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Small Molecular Products of Dealkylation in Soman-Inhibited Electric Eel Acetylcholinesterase†

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ABSTRACT: Product analysis of dealkylation in P(*S*)C(*S*)-soman-inhibited electric eel acetylcholinesterase (AChE) by GC−MS using the selected ion monitoring mode has been carried out. The instrument was calibrated with pure standards of 2,3-dimethyl-1-butene and 2,3-dimethyl-2-butene in the gas phase and methylene chloride extracts of 2,3-dimethyl-2-butanol and 3,3-dimethyl-2-butanol from the aqueous phase. The dealkylation in soman-inhibited AChE at pH 5.0 ± 0.2 and 25 °C produces close to 40% alkenes and 50−60% 2,3-dimethyl-2-butanol. No 3,3-dimethyl-2-butanol could be detected to provide direct evidence of the intervention of a secondary carbenium ion in the reaction path. All the products of the reaction originate from a tertiary carbenium ion. These findings are in good agreement with the results of Michel et al. [(1967) *Arch. Biochem. Biophys. 121*, 29], which were obtained by countercurrent distribution of tritium-labeled products and their identification by scintillation counting. The early experiments were performed with the mixture of the four soman diastereomers, all labeled with tritium in Cα.

The toxic effects of organophosphorus (OP)[1] compounds are predicated on their irreversible inhibition of acetylcholinesterase (AChE) (*1*, *2*) and other serine hydrolases (*3*−*7*). Many of the compounds find applications as pesticides and nerve gases (*8*, *9*). A paradigm of OP inhibition of cholinesterases (ChEs) is with 2-(3,3-dimethylbutyl) methylphosphonofluoridate (soman), one of the nerve gases (*6*, *7*, *10*−*39*). Soman finds a rapid route of entry into vital organs of mammalian systems via the lungs. The toxic effect of soman is aggravated by a secondary reaction, dealkylation (aging) of the pinacolyl group from the pinacolyl methylphosphonate adduct of ChEs (*6*, *7*, *10*−*15*, *21*−*42*). Dealkylation in soman-inhibited ChEs competes very effectively with slow dephosphonylation. In fact, the reaction occurs 10 orders of magnitude faster than a comparable nonenzymic dealkylation reaction and 4 orders of magnitude faster than a similar reaction in soman-inhibited trypsin (*27*). Dealkylation in soman-inhibited AChE results in the production of a phos-

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[1] Abbreviations: AChE, acetylcholinesterase (acetyl hydrolase); BChE, butyrylcholinesterase; ChE, cholinesterase; Ee, electric eel; *Tc*, *Torpedo californica*; soman, 2-(3,3-dimethylbutyl) methylphosphonofluoridate; 2,3-2-ol, 2,3-dimethyl-2-butanol; 3,3-2-ol, 3,3-dimethyl-2-butanol; 2,3-1-ene, 2,3-dimethyl-1-butene; 2,3-2-ene, 2,3-dimethyl-2-butene; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; GC−MS, gas chromatography−mass spectroscopy; OP, organophosphorus; SIM, selected ion monitoring.

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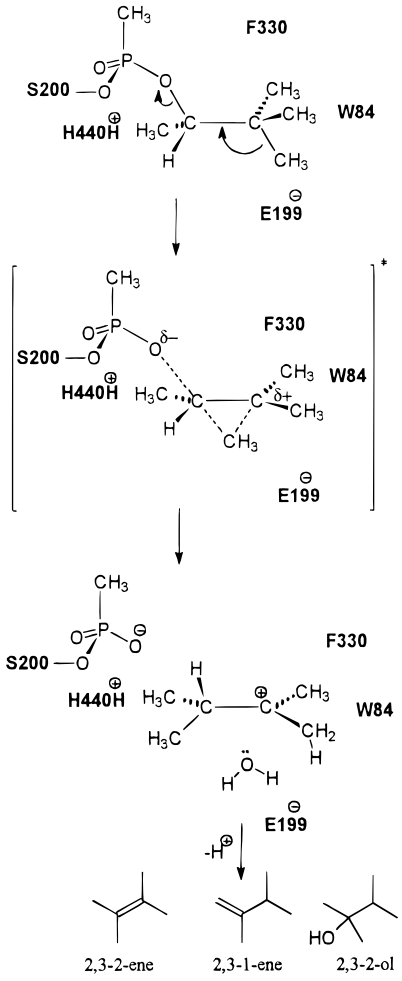
Scheme 1



phonate monoester anion that forms a salt bridge with the protonated His of the catalytic triad (*6*, *29*, *41*, *43*). This structural change has long been known to occur in serine proteases when inhibited with OP compounds (*44*) and recently confirmed for soman-inhibited AChE by X-ray crystallography (*41*, *42*).

The mechanisms of the dealkylation reaction have been studied with increasing frequency in the past three decades (*10*−*15*, *21*−*40*). A systematic investigation on the molecular level has been conducted in this laboratory (*6*, *7*, *21*−*28*, *37*, *39*, *40*). On the basis of studies of the pH dependence, solvent isotope effects, and thermodynamic parameters of the dealkylation reaction in the wild type and E199Q and W84A mutants of AChEs from different species and human butyrylcholinesterase (BChE), the mechanism in Scheme 1 has been proposed (*27*, *28*, *37*, *39*). The essence of the mechanism is that a methyl migration from Cβ to Cα is initiated in the ground state by an electrostatic and steric "push" from E199 and aromatic residues W84 and F330 at the anionic site. Nearly concerted with this migration, the C−O bond breaks which is promoted by the protonated H440 at the active site. Aside from the kinetic and mechanistic support, the bypassing of a secondary carbenium ion is an argument in favor of the mechanism. Molecular dynamics studies of the reaction of soman-inhibited AChE and soman-inhibited E199Q and W84A mutants also support a greater opportunity for a carbenium ion centered on Cβ rather than on Cα to be stabilized by surrounding carboxylates and π electrons (*39*, *40*).

The initial products of dealkylation are the phosphonate monoester anion adduct of the enzyme and a tertiary 2,3-dimethyl butylcarbenium-2 ion. The tertiary carbenium ion has been shown to collapse into alkene and alcohol products. The product distribution was studied by countercurrent distribution of the tritium label originating from soman labeled at Cα (*11*). This study in 1967 reported 60% 2,3-dimethyl-2-butanol (2,3-2-ol), 24% 2,3-dimethyl-1-butene (2,3-1-ene), 14% 2,3-dimethyl-2-butene (2,3-2-ene), and 0.25% 3,3-dimethyl-2-butanol (3,3-2-ol).

It appeared to us that the near absence of products originating from a secondary carbenium ion concurs with the findings of the kinetic studies, i.e., the direct formation of a tertiary carbenium ion. Michel et al. (*11*) expressed some concern about a possible tritium isotope effect on the product distribution resulting from the T-labeled soman. Tritium isotope effects would favor the formation of 1-ene over 2-ene. A more important concern may be that the experiments were performed with the mixture of the four diastereomers of soman, although most probably only the two diastereomers with *S* configuration at P formed covalent adducts with AChE (*16*).

Because of the singularity of this important experiment and the overshadowing questions, we have undertaken the determination of the product composition from the dealkylation reaction in P(*S*)C(*S*)-soman-inhibited electric eel (Ee) AChE. To capture the volatile alkene products, the headspace of the tightly sealed reaction vessel was sampled followed by the extraction of the alcohol product(s) from the aqueous phase, and both were analyzed by high-resolution GC−MS. The results of the experiments fully support the report of Michel et al. (*11*).

MATERIALS AND METHODS

Ee AChE (type VI-S), Bis-Tris propane, 5,5′-dithiobis(2-nitrobenzoic acid), and acetylthiocholine were Sigma Chemical Co. products, and 2,3-1-ene, 2,3-2-ene, 2,3-2-ol, 3,3-2-ol, methylene chloride, and ethyl acetate were purchased from Aldrich Chemical Co.

In preliminary experiments with simulated mixtures, the two expected hexenes were completely rejected by the aqueous phase; thus, their concentration in this phase is negligible. The two expected hexanols remained in the aqueous phase; their concentration in the gas phase was negligible. The alcohol standards were stable in pH 5−7 buffer solutions up to 70 °C in the GC−MS analysis. No rearrangement products could be detected either in the headspace or in the solution of the samples.

Accordingly, the hexenes were analyzed first by sampling only the gas phase and with the instrument calibrated with pure standards. Subsequently, the methylene chloride extract of the liquid phase was analyzed for hexanols. The instrument was calibrated with an extract prepared under the same conditions, that is, using the same ratio of the aqueous phase and the methylene chloride phase. Thus, the GC−MS analysis was carried out in the absence of excess water, enzyme, or different buffer salts.

Mass spectra were acquired on a JEOL SX102 mass spectrometer (Peabody, MA) in SIM mode. Specifically, the

Table 1: Physical Constants of the Expected Products

	MW	density (g/mL)	bp (°C)	P(25 °C) (atm)	p (atm)	B (L/mol)	V_M (L/mol)
2,3-1-ene	84.16	$0.6803^{20/4}$	55.67	0.332	0.163	−1.56	148.5
2,3-2-ene	84.16	$0.7080^{20/4}$	73.2	0.165	0.084	−2.08	289.2
2,3-2-ol	102.18	$0.8236^{20/4}$	118.4	NC[a]	NC	NC	0.12406
3,3-2-ol	102.18	0.8122^{25}	120.4	NC	NC	NC	0.12581

[a] Not calculated.

ion at *m*/*z* 84 ($M^{+\bullet}$) was monitored for the alkenes and *m*/*z* 87 (M − 15) for the alcohols. Samples were injected onto the GC column (0.32 mm × 30 m, Rtx-1, Restek Bellefonte, PA) held isocratically at 40 °C. The injector and the transfer lines were held at 120 °C. The signals for these ions in the sample were compared with those in the standard.

Calibration

Hexenes. A mixture of the two hexenes was prepared in a 200 μL glass vial from 25 μL of each compound. The vial was vortexed, sealed, and kept at room temperature for 10−15 min for equilibration. A 20 μL sample was taken from the gas phase (air saturated with the vapors of the two hexenes) and diluted in air in a 5 mL glass vial. The mixture of the hexenes was considered to be ideal, so their vapor pressure was a function of the temperature only and was calculated using the Antoine equation (*45*):

$$\log P = A - \frac{B}{C + t}$$

where *A*, *B*, and *C* are constants taken from the literature and *t* is the temperature in degrees Celsius.

The partial pressures of the two hexenes were calculated according to Raoul's law, $p_A = X_A P_A$, where X_A is the molar fraction in the liquid phase and P_A is the vapor pressure of the pure component calculated at the corresponding temperature. The temperature of the vapors was relatively close to the boiling points of the hexenes; thus, a nonideal dependence of the volume on the temperature was considered for each component separately. The molar volumes were calculated according to the equation

$$pV_M = RT\left(1 + \frac{B}{V_M}\right)$$

and the second virial coefficients, *B*, were calculated according to the literature (*46*, *47*). The gas mixture from the 5 mL vial was used as a standard to calibrate the instrument for hexenes. After the GC−MS analysis, the response factors were also calculated independently with the equation $f_A = A_A/n_A$, where *A* is the peak area and *n* is the amount of compound injected into the column.

Hexanols. An 8.0×10^{-5} M aqueous solution of the two hexanols was prepared by diluting 5 μL from each in 500 mL of distilled water. The mixture of hexanols was extracted with methylene chloride in a vial similar to the one used for the experiment. This extract was used as a standard to calibrate the GC−MS signals for hexanols.

Because the extractions were performed under the same temperature and same volume ratio of the phases, the ratio of the initial analyte concentrations in the aqueous phase, $(c^{S,R})_{H_2O}$, is equal to the ratio of the analyte concentrations in the methylene chloride phase, $(c^{S,R})_{CH_2Cl_2}$, which is equal to the ratio of the corresponding peak areas:

$$\left(\frac{c^S}{c^R}\right)^0_{H_2O} = \left(\frac{c^S}{c^R}\right)_{CH_2Cl_2} = \frac{A^S}{A^R}$$

When the same volumes are injected, the above ratios are also valid for the number of moles. The response factor can also be expressed as the ratio between the peak areas and the corresponding amount of hexanol in the initial aqueous standard solution. Some physical constants used in the calculations are presented in Table 1.

Product Analysis

Twenty-one milligrams of AChE-lyophilized powder was dissolved in 100 μL of 0.01 M Bis-Tris propane buffer (pH 9.9) in a 0.3 mL screw-top V-vial with a Mininert syringe valve. The enzyme solution (1.8×10^{-4} M) was degassed under vacuum and assayed using Ellman's method (*48*). Twenty-five microliters of 0.24 mg/mL P(*S*)C(*S*)-soman solution in ethyl acetate was added (66% excess), and the solution was assayed for complete inhibition. At these concentrations of the reactants, pH 9.9, and 4 °C, phosphonylation should be complete in a few seconds (*17*) and the excess of soman should hydrolyze in a few hours (*49*). The inhibited enzyme was kept at 4 °C for about 2 h, and it was stored overnight at −10 °C. To initiate the dealkylation reaction, 16 μL of 0.1 M HCl was injected into the mixture through the needle-seal septum of the Mininert valve to bring the pH of the mixture to ∼5. After 15 min, the gas phase was sampled at room temperature several times for hexene analysis by GC−MS. To analyze the hexanols, 50 μL of methylene chloride was injected into the mixture and the mixture stirred vigorously and centrifuged to separate the layers. The bottom layer was sampled several times for GC−MS analysis. The protein precipitated out after ∼15 min. Product recovery was within ±20% of the theoretical value.

RESULTS AND DISCUSSION

Analysis of the Gas Phase. After dealkylation had been complete at pH 5.0 ± 0.2, three major components were found in the headspace of the reaction mixture. They were identified from their retention times as 2,3-1-ene (1.48 min), ethyl acetate (1.62 min), and 2,3-2-ene (1.77 min). Ethyl acetate was the solvent for soman; the other two were dealkylation products. The headspace was sampled several times, but the peak areas decreased in time because of losses; thus, only the first values were used for analysis.

Analysis of the Liquid Phase. The methylene chloride extracts of the aqueous standard solution of the two hexanols and of the reaction mixture were sampled three times, and the averages of the corresponding peak areas were calculated. From the two expected hexanols, only 2,3-2-ol was found

Table 2: Relevant Peak Areas and Results of Analysis of Experiment 1

	A^S	response factor (intensity/nmol)	A^R	amount (nmol)	%
2,3-1-ene	60.4	112	11.4	3.6	15.8
2,3-2-ene	17.0	61.4	8.70	5.0	22.0
2,3-2-ol	20.4	42.3	119	14.0	62.2

Table 3: Relevant Peak Areas and Results of Analysis of Experiment 2

	A^S	response factor (intensity/nmol)	A^R	amount (nmol)	%
2,3-1-ene	45.8	85.0	14.9	2.6	17.4
2,3-2-ene	25.6	92.4	30.8	5.0	33.2
2,3-2-ol	46.2	291	21.7	7.4	49.4

and identified with a retention time of 2.61 min. If 3,3-2-ol (retention time of 2.73 min) is present, it is below the detection limit, that is, $\sim 5 \times 10^{-7}$ M or less than 1% of the total amount of products. The solubility of 2,3-2-ol in water at 25 °C is 4.18% and slightly greater than the solubility of 3,3-2-ol, 2.43% (*50*). It can be expected that 2,3-2-ol is also slightly less soluble in methylene chloride than 3,3-2-ol because it has a larger distribution coefficient K ($[\text{hexanol}]_{H_2O}/[\text{hexanol}]_{CH_2Cl_2}$). Thus, it is slightly more difficult to detect 2,3-2-ol than 3,3-2-ol in the methylene chloride phase. Nevertheless, 2,3-2-ol could be detected and 3,3-2-ol could not. The relevant peak areas for the three products in the standard (A^S) and the reaction mixture (A^R) and the calculated results are presented in Table 2.

The experiment was repeated under similar conditions. The results are presented in Table 3. In this case, the protein was denatured faster by methylene chloride during the extraction and a complete separation of the phases through centrifugation could not be accomplished. The sample from the methylene chloride phase was contaminated by the aqueous phase; consequently, the relative amount of alcohol was smaller, and the mass balance was deficient (82%).

These results are in good overall agreement with those obtained by Michel et al. (*11*), using a completely different technique, countercurrent distribution of the T-labeled products and identification by scintillation counting.

All products of the reaction originate from a tertiary carbenium ion. Their composition is most likely determined by the last step of the reaction which is the breakdown of the tertiary carbenium ion. In contrast, kinetic and previous mechanistic studies provide information about the rate-determining step for the process, resulting in C−O bond breaking and total loss of enzyme activity. An interesting question remains about whether a backside 1,2-methyl shift also occurs in close concert with C−O bond breaking. The unusual facility of the ChE-catalyzed dealkylation reaction may be due to such methyl migration enforcing C−O bond cleavage and thus bypassing formation of an unstable secondary carbenium ion. One may expect quick trapping of a secondary carbenium ion by solvent water. To date, there is no evidence of that. Once the secondary alcohol, 3,3-2-ol, is formed, it is not expected to rearrange to tertiary products. This has been the observation with pinacolyl alcohol standards in our GC−MS studies.

Neither the present results nor the results of mechanistic studies alone may be fully convincing for or against the fleeting occurrence of a secondary carbenium ion that subsequently undergoes methyl migration. A clear distinction between a stepwise and a concerted reaction may require a probe of the stereochemistry of the reaction. An isotopic labeling experiment may shed light on the actual mechanism, but seems too costly and demanding.

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