Vol. 197, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1993 Pages 343-349

THE ROLE OF GLUTAMATE-199 IN THE AGING OF CHOLINESTERASE

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Received October 15, 1993

Aging of organophosphate-conjugated acetylcholinesterase results from the loss of an alkoxy group with concomitant stabilization of the conjugate to spontaneous or nucleophile-induced deacylation. We have examined the kinetics of aging in a pinacolylmethylphosphonofluoridate (soman)-inhibited mutant enzyme in which the glutamate (E_{199}) located at the amino-terminal to the active-site serine (S_{200}) was converted to glutamine (Q). For wild type enzyme, the soman-acetylcholinesterase conjugate aged immediately, giving rise to a form of enzyme resistant to reactivation by oximes. In contrast, the $E_{199}Q$ mutant enzyme was largely resistant to aging and could be reactivated by oximes. Since the pH dependence for aging was not altered appreciably, the primary influence of the loss of charge appears to be on the intrinsic rate of aging. The negative charge on E_{199} likely imparts an inductive effect on the conjugated organophosphate to facilitate removal of the alkoxy group.

Organophosphates (OP), upon reaction with cholinesterase, produce phosphorylated, phosphonylated, and phosphinylated enzyme conjugates with the active-center serine (S_{200}) (1,2). These conjugates, while relatively slow in turnover, have two dominant modes of reaction (2-7). Spontaneous reactivation results from cleavage of the serine-O-P bond forming the reactive serine. Aging results from cleavage of the alkoxy -O-P bond, forming the corresponding $P\text{-}O^-$ containing conjugates which can no longer be reactivated (2,3,8). Analysis of the kinetics and pH profiles of aging suggest two potential mechanisms for aging: a general acid-catalyzed reaction and one catalyzed by a nucleophile/activated H_2O.

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0006-291X/93 $4.00

The acid or low pH-catalyzed rate of aging appears most rapid with OP-acetylcholinesterase (AChE) conjugates containing tertiary alkoxy groups, followed by secondary and primary alkoxy groups. This suggests that a carbonium ion may serve as an intermediate (2,4,9). Formation of the aged enzyme yields a species resistant to spontaneous or nucleophilic attack to achieve reactivation. This may be due, in part, to the negatively-charged phosphate forming a stable ion pair but may also result from a change in conformation of the conjugated species. Evidence for the former mechanism comes from the crystallographic structure of an aged alkylphosphate-chymotrypsin conjugate (10), whereas changes in the fluorescence spectra of pyrenebutylphosphono-AChE and chymotrypsin complexes also suggest a change in conformation of the conjugate (11).

To examine the kinetics of aging in more detail, we have inhibited wild type and mutant AChEs with soman and examined aging under various conditions. This has led to new information on the mechanistic basis of the aging process.

MATERIALS AND METHODS

Materials. Wild type and $E_{199}Q$ mutant of *Torpedo* AChE were expressed and purified by affinity chromatography, as previously described (12). One nmole of wild type AChE was equivalent to 250 units, and one nmole of $E_{199}Q$ mutant AChE was equivalent to 42 units. Soman was obtained from the Chemical Research, Development and Engineering Center (Aberdeen Proving Ground, MD). Soman used in these experiments was 98.6% pure when analyzed by [^{31}P] nuclear magnetic resonance. Concentrations of soman solutions were determined by titration of the solution with a known amount of fetal bovine serum (FBS) AChE and measurement of residual activity (1 nmol of FBS AChE is equivalent to 400 units). The oxime, 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxyaminopyridimium)-dimethyl ether hydrochloride (HI-6), was obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research.

Titration of AChE with Soman. Dilutions of soman in saline were incubated with AChE at room temperature for at least 30 min in 50 mM sodium phosphate, pH 8.0, containing 0.01% BSA. Residual AChE activity was plotted against the concentration of soman added to the reaction mixture to ascertain the stoichiometry between AChE and soman.

Time Course for the Reactivation of Soman-Inhibited AChE with HI-6. Fifty µl aliquots of enzyme (0.8 units/ml, 50 mM phosphate, pH 8.0, containing 0.01% BSA) were incubated at room temperature with various amounts of soman for 30 min; 5 µl was withdrawn for assay of residual AChE activity using the Ellman method (13). To the remaining sample, 3 µl of 40 mM HI-6 solution was added so that the final concentration of oxime was 2.5 mM. Enzyme activity of aliquots of the reactivation mixture were measured at intervals of 0.5, 1, 2, 3, 4, 6, and 24 hr using the Ellman method.

pH Profile for the Aging of AChE. To 108 µl of AChE (0.9 units/ml) were added 12 µl of one of the 0.5 M buffer stock solutions at different pH values. The following buffer solutions were used: sodium acetate at pH 5.0 and 5.5; sodium phosphate at pH 6.0, 6.5, 7.0, 7.5, and 8.0; Tris-HCl at pH 8.5. For each pH, soman was mixed with enzyme at concentrations of 3 ng/ml for wild type AChE and 60 ng/ml for $E_{199}Q$ mutant AChE. These concentrations of soman were slightly in excess of the amounts needed to completely inhibit the wild type and mutant AChE. Parallel samples without soman were used to monitor stability of the enzyme at each pH. Ten

μl aliquots were removed at various time intervals and transferred to tubes containing 10 μl of 5 mM HI-6 in 50 mM phosphate, pH 8.0. Samples were incubated overnight at room temperature before assay for AChE activity using the Ellman method.

RESULTS AND DISCUSSION

Previously we showed that alkylphosphates reacted more slowly with the $E_{199}Q$ mutant than wild type AChE. For example, in the mutant enzyme, diisopropylfluorophosphate and haloxon showed 263-fold and 50-fold reductions in the rate of phosphorylation, respectively (12). In contrast, reductions in the rates of sulfonylation and carbamoylation in the mutant enzyme appear to be smaller (5-fold and 3- to 20-fold, respectively). In the case of carbamoylating substrate, this mutation had little effect on deacylation rates. After phosphorylation with haloxon, appreciable spontaneous reactivation was not evident in the wild type and mutant enzymes (12).

The crystal structure of the *Torpedo* AChE shows E_{199} to reside at the base of the active-center gorge, and its electron cloud appears to reside within 1.5 Å of that of the quaternary ammonium group of the substrate or inhibitor bound to the active center of the enzyme (14, 15).

To examine the role of E_{199} in aging and reactivation we have employed soman-inhibited AChE, whose rate of aging is sufficiently rapid that it occurs concomitantly with the inactivation of enzyme (3,4). At the concentrations of soman employed, the approach to inhibition appeared practically stoichiometric, and the differences in titration curves reflect the 6-fold difference in k_{cat} for acetylthiocholine hydrolysis between wild type and mutant enzymes (Fig. 1). In Fig. 2, we show that only the mutant enzyme was reactivated by the oxime HI-6 at pH 8.0. At least 85% of the wild type enzyme had aged prior to addition of HI-6. In contrast, reactivation of the $E_{199}Q$ mutant enzyme was rapid and virtually complete (Fig. 2), and the presence of excess soman had little influence on the reactivation rate.

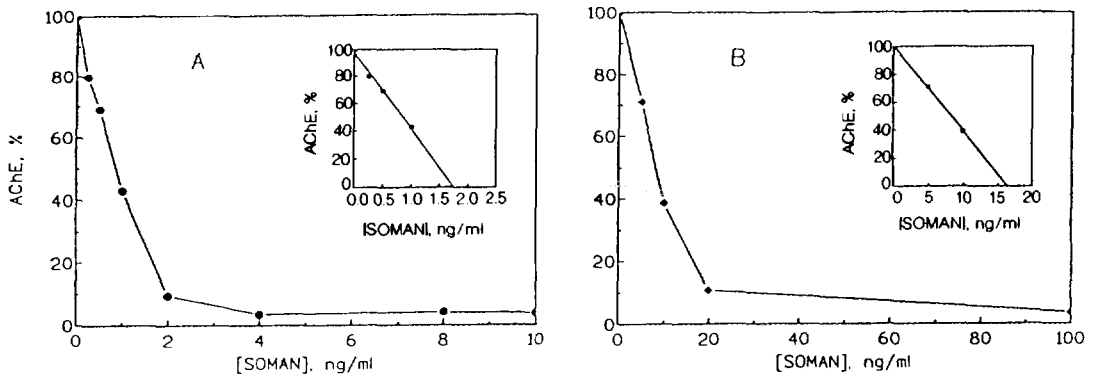

Figure 1. Titration of Torpedo Wild Type and $E_{199}Q$ Mutant AChE with Soman. Data shown are percent of residual AChE activity at various concentrations of soman. Panel A, wild type *Torpedo* AChE; Panel B, $E_{199}Q$ mutant *Torpedo* AChE. Insets: extrapolation of the linear portion of the curve, showing the amount of soman required for inhibition of 100% enzyme activity. Incubation time was 15 minutes.

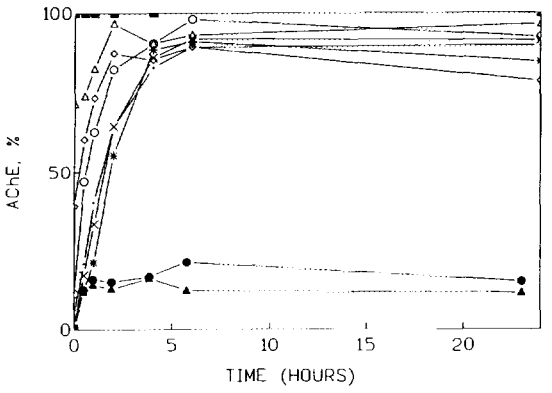

Figure 2. Time Course for the Reactivation of Soman-Inhibited *Torpedo* Wild Type and $E_{199}Q$ Mutant AChE with HI-6. Data are plotted as percent of AChE control activity without soman (■), wild type AChE (closed symbols), and $E_{199}Q$ mutant AChE (open symbols). The various curves represent different concentrations of soman used for inhibiting the enzyme: ▵,▲, 5 ng/ ml; ◊, 10 ng/ml; ○,●,20 ng/ml; · 100 ng/ml; x, 200 ng/ml and *, 500 ng/ml. The enzyme with various concentrations of soman was incubated for 30 minutes prior to transfer to HI-6 solution.

Aging has been proposed to occur by two mechanisms: a pH-dependent, proton-catalyzed dealkylation via a carbonium-ion intermediate and an enzyme or base-catalyzed hydrolytic cleavage. Since the first mechanism shows an increased rate of aging at low pH, we examined the pH dependence of aging by incubation of the enzyme-soman conjugate at various pH's and subsequent measurement of the extent of reactivation by HI-6 at pH 8.0 (Fig. 3). In general, the aging constants for $E_{199}Q$ mutant AChE were 150-700 fold lower at pH 7.0-7.5 than wild type AChE. At pH values below 7.5, aging of the wild type enzyme was too rapid to measure the aging constants accurately. Only a small portion of the kinetic profile was evident therefore, the values reported in this pH range are estimates of the rates of aging. At pH 8.0 and above, the rate of aging of $E_{199}Q$ mutant was so slow that it could not be measured under present experimental conditions (Table I). The soman-$E_{199}Q$ mutant enzyme conjugate showed essentially no aging after 24 hour incubation at pH 8.0 (not shown).

Although the rates of aging differ greatly, wild type and mutant enzymes show an increase in the rate of aging with decreasing pH (Table I). A similar pH dependence for the soman-*Electrophorus* AChE conjugate was observed previously (3). This overall profile for both enzymes is consistent with an aging mechanism involving a carbonium-ion intermediate. The small decrease in the rate of aging of $E_{199}Q$ at pH 5.5 may reflect partial unfolding or a different conformational state of the enzyme. Irreversible denaturation of enzyme is known to occur at this and lower pH values.

Similar pH dependencies suggest that aging in both enzyme conjugates is occurring by a common mechanism. Data also suggest that the carboxylate anion does not serve as attacking nucleophile in aging. It may be involved in influencing polarity of the phosphorus-oxygen dipole

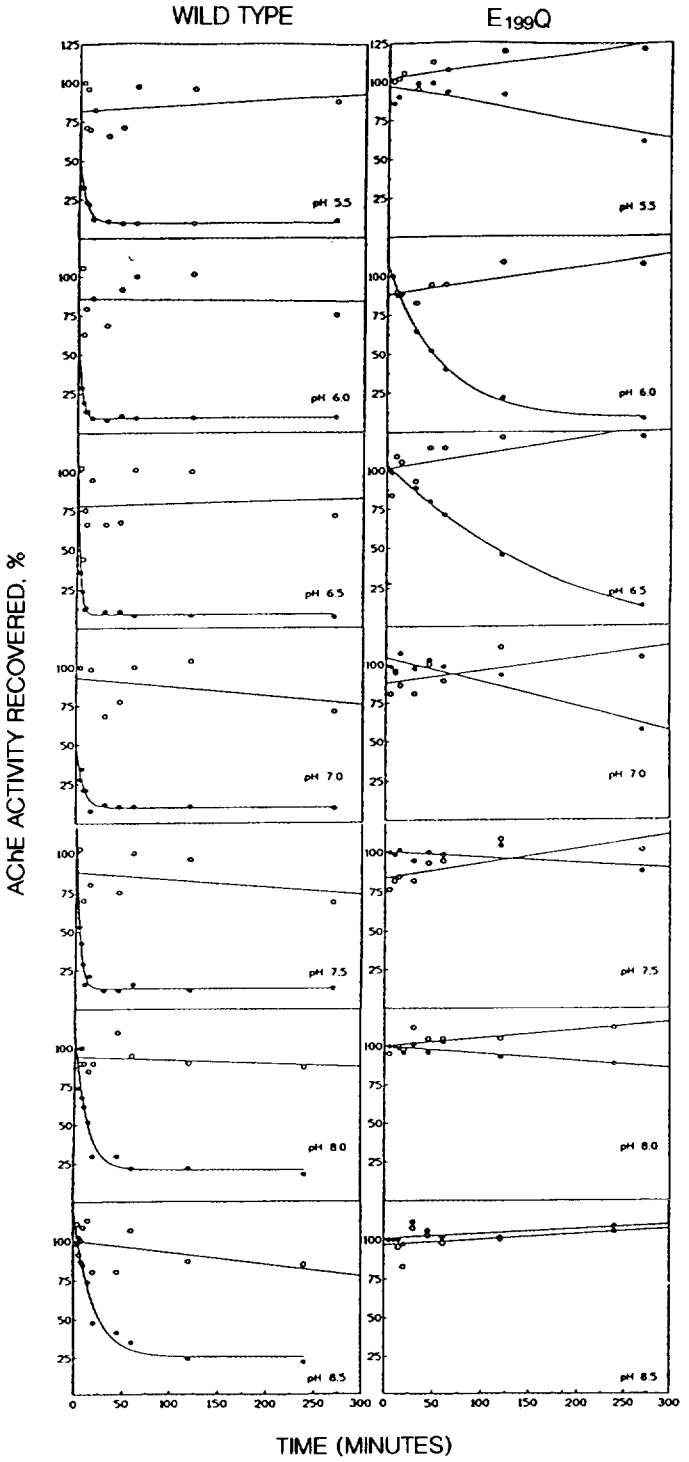

Figure 3. pH Profile for the Aging of *Torpedo* Wild Type and $E_{199}Q$ Mutant AChE. Data for each pH are plotted as percent of AChE control activity without soman (○) and with soman (•) for wild type (left panels) and $E_{199}Q$ mutant AChE (right panels).

Table I. pH DEPENDENCE OF THE RATE CONSTANTS FOR THE AGING OF *TORPEDO* WILD TYPE AND $E_{199}Q$ MUTANT ACETYLCHOLINESTERASES

pH	$k^{\dagger}$ (min^{-1})	
	Wild Type	$E_{199}Q$
5.5	0.14 ± 0.02‡	0.0008 ± 0.0002
6.0	0.34 ± 0.04‡	0.0200 ± 0.0020
6.5	0.37 ± 0.07‡	0.0050 ± 0.0010
7.0	0.15 ± 0.06‡	0.0010 ± 0.0002
7.5	0.18 ± 0.03	0.0003 ± 0.0001
8.0	0.08 ± 0.03	ND
8.5	0.05 ± 0.01	ND

† Determined by non-linear regression analyses of kinetic data shown in Fig. 3.
‡ Estimates from the residual portion of the kinetic profiles (Fig. 3).
ND: Aging not detected over a 24-hr period.

or stabilization of the carbonium ion intermediate in the aging reaction. The carboxylate side chain of E_{199} may reside near the effective positive charge on the phosphorus-oxygen dipole, thereby facilitating proton addition. Alternatively, it may ion pair with and effectively facilitate carbonium ion formation on the corresponding carbon of the pinacolyl moiety.

Aging has proven to be the major barrier to achieving oxime reactivation of AChE inhibited by the more potent organophosphates (16). Recombinant enzymes without this liability would confer a superior characteristic in the development of catalytic scavengers of organophosphates.

ACKNOWLEDGMENTS: Authors wish to thank Ms. Mary Kay Gentry for the preparation of and Dr. Yacov Ashani (visiting scientist at the Walter Reed Army Institute of Research) for critical review of this manuscript. This work was supported in part by Contract No. DAMD 17-9C-1058 to PT.

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