reactivation. The reactivatable (non-aged methylphosphono conjugate) fraction was determined by reactivation with 0.5 mM HI-6 under conditions where the rate of reactivation is higher than the rate of aging (see Scheme 1) [28].

2.3. 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 HuAChE-bac and of the phosphonyl-HuAChE conjugates, prepared by mixing the enzyme (30–40 pmol/μl) with an excess of appropriate phosphonofluoridate, were introduced directly into the ion source in a 50 : 50 acetonitrile-water solutions. The flow rate of the sample solution into the ion source was 20 μl/min. The mass spectrometer was scanned, in positive ion mode, from m/z 500 to 1900 in 10 s and several scans were summed to obtain the final spectrum. Mass-scale calibration employed the multiply charged ions from a separate introduction of myoglobin.

The multiply charged electrospray ionization mass spectra were converted to the true molecular mass spectra using the VG MaxEnt algorithm of the MassLynx NT software. Molecular mass calculations of homogeneous samples were carried out over a mass range of 50 000–80 000 Da (see Figs. 1 and 3) whereas a narrower mass range (64 000–66 000 Da) was used for the case of 1,2-dimethylpropyl methylphosphono-HuAChE (see Fig. 2). At least 5 mass determinations were carried out for each of the molecular species reported, yielding every time exactly the same values (within the experimental resolution of 1 Da).

Similar experiments aimed to obtain molecular masses for either the WT HuAChE or the monomeric C580A mutant, expressed in mammalian cells, were unsuccessful. Scanning in positive or negative ion modes and at higher resolution (peak width at half height of ≤0.75 Da) failed to produce spectra in which discrete multiply charged species could be isolated.

3. Results and discussion

ESI-MS is a new mass spectrometric method that has been recently applied to the detection of covalent enzyme-substrate and enzyme-inhibitor complexes as well as for probing protein-ligand non-covalent interactions [40–42]. One of its unique features is that the protein sample is introduced directly from solution, in its biologically active form, for mass spectrometry analysis. The ESI is not expected to facilitate chemical changes of covalent conjugates during the ionization process and, therefore, is suitable to monitor the post-inhibitory behavior of phosphyl-AChE adducts.

To examine the potential utility of ESI-MS to such study, we attempted to determine the molecular mass of the HuAChE recombinant enzyme expressed in human embryonal 293 cells [5], by direct introduction into the ion source. However, the resulting signal could not be resolved into discrete masses, indicating a mixture of molecular species which may originate from oligomerization and post-translational modifications of the protein [1]. To overcome this problem, we measured, in the same manner, the mass spectrum of the C580A mutant enzyme in which replacement of the C-terminal cysteine prevents oligomerization and the protein is monomeric [5]. In this case, we still could not obtain a discrete molecular mass, probably due to the variable sugar composi-

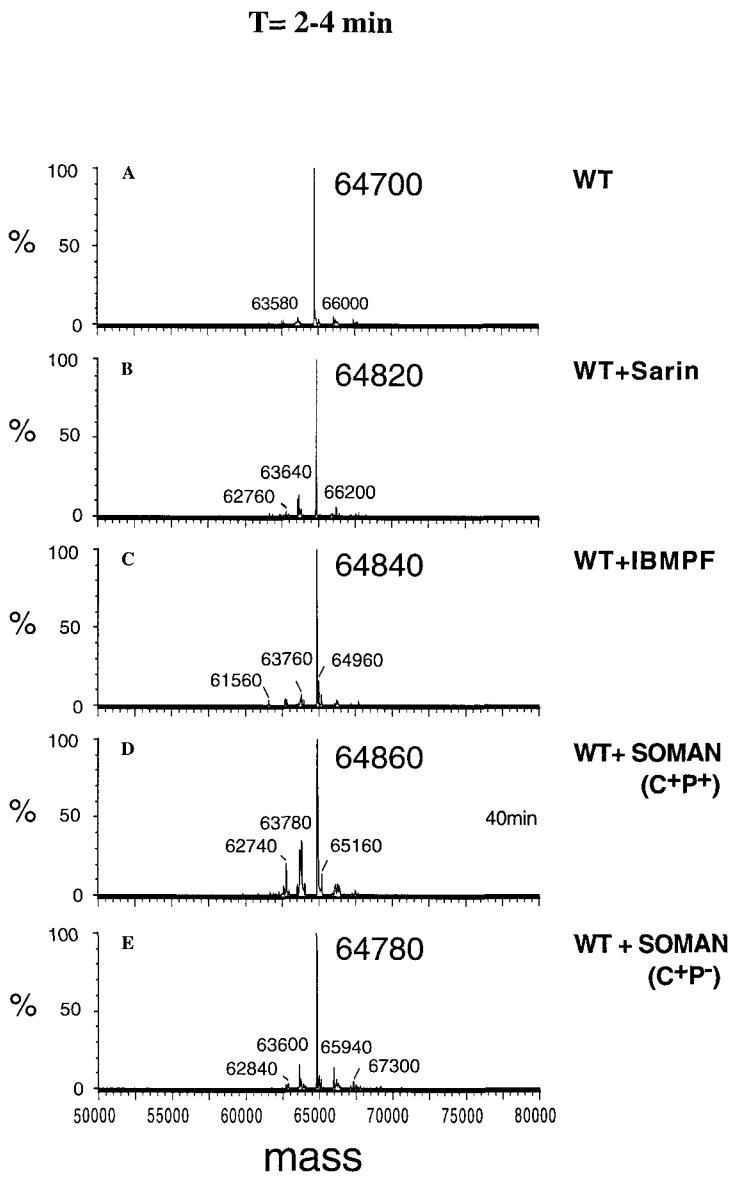

Fig. 1. Positive-ion ESI-MS mass spectra of HuAChE-bac and of its alkyl methylphosphono conjugates, after processing by VG MaxEnt software. Spectra of the adducts were measured within 2–4 min of mixing the enzyme with excess of the phosphonylating agent, as shown in each of the panels: (A) free C580S HuAChE-bac (mass calculated from sequence 64 695 Da); (B) 2-propyl methylphosphono-HuAChE-bac (mass calculated 64 821 Da); (C) 2-butyl methylphosphono-HuAChE-bac (calculated 64 835 Da); (D) product of phosphonylation with (C+P+)-1,2,2-trimethylpropyl methylphosphono-fluoridate (calculated 64 863 Da), spectrum taken after 40 min since in this case adduct formation is very slow; (E) product of phosphonylation with (C+P−)- 1,2,2-trimethylpropyl methylphosphonofluoridate (calculated 64 778 Da for the aged adduct, see text and Fig. 3). The minor peaks in the spectra may reflect the inherent 'noise' of the method. It should be pointed out that the peak height is a reflection of the precision as well as of the abundance of the corresponding molecular species in the sample.

tion of the enzyme glycosyl substituents and variability of the glycosylation pattern [43]. Such variability appears to be characteristic to HuAChEs where all the three potential N-glycosylation sites are utilized but not all the secreted molecules are fully glycosylated [44,45]. Consequently, we decided to examine the monomeric form of HuAChE (C580S) expressed in *E. coli* (HuAChE-bac) which is not glycosylated. In the past, it was demonstrated that oligomerization or the extent of glycosylation of HuAChE has practically no effect on either catalytic activity or on reactivity towards active center ligands [45]. For the HuAChE-bac, the molecular mass could indeed be determined by ESI-MS (see Fig. 1A). The measured molecular mass (64 700 Da) is consistent with that calculated (64 695 Da) for a bacterial HuAChE (C580S) sequence containing methionine at the N-terminus.

Following the determination of HuAChE-bac molecular mass, we examined the feasibility of measuring the composition of its phosphonyl adducts. Since for some methylfluorophosphonates (like soman or DMPF, see Scheme 1) the initial phosphonyl-HuAChE conjugate could be expected to undergo fairly rapid post-inhibitory transformations [46,47], the mass spectrometric experiments had to be carried out immediately after the phosphonylation. In addition, the reaction had to be carried out under conditions where the phosphonylation reactions were rapid but the development of non-reactivability was relatively slow. To determine accurately these boundary

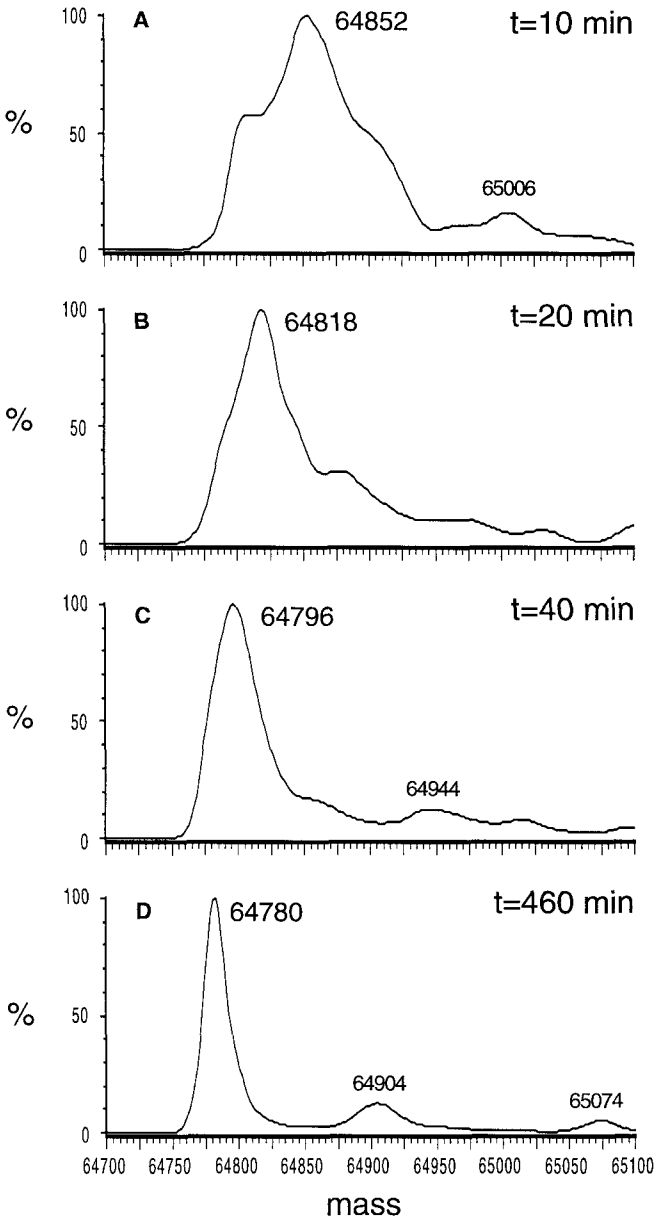

Fig. 2. Positive-ion ESI-MS mass spectra of the reaction mixture DMPF+HuAChE-bac sampled at different times after the reaction onset. Mean molecular mass of the mixture components was obtained for each spectrum after processing by the VG MaxEnt software. Although the value after 10 min corresponds closely to that calculated for 1,2-dimethylpropyl methylphosphono-HuAChE-bac (64 849 Da), the asymmetric shape of the peak indicates an additional component with a lower molecular mass. Both the shapes and the mean values of the peaks, obtained after 20 and 40 min, suggest a gradual transition from a species with molecular mass of 64 852 Da to one with molecular mass of 64 780 Da (see also Fig. 3B).

conditions, the rates of HuAChE phosphonylation with the homologous series of alkyl methylphosphonofluoridates (soman, DMPF, IBMPF, sarin) and the stabilities of the corresponding phosphonyl-HuAChE conjugates were measured (see Table 1). The actual measurements were carried out by reacting the enzyme with excess diastereomeric mixtures of DMPF, IBMPF and sarin and subjecting the reaction solutions to mass spectrometric analysis 2–4 min after the reaction onset. On the other hand, in the case of soman, measurements were carried out with each of the purified diastereomers (C+P− and C+P+) since for the diastereomeric mixture only the aged product is expected to be formed within a few minutes after the phosphonylation (see Table 1 [28]).

The mass spectrometric results for some of the phosphonyl-HuAChE-bac adducts are presented in Fig. 1. For the isopropyl methylphosphonyl-HuAChE-bac (derived from reaction with sarin), the molecular mass (Fig. 1B) corresponds exactly to the sum of previously measured mass of the free enzyme and that of the phosphonyl moiety (64 700+121). Mass measurement of the reaction product with IBMPF (Fig. 1C), which differs from sarin by one methyl group, gave a molecular mass of 64 840 (compared to the calculated mass 64 700+135). For the (P+)-1,2,2-trimethylpropyl methylphosphono-HuAChE-bac (Fig. 1D), the observed molecular mass – 64 860 Da – corresponds to an intact somanyl adduct (calculated 64 700+163 Da) which is different by two and

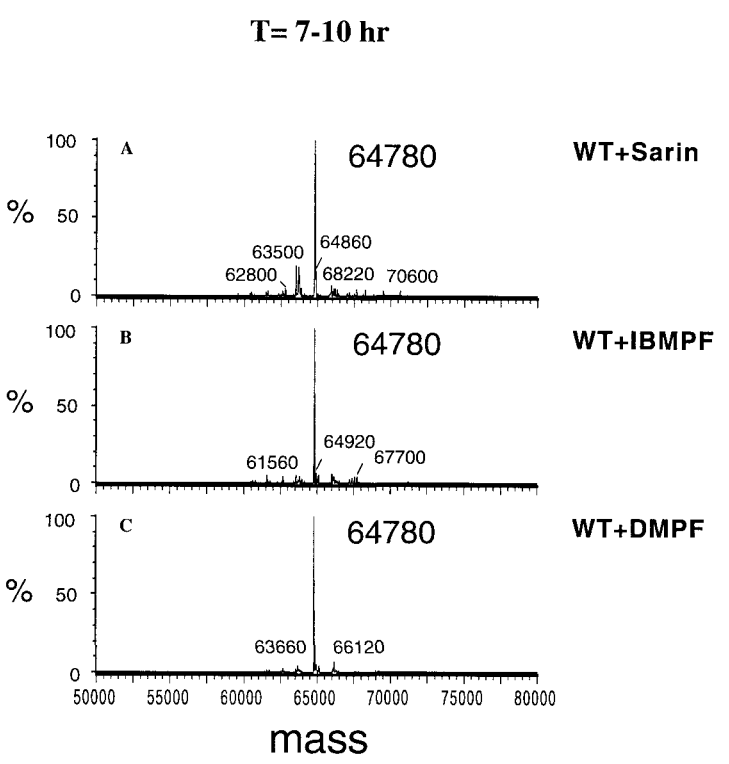

Fig. 3. Positive-ion ESI-MS mass spectra of phosphono-HuAChE-bac conjugates, 7–10 h after phosphonylation. For all the cases shown, the molecular mass corresponds to a common product resulting from dealkylation the respective phosphonyl moieties (calculated 64 778 Da). Note that these spectra are equivalent to that obtained for the phosphonylation product with (C+P−)-1,2,2-trimethylpropyl methylphosphonofluoridate (see Fig. 1E).

three methyl groups from the corresponding adducts of IBMPF and sarin, respectively. These results demonstrate again the utility of the ESI-MS in monitoring chemical changes in enzymes, like HuAChE, and its accuracy in measuring such high molecular masses with deviations of ≤5 Da (corresponding to the precision of 0.01% achievable by this method [42]). Furthermore, in accordance with the kinetic results (see Table 1), intact (non-'aged') phosphonyl-HuAChE adducts were observed for all the cases where the development of non-reactivability was slow. On the other hand, for the phosphonylation product with the (C+P−)-stereoisomer of soman, only an entity with molecular mass of 64 780 Da could be observed after 4 min (Fig. 1E). This molecular mass corresponds almost exactly to that calculated (64 700+78 Da) of a dealkylated methylphosphonyl-HuAChE adduct resulting in the 'aging' process (see Scheme 1). Assuming that the *progressive* non-reactivability of the other phosphonyl adducts originates from analogous dealkylation mechanism, the same dealkylation product (64 780 Da) should result in all cases (see Scheme 1). This point is clearly demonstrated by the behavior of 1,2-dimethylpropyl methyl-phosphono-HuAChE-bac where the time frame of the development of non-reactivability (Table 1) makes it possible to actually follow the process (see Fig. 2). Although the mass difference between the intact and aged adducts is too small to permit observation of two discrete masses, the molecular mass measured after 10 min – 64 852 Da (calculated for the intact adduct 64 700+149 Da, see Fig. 2A) and that measured after 7.5 h – 64 780 Da (calcualted 64 700+78 Da, see Fig. 2D), as well as the continuous shift of the mean mass (Fig. 2B,C) all indicate the progression of the dealkylation process.

The notion that aging results in a common product for all the methylphosphonyl-HuAChE conjugates is further supported by measuring the composition of the sarin and IBMPF adducts after 7–10 h. In both cases, the adducts underwent conversion to molecular entities with the same mass (64 780 Da, see Fig. 3A,B) as those resulting from (P−)-soman-HuAChE and the DMPF conjugates (see Figs. 1E and 3C). Since for all the adducts the time scale of this change is comparable to that of the kinetically observed aging process (Table 1), we can conclude that the aging indeed involves loss of alkyl group from the phosphonyl conjugate, as has been shown in the cases of trypsin [32] and chymotrypsin [33]. Furthermore, no other molecular species could be observed, indicating that

Table 1
Rate constants of phosphonylation (k_i) and $t_{1/2}$ for the aging of soman, DMPF, IBMPF or sarin-inhibited HuAChE[a] at pH 8.0, 24°C

	Soman		DMPF	IBMPF	Sarin
	C(+)P(−)	(C(+)P(+)			
k_i ($\times 10^{-5}$ min^{-1} M^{-1})	1500	<0.05	2000	1400	230
$t_{1/2}$ (min)	5.3	>40 000	130	255	2300

[a]Expressed in mammalian cells.

in case of phosphonyl adducts, aging is the only mechanism responsible for non-reactivability. This point is further exemplified by both, the stability of the aged (P−)-soman-HuAChE adduct (Fig. 1E) and by the continuous change observed in the case of DMPF-HuAChE adduct, where the only masses observed correspond to the transition between the non-aged and the aged (dealkylated) forms. On the other hand, the molecular mass of the P(+)-soman-HuAChE adduct remained unchanged after 10 h, in agreement with the already reported stability of the (P+)-soman-AChE adducts [48].

The ability to observe and sometime even to monitor the chemical transitions of HuAChE covalent adducts by a direct determination of chemical composition may help to examine other cases where the post-inhibitory behavior of the adducts is not clear. The fact that the current ESI-MS method is applicable to enzyme preparations that are either non-glycosylated or have a homogenous carbohydrate profile should not limit its utility for investigating various aspects of HuAChE function. Such aspects include, for example, the reactivation properties of tabun-AChE adducts or the incomplete regeneration of enzymatic activity which is often observed following inhibition of HuAChE enzymes by the presumably reversible active center inhibitor – *m*-(*N,N,N*-trimethylammonio)-trifluoroacetophenone [49]. The findings also exemplify the great potential of ESI-MS and related mass spectrometric techniques to the study of structure–function and reactivity characteristics of enzymes.

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