Organophosphorus (OP) esters inhibit acetylcholinesterases (AChE; EC 3.1.1.7) and butyrylcholinesterases (BChE; EC 3.1.1.8) by forming a stoichiometric (1:1) conjugate between the OP and the enzyme at the active-site serine (Aldrich & Reiner, 1972). In general, OP-inhibited cholinesterases (ChE) can be reactivated by various oxime nucleophiles (Aldrich & Reiner, 1972); however, for certain OPs, the displacement of the phosphoryl moiety from the active site to restore enzyme activity is impossible due to a parallel aging reaction (Hobigere, 1955; Berenda et al., 1959) (Figure 1). Thus, aging of phosphorylated ChEs is defined as the process that converts the inhibited enzyme into a form that can no longer be regenerated by commonly used reactivators. The inability to reactivate aged OP-AChE conjugates renders oxime treatment of the inhibited enzyme into a form that can no longer be regenerated by commonly used reactivators. This indicates that the unusual resistance to reactivation of the aged enzyme cannot be ascribed to simple electrostatic repulsion of an approaching nucleophile. The broadening of the $^{31}$P NMR signal of native OP-ChEs relative to that of OP-Ch is in agreement with the crystal structure of AChE, showing that the active site region of ChEs in solution resides in a deep, narrow gorge.

**Abstract:** $^{31}$P NMR spectroscopy of butyrylcholinesterase (BChE), acetylcholinesterase (AChE), and chymotrypsin (Cht) inhibited by pinacolyl methylphosphonofluoridate (soman), methylphosphonodifluoridate (MPDF), and diisopropyl phosphorofluoridate (DFP) allowed direct observation of the OP-linked moiety of aged (nonreactivatable) and nonaged organophosphorus (OP)--ChE conjugates. The $^{31}$P NMR chemical shifts of OP-ChE conjugates clearly demonstrated insertion of a P-O bond into the active site of aged OP-ChE adducts. The OP moiety of nonaged OP-ChEs was shown to be unchanged. The OP-bound pinacolyl moiety of soman-inhibited and aged AChE was detached completely, whereas only partial dealkylation of the pinacolyl group was observed for soman-inhibited BChEs. This suggests that the latter enzyme reacted with the less active stereoisomer(s) of soman. In the case of soman-inhibited Cht, no dealkylation could be experimentally detected for any of the four stereoisomers of OP-ChE adducts. Results are consistent with the contention that the phenomenon of enzyme-catalyzed dealkylation of OP adducts of serine hydrolyses strongly depends on the orientation of both the catalytic His and the carbonyl side chain of either Glu or Asp positioned next to the catalytic Ser. The denatured protein of aged OP--ChE or OP--Ch is a convenient leaving group in nucleophilic displacements of tetrahedral OP compounds despite the presence of a P-O bond. This indicates that the unusual resistance to reactivation of the aged enzyme cannot be ascribed to simple electrostatic repulsion of an approaching nucleophile. The broadening of the $^{31}$P NMR signal of native OP-ChEs relative to that of OP-Ch is in agreement with the crystal structure of AChE, showing that the active site region of ChEs in solution resides in a deep, narrow gorge.
of the aged and nonaged OP conjugates of various serine proteases (Gorenstein & Findlay, 1976; van der Drift et al., 1985; Gorenstein et al., 1989; Adebodun & Jordan, 1989a,b; Grunwald et al., 1989; Kovac et al., 1993). Recent X-ray crystallographic data on a homologous pair of aged and nonaged OP conjugates of chymotrypsin (Cht; EC 3.4.21.1) were reported to be in excellent agreement with the data obtained by 31P NMR spectroscopy for OP–Cht conjugates in solution (Harel et al., 1991). Thus, it was established that the aged OP–Cht conjugate contains a P–O–P bond which forms close interaction with the N\textsuperscript{2} atom of the catalytic amino acid His 57. Although the structure of AChE from Torpedo californica (TvAChE) has been determined by X-ray crystallography (Sussman et al., 1991), the three-dimensional (3D) geometry of its phosphorylated active site has not been reported. The lack of sufficient quantities of purified cholinesterases (Cht) until recently precluded proper utilization of the 31P NMR technique for the determination of the structure of aged and nonaged forms of OP-inhibited ChEs.

In this study we have characterized the OP moiety of phosphorylated ChEs by both direct and comparative 31P NMR spectroscopy. We report here on the elucidation of the structure of the aged and nonaged OP conjugates of AChE and BChE obtained using soman, methylphosphonodifluoridate (MPDF), and diisopropyl phosphorofluoridate (DFP). This, together with the characterization of a homologous pair of OP–Cht conjugates, permitted speculation on the mechanism of the reactivation and aging of OP–ChEs and offered a partial explanation for the unusual resistance of aged OP-ChEs to reactivation.

MATERIALS AND METHODS

- **Materials.** BChE from either human (HuBChE) or equine (EqBChE) serum was purified by an affinity chromatography technique to be published elsewhere. AChE from fetal bovine serum (FBS) was purified according to De La Hoz et al. (1986). One milligram of pure enzyme contained approximately 11 and 14 nmol of active site of BChE and AChE, respectively, with the following specific activities: HuBChE, 750; EqBChE, 950; and FBS–AChE, 5000 units/mg. Bovine pancreatic \( \alpha \)-Cht (type II, \( 3\times \) crystallized, salt-free, and lyophilized), N-succinyl-Ala-Ala-Pro-Phe-\( p \)-nitroanilide, and stereoisomers of soman, Cht was dissolved in distilled water, approximately 11 and 14 nmol of active site of BChE and AChE, respectively.

Preparation of OP–ChE Conjugates. Large quantities of OP–ChE conjugates were obtained by adding freshly prepared concentrated solutions (1–5 mM) of either soman (in 50% propylene glycol/water) or DFP (in 50% isopropanol/water) to 10–20 mg of purified ChE in 1–5 mL of 25 mM Tris buffer, pH 8.0. Assuming molecular masses of approximately 65 and 95 kDa for AChE and BChE, respectively, the ratio of the molar concentration of OP to ChE ranged between 1 and 5. The decrease in the enzymic activity was monitored until inhibition was complete. MPDF-inhibited BChE was obtained by sequential addition of 0.1 M MPDF in CH\textsubscript{3}CN to a solution of 10 mg of enzyme in 1 mL of 50 mM phosphate buffer, pH 8.0. A total of 25 \( \mu \)L was required to complete the inhibition of enzyme.

To remove traces of phosphorus-containing low molecular weight compounds, the inhibited enzyme was dialyzed against 2 L of 0.1 M Tris buffer, pH 8.0, for 48 h at 6 \( ^\circ \)C. Finally, volume of the OP–ChE conjugate was reduced to approximately 0.4 mL by ultrafiltration (Millipore Ultrafree-MC, Bedford, MA). The protein content of the concentrated solution (15–30 mg/mL) was determined spectrophotometrically at 280 nm using absorption coefficients of \( \varepsilon^\text{BChE} = 19 \) and 16 for BChE and AChE, respectively.

Enzymatic Assays. AChE and BChE activity were determined by the method of Ellman et al. (1961), using 0.45 mM BTC and ATC as substrates, respectively. Cht was assayed using the chromogenic substrate N-succinyl-Ala-Ala-Pro-\( p \)-nitroanilide (Delmar et al., 1979).

Titration of ChEs by Soman and DFP. To approximately 4–180 \( \mu \)M (active site concentration) of a ChE in either 5 mM phosphate or 10 mM Tris buffer, pH 8.0, various amounts of OP inhibitor solution (0.1–2 times enzyme equivalent) were added. The mixtures were incubated at 25 \( ^\circ \)C. Inhibition was allowed to proceed to completion, and residual enzyme activity was assayed by Ellman’s method, as described above. The percentage of residual enzyme activity was plotted against the number of equivalents of OP.

Preparation of OP–Cht Conjugates. One hundred to 250 \( \mu \)L of either 0.08 M soman in 50% propylene glycol/water or DFP in isoPrOH was added at room temperature to a stirred solution of 100–300 mg of Cht in 4 mL of 0.1 M Tris buffer, pH 7.6. The molar ratio of OP to Cht ranged between 2 and 5. To obtain soman-inhibited Cht that consisted of all four stereoisomers of soman, Cht was dissolved in distilled water, the pH was adjusted to 7.8 with 0.1 N NaOH, and the molar ratio of soman to Cht was adjusted to 1:1. The inhibitor–enzyme mixture was allowed to incubate until 99% inhibition of enzyme activity was obtained. To remove traces of phosphorus-containing low molecular weight compounds, the solution of inhibited enzyme was dialyzed against Tris buffer as described above. To obtain the assumed dealkylated form of soman-inhibited Cht, 4 mL of 1 mM Cht in distilled water was adjusted to pH 8.0 and mixed with 10 \( \mu \)L of 0.65 M MPDF in CH\textsubscript{3}CN. Approximately 80% of enzyme activity was inhibited. The pH (6.5) was readjusted to 8.0, and the remaining activity was inhibited with an additional 3 \( \mu \)L of MPDF stock solution. Inhibited enzyme was dialyzed against 0.1 M Tris buffer as described above.

Monitoring the Aging of OP–Inhibited ChEs and Cht. Inhibited ChEs were diluted 1000-fold at selected time intervals into a reactivation medium containing 1 mM of either 2-(hydroxyiminomethyl)-1-methylpyridinium methyl methane sulfonate (P\textsubscript{S}S) or 1,1'-(trimethylenediamine)bis(4-hydroxyiminomethyl) pyridinium dibromide (TMB\textsubscript{S}) (Gray, 1984) in 50 mM phosphate buffer, pH 8.0. OP–Cht conjugates were diluted 1000-fold into reactivation medium (0.1 M Tris–0.01 M CaCl\textsubscript{2}, pH 7.8) containing 0.1 M 3-(hydroxyiminomethyl)
NMR of Phosphorylated Cholinesterases

![Figure 2: Time course of the aging of soman-inhibited EqBChE (I), HuBChE (II) and FBS-AChE (a) in 50 mM phosphate buffer, pH 8.0, at 25 °C. (Inset) Plot of reactivation of enzyme activity vs time for the nonaged portion of soman-inhibited HuBChE. The line was fitted in accordance with first-order reaction kinetics in presence of 1 mM PSe.](image)

**Table I: **31P NMR Chemical Shifts of Soman-Related OP Moieties of Model Compounds and Phosphorylated ChEs and ChT Obtained with Soman

<table>
<thead>
<tr>
<th>OP model compounds</th>
<th>status</th>
<th>chemical shifts</th>
</tr>
</thead>
<tbody>
<tr>
<td>soman</td>
<td>CH3P(O)(O)OH</td>
<td>+30.81, +30.45</td>
</tr>
<tr>
<td>MP(Pin)2</td>
<td>CH3P(O)(O)OH2</td>
<td>+29.94, +29.46</td>
</tr>
<tr>
<td>DEMP</td>
<td>CH3P(O)(O)OH2</td>
<td>+31.39, +30.98</td>
</tr>
<tr>
<td>FinMP-OH</td>
<td>CH3P(O)(O)OH2</td>
<td>+22.27, +22.83</td>
</tr>
<tr>
<td>MP-OH</td>
<td>CH3P(O)(O)OH2</td>
<td>+17.44, +17.87</td>
</tr>
<tr>
<td>OP-enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FinMP-Cht</td>
<td>CH3P(O)(O)OH-Cht</td>
<td>+35.96, +33.02</td>
</tr>
<tr>
<td>FinMP-Cht</td>
<td>CH3P(O)(O)OH-Cht</td>
<td>+35.96, +33.02</td>
</tr>
<tr>
<td>MP-Cht</td>
<td>CH3P(O)(O)OH-Cht</td>
<td>+24.63, +24.69</td>
</tr>
<tr>
<td>MP-BChE</td>
<td>CH3P(O)(O)OH-BChE</td>
<td>+24.63, +24.69</td>
</tr>
<tr>
<td>MP-BChE</td>
<td>CH3P(O)(O)OH-BChE</td>
<td>+24.63, +24.69</td>
</tr>
</tbody>
</table>

*δ ppm relative to external TMP. Positive signs denote downfield shifts relative to TMP. Unless indicated, the 31P NMR line is a singlet. Error estimate is less than 0.05 ppm. † FinCM, –CH(CH3)2. Center of a doublet. ‡ δF, 973 Hz. § δP, 0.03 upfield to TMP, at pH 8.0. ¶31P NMR signal at -6.24 ppm. ‣ Chemical shift estimated from the center of a broad peak.

**Table II: **31P NMR Chemical Shifts of DFP-Related OP Moieties of Model Compounds and Phosphorylated ChEs and ChT

<table>
<thead>
<tr>
<th>OP</th>
<th>status</th>
<th>chemical shifts</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFP</td>
<td>(isoPrO)P(O)(O)2</td>
<td>-13.55 ‡</td>
</tr>
<tr>
<td>TIP</td>
<td>(isoPrO)P(O)</td>
<td>-6.30 ‡</td>
</tr>
<tr>
<td>DIP-OH</td>
<td>(isoPrO)P(O)OH</td>
<td>-3.97 ‡, -4.05 ‡</td>
</tr>
<tr>
<td>MEP-OH</td>
<td>(isoPrO)P(O)(O)OH2</td>
<td>-0.06 ‡</td>
</tr>
<tr>
<td>OP-enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIP-Cht</td>
<td>nonaged (isoPrO)P(O)-Cht</td>
<td>-3.55 ‡, -6.20 ‡</td>
</tr>
<tr>
<td>DIP-AChE</td>
<td>nonaged (isoPrO)P(O)-AChE</td>
<td>-5.99 ‡</td>
</tr>
<tr>
<td>MEP-Cht</td>
<td>aged (isoPrO)P(O)-Cht</td>
<td>-1.09 ‡, -3.43 ‡</td>
</tr>
<tr>
<td>MEP-BChE</td>
<td>aged (isoPrO)P(O)-BChE</td>
<td>-1.20 ‡, -3.35 ‡</td>
</tr>
</tbody>
</table>

1-methylpyridinium iodide (3-PAM) (Cohen & Erlanger, 1960). To ensure the absence of residual inhibitor, the activity of ChE or ChT freshly added to the corresponding OP-enzyme concentration range of 1-5 mM. The concentration of OP-enzyme solutions containing 10-20% of a doublet, JpF = 973 Hz. a Center of a doublet, JpF = 973 Hz. b Center of three lines. c Center of a singlet. Error estimate is less than 0.05 ppm. d isoPrCM, –(CH(CH3)2). e Center of a doublet. f δF, 973 Hz. g δP, 0.03 upfield to TMP, at pH 8.0. h31P NMR signal at -6.24 ppm. i Chemical shift estimated from the center of a broad peak.

**RESULTS**

**Titration of ChEs with Soman and DFP.** The stoichiometric amount of soman needed to inhibit 100% enzyme activity of either EqBChE or HuBChE was 20-25% higher.
Aging of OP Conjugates of ChEs and Cht. Following inhibition of either HuBChE or EqBChE with approximately 1.2-fold stoichiometric excess of soman, 60% of the inhibited enzyme aged within less than 30 min (Figure 2). The remaining 40% could be reactivated completely, even after 28 h of incubation at 25 °C prior to the dilution into the reactivation medium (Figure 2, inset). These results are in agreement with the observations of Keijer and Wolring (1969) who reported a t1/2 of approximately 9 and 60 min for the aging of EqBChE inhibited by the two potent stereoisomers of soman, P(-)C(+) and P(-)C(-) and (b) EqBChE inhibited by a third stereoisomer with a P(+) configuration did not undergo detectable aging when incubated 24 h at 25 °C (pH 7.5). In contrast to BChEs, more than 98% of soman-inhibited FBS-AChE was converted to a nonreactivatable form. The time course of the aging of soman-inhibited FBS-AChE displayed a distinct biphasic behavior (Figure 2), and aging was completed only after 120 min of incubation in 50 mM phosphate buffer (pH 8.0) at 25 °C.

No decrease in the ability to reactivate soman-inhibited Cht could be observed even after 96 h of incubation in 0.1 M Tris buffer, pH 8.0, at 25 °C. By adjusting the experimental conditions to enable all four stereoisomers of soman to phosphonylate Cht (see above), the inhibited enzyme could be reactivated only to 72–75% of its original activity. The inability to observe complete reactivation was independent of the time of incubation prior to dilution into the reactivation medium. In contrast to soman-inhibited Cht, no reactivation could be detected after 48 h of incubation of MPDF-inhibited Cht in the presence of 0.1 M 3-PAM. MPDF was expected to produce the aged form CH$_3$P(O)(OCht)O$^-$ due to rapid hydrolysis of the P-F bond of the phosphorylated enzyme [CH$_3$P(O)(OCht)F].

DIP-BChE and DIP-Cht could be reactivated only partially (25–65%). Since the reactivation proceeded at slow rates ($t_{1/2}>20$ h), it was assumed that other side reactions occurred in parallel to the reactivation (e.g., aging, autolysis, denaturation). To convert DIP-BChE and DIP-Cht completely into the aged forms, the inhibited enzymes were either dialyzed
at room temperature against 0.1 M Tris buffer, pH 7.0, for 96 h (DIP-BChE) or incubated at 37°C for 6 days (DIP-Ch).

Reactivation of Nonaged Soman-Inhibited BChE and Ch.
The nonaged portion of soman-BChE conjugate was reactivated by 1 mM P2S at a rate of 0.23 ± 0.02 h⁻¹, in accordance with a single-exponential decay equation (Figure 2). More than 98% of OP-Ch obtained by >2-fold stoichiometric excess of soman could be reactivated by 0.1 M 3-PAM. The data of the time course of reactivation of soman-inhibited Ch were best fitted by a biexponential kinetic equation with the following parameters: 59% reactivated at a rate of 0.072 h⁻¹ and 39% at 0.42 h⁻¹. The ratio of the two amplitudes (59/39) may reflect, in part, the relative anti-Ch activity of the two potent epimers P(−)C(±) (Ooms & van Dijk, 1961; Schoene, 1971).

The time course of the reactivation of Ch inhibited by all four stereoisomers of soman could be reasonably described by a triexponential kinetic equation with the following computer-fitted values: 24% at 0.060 h⁻¹, 22% at 0.062 h⁻¹, and 26% at 0.52 h⁻¹ (not shown). The inability to obtain full reactivation is attributed to steric hindrance rather than dealkylation (see below).

**31P NMR Spectroscopy.** (a) **31P NMR Chemical Shifts of Model Compounds.** Tables I and II list names, structures, and 31P NMR chemical shifts of soman- and DFP-related OPs, respectively. The characterization of differences between the aged and nonaged OP moieties was based in part on direct identification of the phosphoryl ligand obtained after hydrolysis of the OP-protein adduct in NaOH (Figure 1). Therefore, it was important to determine the pH dependence of the 31P NMR chemical shift of phosphoric and methylphosphonic...
 acids. Above pH 9, the chemical shifts of the esters were well separated from the mono- and diacid model compounds (Tables I and II). No changes in the $^{31}$P NMR spectra could be observed after 5 days of incubation of either the mono- or the diacids in 0.2 N NaOH. This finding validated the reaction pathway depicted in Figure 1 and permitted assignment of the $^{31}$P NMR signals of inhibited enzymes to the structure of the OP-bound moiety.

Figure 3 shows differences in both the chemical shifts and line multiplicity of the $^{31}$P NMR signals of soman and DFP. The P–F bond couples the $^{31}$P NMR line of the achiral DFP into a doublet. In the case of soman, which contains two chiral centers, CH$_3$P*(O)[OC*H(CH$_3$)$_2$C(CH$_3$)$_3$]F, a doubling of the resonance occurred. The two doublets of the $^{31}$P NMR signal of soman originate from two pairs of diastereomers, P(-)C(+); P(+)(-) and P(-)C(-); P(+)(+). These pairs are distinguishable by their $^{31}$P NMR chemical shifts. Due to compensation produced by the opposing chemical environment of the individual constituents, each pair displayed only one doublet. Thus, it is likely that OP adducts of soman will give rise to two $^{31}$P NMR signals. Kovach et al. (1993) have recently demonstrated the formation of two diastereomeric OP-Cht adducts by $^{31}$P NMR spectroscopy.

The line multiplicity of the NMR signal (Figure 3, upper traces; proton decoupler off), further demonstrates the applicability of the $^{31}$P NMR spectroscopy to determine the nature of the substituents around the P atom.

(b) $^{31}$P NMR Chemical Shifts of DFP- and Soman-Inhibited Chs. The $^{31}$P NMR chemical shifts of the native and unfolded forms of DIP-Cht, MIP-Cht, and PinMP-Cht (reported here for the first time) were used to assign the $^{31}$P NMR chemical shifts of homologous OP-ChE conjugates.

The two NMR signals associated with a fully reactivatable PinMP-Cht, obtained with a 2.2 molar excess of soman over Cht (Figure 4A, lower trace), are consistent with the insertion of the two potent stereoisomers of soman, i.e., P(-)C(+) and P(-)C(-) (Ooms & van Dijk, 1966; Schoene, 1971). A third peak appeared in the spectrum of a PinMP-Cht obtained with a 1:1 molar ratio of soman to Cht (Figure 4A, upper trace). This conjugate was demonstrated above to contain also the less active P(+) epimers. Similar multiplicity of the $^{31}$P NMR line was observed for the racemic model compound MP(Pin)$_2$ that contains three chiral centers (Table I). Since the P(+) containing OP-Cht preparation released only the monoacid PinMP-OH (Figure 4C), it is suggested that it did not undergo dealkylation at all.
Figure 6: $^{31}$P NMR spectra of approximately 0.2 mM soman- and MPDF-inhibited BChE in 0.1 M Tris, pH 8.0. Negative signs indicate an upfield chemical shift relative to external TMP (0 ppm). (Panel A) soman-inhibited EqBChE; (lower trace) in 0.1 M Tris-6 M Gdn-HCl, pH 8.0 (21 000 scans); (middle trace) NaOH-released OP ligands from a conjugate obtained by reacting 1:1 molar ratio of soman to BChE (25 000 scans); (upper trace) NaOH-released OP ligands from a conjugate obtained by a 5:1 molar ratio of soman-to-BChE (25 000 scans). (Panel B) Spectra of MPDF-inhibited HuBChE and 0.15 mM homologous conjugate of Cht; (lower trace) native MPDF-Cht (21 000 scans); (middle trace) native MPDF-HuBChE (96 000 scans); (upper trace) NaOH-released OP ligand from MPDF-inhibited HuBChE. Lines assignment (for definitions see Table I): 1, PinMP-BChE; 2, HMPA (internal standard); 3, MP-BChE; 4, PinMP-OH; 5, MP-OH; 6, MP-Cht; 7, phosphoric acid (internal standard).

When either PinMP-Cht was transferred to 0.1 M Tris-6 M Gdn-HCl, pH 8.0, the NMR peaks narrowed and moved upfield relative to the native enzyme by 1.8-4.7 ppm (Figure 4B). On the basis of previous reports with other OP-Cht conjugates (van der Drift, 1985; Grunwald et al., 1989), these changes were expected upon unfolding of the enzyme that perturbed the environment of the P atom toward that of the model compounds (Table I). Following the addition of NaOH to the native OP-Cht adduct, the broad signals disappeared with the concomitant rise of a single narrow peak which corresponded to the chemical shift of O-pinacolyl methylyphosphonic acid (PinMP-OH) in 0.2 N NaOH (+22.27 ppm; Figure 4C and Table I). Spiking with an authentic sample of PinMP-OH (not shown) and the multiplicity of the $^{31}$P NMR line (Figure 4C, lower trace) confirmed the exclusive release of PinMP-OH. Similar findings were obtained for the least potent P(+)-containing OP-Cht adducts that were incubated 150 h in 50 mM acetate buffer at pH 6.0. Dealkylation could not be detected even after 96 h of incubation at 35 °C (0.1 M Tris, pH 8.0).

To characterize the CH$_3$P(O)(O$^-$)-containing moiety of Cht (MP-Cht), the difluoridate MPDF was used to instantaneously obtain the aged enzyme. The $^{31}$P NMR of the latter conjugate clearly demonstrated that the NaOH-released OP was MP-OH (Figure 4D, upper trace). This enabled assignment of the $^{31}$P NMR chemical shifts of the native and the unfolded forms of MP-Cht (Figure 4D, lower trace and middle trace, respectively).

(c) $^{31}$P NMR Spectra and Identification of the OP-Containing Moieties of DFP- and Soman-Inhibited ChEs. In marked contrast to OP-Cht conjugates, 0.2 mM adducts obtained by reacting ChEs with either soman, MPDF, or DFP showed a broad $^{31}$P NMR signal of low intensity even after accumulation of 100 000 transients/spectrum. However, unfolding in 0.1 M Tris-6 M Gdn-HCl gave rise to an intense narrow signal already detectable after 20 000 scans (Figures 5-7). The $^{31}$P NMR chemical shifts of the unfolded form of aged MIP-ChE conjugates (~3.35 ppm; Figure 5A, middle trace) were similar to that observed for aged MIP-Cht in 0.1 M Tris-6 M Gdn-HCl (~3.43, Table II). Following dialysis against 0.1 M Tris buffer, pH 8.0, the $^{31}$P NMR peak disappeared and appeared again at the same position upon transfer of the dialyzed solution into 0.1 M Tris-6 M Gdn-HCl (Figure 5A, upper trace). These results demonstrate that the phosphoryl moiety is covalently bound to the enzyme. When the pH of a solution of native aged MIP-ChE conjugates was elevated to ~12, a new peak with a relatively high intensity was observed after 4000 transients at ~0.06 ppm upfield to external TMP (Figure 5B, lower trace). This pointed at the release of isoPrOP(O)(O$^-$)$_2$ (MIP) from the aged conjugate.

The $^{31}$P NMR normal mode (Figure 5B, middle trace) showed a doublet that is consistent with one hydrogen coupling the $^{31}$P NMR signal [(CH$_3$)$_2$CHOP(O)(O$^-$)$_2$]. Finally, spiking with authentic isoPrOP(O)(OH)$_2$ (Figure 5B, upper trace) confirmed the assignment of the structure of the aged form to isoPrOP(O)(O$^-$)-ChE. Similar conclusions were...
Tris-6 M Gdn.HCl produced two distinct narrow signals at related ChEs upon unfolding in 6 M Gdn.HCl not only the nonaged form. Consequently, release of applied to the nonaged DIP-ChE conjugates, the exclusive reached with MIP-AChE. When the same procedure was residue. See Tables I and Gdn.HCI, pH 8.0 (22 000 scans). Lines assignment (for definitions FBS-AChE in 0.1 M Tris-6 M Gdn.HCI (Figure 7B and (20 000 scans). (Panel B) DFP-inhibited AChE in 0.1 M Tris-6 M The dramatic change in the NMR spectra of the phospho-

FIGURE 7: 31P NMR spectra of approximately 0.25 mM OP-inhibited FBS–AChE. Negative signs indicate an upfield chemical shift relative to external TMP (0 ppm). (Panel A) Soman–AChE conjugate. (Lower trace) native conjugate in 0.1 M Tris, pH 8.0 (91 000 scans); (middle trace) unfolding in 0.1 M Tris-6 M Gdn.HCI, pH 8.0 (22 000 scans); (upper trace) NaOH-released OP ligand from soman-inhibited AChE (20 000 scans). (Panel B) DFP-inhibited AChE in 0.1 M Tris-6 M Gdn.HCI, pH 8.0 (22 000 scans). Lines assignment (for definitions see Tables I and II): 1, HMPA (internal standard); 2, MP-OH; 3, MP-AChF; 4, phosphoric acid (internal standard); 5, DIP–AChE. were identified unequivocally as PinMP-OH (CH3P(O)-(OPin)(OH), +22.27 ppm) and MP-OH (CH3P(O)(OH)2, +17.44 ppm) (Figure 6A, middle and upper traces). It should be pointed out that the relative intensity of the NMR signals of the two latter acids did not change over 5 days in 0.2 N NaOH at room temperature. This is consistent with the stability of the authentic individual acids in 0.2 N NaOH. Thus, the two OP acids represent different OP conjugates rather than undergoing chemical modification after being detached from the OP-protein adduct (see Figure 1). Since it appeared that the low intensity signal corresponds to the 40% reactivatable portion of soman-inhibited BChE (Figure 6A, lower trace), it was of interest to examine the effect of the initial soman to BChE ratio on the relative height of the two peaks. When the 31P NMR spectra of two conjugates obtained by 1:1 and 5:1 soman to BChE molar ratios were compared (Figure 6A, middle and upper traces, respectively), the latter showed clear enrichment of the isomers that eventually produced a higher MP-OH to PinMP-OH ratio. These findings are in agreement with the preference of BChE for the potent P(−) epimers of soman that undergo rapid dealkylation compared to the least potent P(+) stereoisomers (Keijer & Wolring, 1969).

To further demonstrate the broadening of the 31P NMR signal of native OP–ChE conjugates relative to OP-ChT adducts, the spectra of aged MP–BChE and aged MP–ChT obtained with MPDF were compared in Figure 6B. As shown, the number of scans required to produce similar peak intensity of equimolar concentration of MP–enzyme conjugates was at least 5-fold higher for MP–BChE (middle trace) than for MP–ChT (lower trace). The structure of MP–BChE was further confirmed by the conversion of the OP-bound moiety to the diisopropylphosphonyl diacid MP-OH (Figure 6B, upper trace).

Essentially similar observations were made with soman- and DFP-inhibited AChE. The native aged conjugate obtained by inhibition with soman showed a broad-low intensity 31P NMR signal after 91 000 scans (Figure 7A, lower trace). Unfolding in 0.1 M Tris–6 M Gdn-HCl revealed the presence of a methylphosphonyl moiety at 24.63 ppm downfield to TMP (Figure 7A, middle trace). A similar chemical shift was assigned to MP–ChT in 6 M Gdn-HCl (Figure 4D; Table I). Dialysis and refolding in Gdn-HCl clearlyed that the 31P NMR of soman-inhibited AChE originated from a covalently bound OP moiety (not shown). The OP ligand released by NaOH was identified as the methylphosphonic diacid, MP-OH (Figure 7A, upper trace).

The 31P NMR chemical shift of the nonaged DFP-inhibited FBS–AChE in 0.1 M Tris–6 M Gdn-HCl (Figure 7B and Table II) was similar to that assigned to the nonaged DIP–ChT (Table II). These findings suggest that the OP moiety of the former adduct consists of a diisopropylphosphoryl residue.

DISCUSSION

Structure of the OP Moiety of Aged and Nonaged ChEs. The dramatic change in the NMR spectra of the phosphorylated ChEs upon unfolding in 6 M Gdn-HCl not only demonstrated the covalent attachment of the OP residue to the BChE and AChE but also enabled the elucidation of the structure of the OP moiety of aged and nonaged OP–ChEs, by comparing the chemical shifts of the unfolded OP adducts with those of OP–ChT conjugates with known structures. The aged OP–ChE conjugates were shown here to contain a P–O– bond, whereas the OP of the homologous nonaged form is
Glu 199 (next to the catalytic amino acid Ser200), which is next to the catalytic serine (Cygler et al., 1993), do not age 3.4 Å apart from the charged carbon of the assumed carbonium readily (Sterri & Fonnum, 1987). It is suggested that, in (Benschop & Keijer, 1966), it has been recently argued that sequences, the catalytic triad Ser-His-Glu and a Glu residue the basis of the mechanism of the dealkylation reaction of proteins to ChEs that contain, among other conserved deep and narrow cavity. Finally, it is of interest to point out that OP conjugates of active site of ChEs in solution is also located inside a relatively broad cavity. It can not offer reasonable interaction with the carbonium structure of the latter enzyme which predicted a similar ion, as might be the case in ChEs. Using the same rationale the enzyme (Sussman, 1991). Sequence homology between Ser195) is projected >9 Å away from the same carbon. Thus, constituted from an uncharged OP triester moiety. This assignment is further supported by the identification of the protein-bound OP ligands released by NaOH (Figure 1).

The denatured form of the aged conjugated proteins could be completely hydrolyzed within 1 h at pH 12. This finding suggests that, in contrast to native aged OP-ChEs or aged OP-Cht, the unfolded protein is the preferred leaving group in a nucleophilic displacement at the P-O− center. Simple electrostatic repulsion of an approaching nucleophile does not play a major role in the unusual resistance to reactivation of native aged OP adducts of serine hydrolases.

Broadening of the 31P NMR Signal of OP-ChE Conjugates.

The 31P NMR signal in a high magnetic field is mainly controlled by the chemical shift anisotropy relaxation mechanism and, to a lesser extent, by the dipole–dipole interactions (Brauer & Sykes, 1984). For native OP-ChEs it is envisaged that the narrow and crowded active site gorg will increase the chemical shift anisotropy of a covalently bound 31P atom, compared to the homologous OP moiety of Ch conjugates that reside close to the surface of the enzyme (Sigler et al., 1968; Harel et al., 1991). Unfolding was expected to decrease significantly the differences between the two OP–enzyme conjugates in terms of (a) chemical shift anisotropy, (b) rotational correlation time, and (c) dipolar interactions. Indeed, the 31P NMR spectrum of both OP-ChE or OP-Cht in 6 M Gdn-HCl showed narrow signals with similar intensity.

It has been recently shown that the active site of TcAChE is structured in a deep, narrow gorge that penetrates 20 Å into the enzyme (Sussman, 1991). Sequence homology between TcAChE and HuBChE enabled the modeling of the 3D structure of the latter enzyme which predicted a similar location for the catalytic region of HuBChE (Harel, 1992) and presumably for that of EqBChE and other AChEs. The apparent broadening of the 31P NMR signal suggests that the active site of ChEs in solution is also located inside a relatively deep and narrow cavity.

Possible Mechanism of Dealkylation of OP-ChEs. On the basis of the mechanism of the dealkylation reaction (Benschop & Keijer, 1966), it has been recently argued that Glu199 (next to the catalytic amino acid Ser200), which is 3.4 Å apart from the charged carbon of the assumed carbonium moiety (Figure 8), may facilitate its rate of dealkylation by means of electrostatic forces operating between the carboxylate side chain and the positively charged carbon (Qian and Kovach, personal communication).

The data on the remarkable stability to dealkylation of the OP moiety of soman-inhibited ChE suggest that comparative analysis of the 3D structure of OP conjugates of Ch and AChE that contain the same phosphoryl moiety might help us to understand the role of a carboxyl side chain in the dealkylation process. Since the 3D geometry of (C5H9O)3P−O−Cht has been shown by X-ray crystallography (Harel et al., 1991), we chose to model the diethylphosphoryl residue into the known coordinates of TcAChE (Sussman et al., 1991) and to compare interatomic distances between key amino acid side chains and the scissible P–O−alkyl bond. It was assumed that the structural variations in the alky residue will not cause significant changes in the relative positioning of the oxygen atom of the P–O−alkyl link.

As shown in Figure 8, the distance between the oxygen atom of a proximal ethoxy group and the N2 atom of the catalytic His and the oxygen atom of a proximal P–O−ethyl group.

FIGURE 8: Active site residues and phosphoryl moiety of diethylphosphoryl conjugates of Cht and TcAChE. (Panel A) Extracted from the X-ray structure of (C5H9O)3P−O−Cht (Harel et al., 1991). (Panel B) Computer modeling of the diethylphosphoryl moiety into the active site of TcAChE. Dashed lines show interatomic distances (Å) between labeled atoms. ---H--- depicts hydrogen bond between the protonated N2 atom of the catalytic His and the oxygen atom of a proximal P–O−ethyl group.
addition to the structure of the P–O–alkyl moiety, its rate of
detachment strongly depends on the availability of both the
imidazole of the catalytic His and the carboxyl side chain
to interact in concert with the atoms of the P–O–alkyl bond.

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ABSTRACT: $^{31}$P NMR spectroscopy of butyrylcholinesterase (BChE), acetylcholinesterase (AChE), and chymotrypsin (Cht) inhibited by pinacolyl methylphosphonofluoridate (soman), methylphosphonodifluoridate (MPDF), and diisopropyl phosphorofluoridate (DFP) allowed direct observation of the OP-linked moiety of aged (nonreactivatable) and nonaged organophosphorus (OP)-ChE conjugates. The $^{31}$P NMR chemical shifts of OP-ChE conjugates clearly demonstrated insertion of a P-O- bond into the active site of aged OP-ChE adducts. The OP moiety of nonaged OP-ChEs was shown to be uncharged. The OP-bound pinacolyl moiety of soman-inhibited and aged AChE was detached completely, whereas only partial dealkylation of the pinacolyl group was observed for soman-inhibited BChEs. This suggests that the latter enzyme reacted with the less active stereoisomer(s) of soman. In the case of soman-inhibited Cht, no dealkylation could be experimentally detected for any of the four stereoisomers of OP-Cht adducts. Results are consistent with the contention that the phenomenon of enzyme-catalyzed dealkylation of OP adducts of serine hydrolases strongly depends on the orientation of both the catalytic His and the carboxyl side chain of either Glu or Asp positioned next to the catalytic Ser. The denatured protein of aged OP-ChE or OP-Cht is a convenient leaving group in nucleophilic displacements of tetrahedral OP compounds despite the presence of a P-O- bond. This indicates that the unusual resistance to reactivation of the aged enzyme cannot be ascribed to simple electrostatic repulsion of an approaching nucleophile. The broadening of the $^{31}$P NMR signal of native OP-ChEs relative to that of OP-Cht is in agreement with the crystal structure of AChE, showing that the active site region of ChEs in solution resides in a deep, narrow gorge.

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