^{31}P NMR and Mass Spectrometry of Atropinesterase and Some Serine Proteases Phosphorylated with a Transition-State Analogue

A. C. M. van der Drift,*,‡ H. C. Beck,§ W. H. Dekker,§ A. G. Hulst,§ and E. R. J. Wils§
Medical Biological Laboratory TNO and Prins Maurits Laboratory TNO, 2280 AA Rijswijk, The Netherlands
Received March 22, 1985

ABSTRACT: The serine residue in the active center of atropinesterase (AtrE), α-chymotrypsin (Chymo), and subtilisin A (Sub) and in α-chymotrypsinogen (Chymogen) was labeled with a diisopropylphosphoryl (DP) group. The labeled proteins were studied in buffered aqueous solution under various native and denaturing conditions with ^{31}P NMR before and after being subjected to "ageing", a process leading to conversion of the DP group into a monoisopropylphosphoryl (MP) group. Besides, the model compounds Gly-Ser(DP), Gly-Glu-Ser(DP)-Gly-OEt, and diisopropyl hydrogen phosphate were investigated under similar conditions and in other solvents with different hydrogen-bonding capacity. Mass spectrometry was used to analyze products resulting from ageing in the presence of $H_2{}^{18}O$. The ^{31}P chemical shift of the DP proteins increases according to a simple titration curve upon lowering the pH from 9.0 to 5.0. This is ascribed to protonation of a particular histidine residue in the active center that interacts with a nearby isopropoxy group by hydrogen bonding with the ester oxygen. In DP-AtrE, hydrogen bonding at the phosphoryl oxygen dominates the interaction between substituent and protein; in the other DP proteins, nonbonding interactions become more dominant in the order Chymogen < Chymo < Sub. DP-AtrE, DP-Chymo, and DP-Sub age according to first-order kinetics. The pH dependence of the reaction rate constant k_a indicates that ageing is catalyzed by the protonated histidine, which is responsible for the increase in chemical shift. The direct interaction between the phosphoryl group and the histidine is lost upon ageing whereas there is an increase in the nonbonding interaction of the remaining isopropyl group with the protein in the order Chymo < Sub < AtrE. The maximum value of k_a when the histidine is fully protonated ($k_a{}^m$) increases in the same order. Ageing of the DP enzymes occurs exclusively by C–O fission, yielding 2-propanol and propene. Since the amount of 2-propanol decreased and that of propene increased in the order Chymo to Sub to AtrE, the increase in $k_a{}^m$ has been interpreted as a shift in character of ageing from mainly S_N2 for Chymo to considerably S_N1 for AtrE and Sub. This has been attributed to preferential stabilization of the S_N1 transition state by an interplay of hydrogen-bonding and nonbonding interactions between the phosphoryl group and the protein. The results indicate that the active center region in AtrE resembles that of serine proteases with regard to the so-called charge-relay system, the oxyanion hole, and the nonpolar binding crevice, suggesting that esterolysis by AtrE proceeds according to a mechanism similar to that of proteolysis by Chymo or Sub.

Serine proteases and serine esterases are characterized by an extremely nucleophilic serine residue at the active site. A generally accepted criterion for identification of these enzymes is the inhibition by diisopropyl phosphorofluoridate (DFP)[1] (Hartley, 1960), which results from covalent binding of a diisopropylphosphoryl (DP) group to the oxygen atom in the side chain of this serine residue (Cohen et al., 1955; Oosterbaan et al., 1955). Such a phosphorus-containing substituent opens the possibility of using ^{31}P NMR spectrometry to study these serine hydrolases (Gorenstein & Findlay, 1976; Reeck et al., 1977; Markley, 1979).

Since the magnetic properties of a ^{31}P nucleus depend on its electronic configuration as determined by the substituents and their bonding and nonbonding interactions with the immediate environment (Letcher & Van Wazer, 1967; Ionin, 1968; Mavel, 1973), the DP group may be a suitable label to compare the structure of the active site region in different serine hydrolases. Investigations on DP-serine proteases [cf. Markley (1979) and Steitz & Shulman (1982)] have already shown that the chemical shift of the ^{31}P nucleus is a sensitive criterion for the detection of variations in the interaction between the DP group and its environment, but the data are still insufficient to allow a detailed correlation between chemical shift and the corresponding interactions.

Further, the catalytic activity of serine hydrolases toward hydrolysis of peptide or ester bonds is based on their ability to act as a mold for binding and stabilizing the so-called tetrahedral intermediate (Kraut, 1977). In this transition state of the enzyme–substrate complex, the carbonyl carbon of the scissile bond in the substrate has a tetrahedral configuration and is covalently linked to the O^γ of the active serine, while the carbonyl oxygen resides in the "oxyanion hole" on the enzyme surface, stabilized by hydrogen bonding with the protein. Many inhibitors of serine hydrolases also form tetrahedral adducts, which, presumably, closely resemble this transient intermediate. The DP group is considered to be such a transition-state analogue (Stroud et al., 1974; Kraut, 1977), and thus, the magnetic properties of the ^{31}P nucleus may reflect interactions that stabilize the transition state of the enzyme–substrate complex.

Finally, phosphorylated serine hydrolases in solution may be converted into a nonreactivatable form by a process called ageing [cf. Usdin (1970)]. As was shown for α-chymotrypsin and butyrylcholinesterase, ageing of DP enzymes involves the

‡ Medical Biological Laboratory TNO.
§ Prins Maurits Laboratory TNO.

[1] Abbreviations: AtrE, atropinesterase; Chymo, α-chymotrypsin; Chymogen, α-chymotrypsinogen; Sub, subtilisin A; DFP, diisopropyl phosphorofluoridate; DP, $(i\text{-PrO})_2P(=O)-$ (called diisopropylphosphoryl in this paper); MP, $(i\text{-PrO})OP(=O)-$ (called monoisopropylphosphoryl in this paper); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-6894$01.50/0

conversion of the tertiary phosphate ester into a secondary ester, i.e., the DP group loses one isopropyl group and becomes a monoisopropylphosphoryl (MP) group (Berends, 1964). It has been suggested that elements of the enzyme structure responsible for the catalytic activity play an essential role in ageing (Berends, 1964). Because of its negative charge and its geometry, the MP group may be regarded as an even better transition-state analogue than the DP group (Kossiakoff & Spencer, 1981). Alterations in the interactions with the protein resulting from such a change in transition-state analogue will affect the magnetic properties of the ^{31}P nucleus, and therefore, investigation of this conversion by ^{31}P NMR may give additional information about the transition state and the structural elements involved in ageing. Besides, investigation of the ageing mechanism, e.g., by mass spectrometric characterization of the formed products, may supplement this structural information.

Previously, the active site region of atropinesterase from *Pseudomonas putida* strain L of the biotype A (strain PMBL-1),[2] a pure serine esterase, was compared with that of the serine proteases α-chymotrypsin and subtilisin A with respect to inhibition kinetics and by fluorescence and ESR spectrometry (Van der Drift et al., 1981, 1985; Van der Drift, 1983). These investigations gave strong indications that some of the structural elements forming the basis of the catalytic activity of the proteases, i.e., a nonpolar primary substrate binding site and a particular histidine as part of a so-called charge-relay system, are also present in atropinesterase. The present study concerns a comparison of the DP-labeled active sites of the same enzymes and of α-chymotrypsinogen, the inactive precursor of α-chymotrypsin, by means of ^{31}P NMR and mass spectrometry with special attention to ageing. It supplements the above-mentioned investigations. A more detailed account has been given elsewhere (Van der Drift, 1983).

Materials and Methods

Proteins and Model Compounds. Salt-free α-chymotrypsin (Chymo; EC 3.4.21.1) from bovine pancreas (3 times crystallized and lyophilized; lots 58C-8135, 40F-8051, and 49C-8015) and salt-free α-chymotrypsinogen A (Chymogen) from bovine pancreas (6 times crystallized; type II; lot 29C-8010) were obtained from Sigma Chemical Co.; dialyzed and lyophilized subtilisin A (Sub; EC 3.4.21.14) was from NOVO Industri A/S (batches 70-4, 73-1, A 8003-75, A 9001-75, and A 9005-75). Atropinesterase (AtrE) was obtained from *Pseudomonas putida* PMBL-1 according to the procedure of Rörsch et al. (1971) as modified by R. A. Oosterbaan et al. (personal communication). Characterization of the enzymes and determination of enzyme activities were performed as described elsewhere (Van der Drift, 1983).

The phosphorylated model peptides Gly-Ser(DP) and Gly-Glu-Ser(DP)-Gly-OEt were kindly provided by Dr. H. Kienhuis (Prins Maurits Laboratory TNO); diisopropyl hydrogen phosphate and diisopropyl phosphorofluoridate (DFP) were obtained from C. de Borst (Prins Maurits Laboratory TNO).

Chemicals. All chemicals were reagent-grade. Organic solvents (Merck) were distilled shortly before use. Deuterium oxide (D_2O; 99.8% isotopically pure; Uvasol, Merck), $H_2{}^{18}O$ (91% isotopically pure, Ventron), citric acid (Merck), sodium citrate (Merck), Tris (J. T. Baker Chemicals B.V.), urea (Union Chimique Belge, s.a.), sodium dodecyl sulfate (SDS; specially purified for biochemical work, British Drug House), guanidine hydrochloride (Gdn-HCl; Fluka, AG), and 1,4-dithiothreitol (Merck) were used without further purification. Buffers for calibration of pH meters were obtained from Radiometer A/S and Beckman.

Labeling with a DP Group. Chymo, Chymogen, or Sub (30–90 mg) was dissolved in 1 mL of 0.1 M Tris-HCl, pH 7.6 (protein concentration 1–3 mM). AtrE solutions of similar concentration were prepared at 4 °C from stock solutions of 0.01–0.10 mM active enzyme by concentration with Amicon magnetically stirred cells (Models 202 and 52) equipped with a PM-10 filter, followed by a further concentration with Minicon macrosolute concentrators (type A-25, Amicon).

To achieve complete inhibition (>99.9%), 20 μL of 0.5 M DFP in dry, peroxide-free 2-propanol (isopropyl alcohol) was added (final DFP concentration 10 mM), and the mixture was incubated at room temperature for about 6 h or, in the case of AtrE and Chymogen, for 20 h. Nonspecific labeling was not observed. Sometimes, e.g., at high concentrations of AtrE, a slight precipitate was formed. After inhibition, this was removed by centrifugation.

The remaining DFP and its hydrolysis product were removed by dialysis at 4 °C against 140 mL of buffer for at least 36 h, under continuous stirring and frequent renewal. Finally, the sample was dialyzed against 12 mL of buffer containing about 20% D_2O as internal standard for field-frequency locking. Buffers employed were 0.1 M Tris-HCl, pH $\geq$7.6, and 0.1 M citric acid–sodium citrate, pH <7.6. Concentrations of phosphorylated proteins were obtained by determination of the amount of phosphorus according to a modified method of Gerlach & Deuticke (1963; Kienhuis & Baar, 1964). To improve the signal-to-noise ratio, dialyzed protein solutions were sometimes concentrated at 4 °C by means of Minicon macrosolute concentrators (type A-25, Amicon). Usually, concentrations ranged from 1.5 to 3.0 mM.

Ageing and Denaturation. Unless stated otherwise, 0.5 mL of DP enzyme was allowed to age at 36 °C in a dialysis tube to remove released low molecular weight materials. To stop the reaction, the sample was rapidly cooled to 4 °C and brought to the conditions required for NMR measurements (see above).

For mass spectrometric analysis of reaction products, ageing of 0.5-mL samples (mostly in a dialysis tube) was run to completion in sealed glass capillaries under similar conditions but in the presence of about 50% $H_2{}^{18}O$ (introduced into the tube by dialysis).

Denaturations were carried out with 0.2 M SDS, 8 M urea, or 6 M Gdn-HCl, containing 10 mM 1,4-dithiothreitol. In the first case, 1.0 M SDS in a buffer containing 20% D_2O was added to the protein solution (4 °C); the mixture was slowly (4–8 h) brought to room temperature and kept for at least 24 h before NMR measurements were done. In the other two cases, the solutions were dialyzed at 4–8 °C for 24 h and subsequently at room temperature for at least 48 h against 140 mL of buffer containing the denaturant, followed by dialysis for at least 24 h against 12 mL of the same solution containing D_2O. By following these denaturation procedures, no additional ageing was observed during denaturation.

NMR Spectrometry. ^{31}P NMR spectra were recorded at a frequency of 40.5 MHz in the Fourier-transform mode on a Varian XL-100-12 NMR spectrometer system; generally, 5-mm cylindrical sample tubes (Wilmad 507 PP) containing 0.5-mL samples were used. Because of the limited amount of AtrE available, measurements with this enzyme were usually performed with cylindrical microtubes (Wilmad, 508 CP) with

[2] This notation refers to the culture collection of the Medical Biological Laboratory TNO.

0.2-mL samples. Proton noise decoupling was provided at high power by 90-Hz square wave modulated irradiation. A spectral width of 6 kHz, a 90° pulse (pulse width 35 μs), an acquisition time of 1 s, and a pulse delay of 2 s were applied. Data accumulation in the time domain and Fourier transformation of the free induction decay were carried out by a Varian 620/L-100 computer interfaced to the spectrometer. In order to enhance sensitivity, the time domain data were exponentially weighted by using the optimal signal-to-noise filter [cf. Ernst (1966)] with a time constant of 0.1 s, on the basis of the narrowest line in the spectra of model compounds and denatured enzymes. Optimization of field homogeneity was performed with trimethyl phosphate, yielding line widths smaller than 0.4 Hz.

Dependent on the enzyme concentration, 20000–30000 transients were accumulated for a spectrum. In some cases 50000–80000 transients were accumulated for the more dilute AtrE solutions. For the low molecular weight model compounds (concentrations 1–10 mM), 100–500 transients were made. To calculate the chemical shift, 10–20 transients of a 85% phosphoric acid standard solution in a thin capillary, centered in a sample tube with water, were added. Shifts to higher frequencies (lower field) with respect to this external standard were taken as positive.

Results obtained with either external fluorine or internal deuterium field-frequency lock did not differ. In the latter case, samples contained about 20% D_2O, unless urea or Gdn-HCl was present. Then, it amounted to at least 50%. If required for accurate comparison between various solvents, observed chemical shifts were corrected in the usual way for differences in magnetic susceptibility between solution and reference (Crutchfield et al., 1967). Spectra of denatured enzymes and model compounds were measured at 25.0 °C and the other spectra at 4.0 °C to avoid ageing (see below). Reproducibility was within 0.1 ppm for the proteins and within 0.05 ppm for the model compounds.

Mass Spectrometry. After completion of ageing, the contents of the capillary were analyzed for reaction products with a VG 7070 F mass spectrometer combined with a Varian Model 1400 gas chromatograph using a glass column (100 × 0.2 cm) filled with Porapak Q. For characterization of gaseous products, 100 μL of the vapor was injected and analyzed at a column temperature of 110 °C; for characterization of dissolved products, 1–5 μL of liquid, freed from protein by dialysis, was injected and analyzed at a column temperature of 80–130 °C, increasing at 4 °C/min. Data acquisition and spectral analysis were performed with a VG 2050 data system.

pH Measurements. Measurements of pH were performed with various Radiometer pH meters (pH Meter 22, type PHM 22q, pH Meter 26, and PHM 64 Research pH meter). Calibration was performed shortly before use with standard buffers. Prior to the NMR experiment, the pH of the protein solution was determined in the dialyzate and immediately after completion of the experiment in the solution itself. NMR results were accepted if the difference between the two measurements was within 0.05 pH unit.

Results

Native and Denatured DP Enzymes. Around pH 7.6 the ^{31}P NMR spectra of the DFP-inhibited enzymes showed a single line with a width at half-height ($W_{1/2}$) between 10 and 20 Hz and with substantially different chemical shifts (Figure 1). The chemical shift did not depend on protein concentration; $W_{1/2}$ showed a small increase with concentration. Lowering the pH from 9 to 5 resulted in an increase in the chemical shift according to a simple titration curve of a group

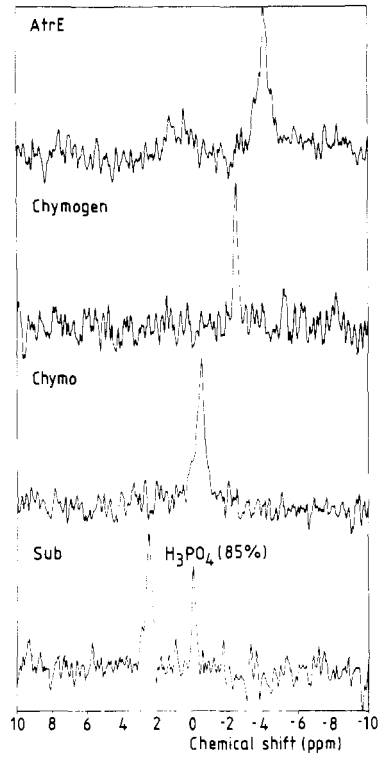

FIGURE 1: ^{31}P NMR spectra of DP-serine enzymes in buffered aqueous solution at 4 °C. AtrE = atropinesterase (pH 7.76), Chymogen = α-chymotrypsinogen (pH 7.52), Chymo = α-chymotrypsin (pH 7.60), and Sub = subtilisin A (pH 7.65). H_3PO_4 reference peak is shown in the spectrum of Sub.

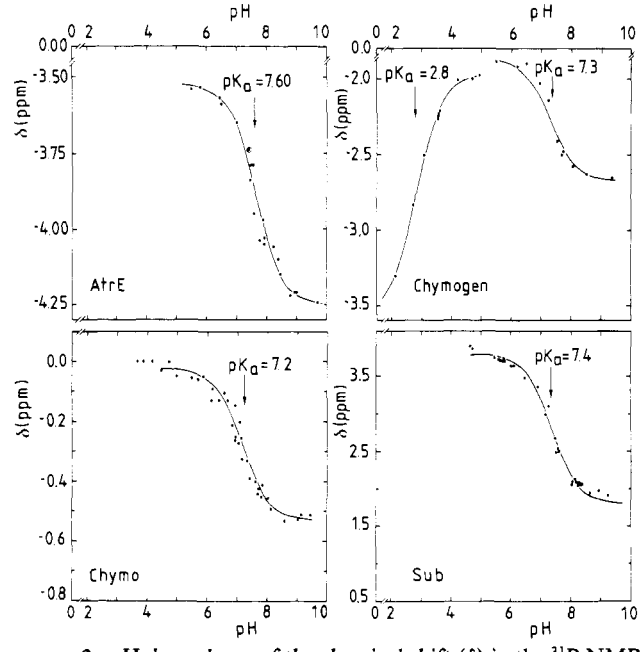

FIGURE 2: pH dependence of the chemical shift (δ) in the ^{31}P NMR spectra of DP-serine enzymes. Solid lines are theoretical curves calculated according to the Henderson–Hasselbach equation [cf. Edsall & Wyman (1958)]. For abbreviations, see legend to Figure 1.

with pK_a between 7.2 and 7.6 (Figure 2), whereas $W_{1/2}$ was not significantly affected. The limiting values of the chemical shift characterizing these curves at low and high pH are given in Table I. The chemical shift of phosphorylated Chymogen showed a second titration curve at low pH (Figure 2).

The variation in chemical shift with pH was reversible. At the lowest pH values, sometimes a slight precipitation occurred, but this did not influence the chemical shifts. Spectra of native

Table I: ^{31}P Chemical Shifts (δ) of DP Enzymes in Different Aqueous Systems[a]

system	δ (ppm) chymotrypsin	subtilisin	atropinesterase	chymotrypsinogen
buffer				
low pH[b]	−0.0[2]	3.8[0]	−3.5[2]	−1.8[7]
high pH[b]	−0.5[5]	1.8[0]	−4.2[5]	−2.6[7]
buffer with denaturant				
pH 3.5				
0.2 M SDS	−3.1	−2.9	−2.8	−3.0
8.0 M urea	−3.6	−3.7	−3.6	−3.6
8.0 M urea + 0.2 M SDS	−3.3	−3.1	−3.0	−3.3
pH 7.5				
0.2 M SDS	−3.1	−2.8[c]	−2.8	−3.1
8.0 M urea	−3.3	−3.2	−3.2	−3.2
8.0 M urea + 0.2 M SDS	−3.2	−2.8	−2.7	−3.1
6.0 M Gdn-HCl[d]	−4.0[e]	−4.1	−4.1	−4.0

[a] At room temperature, unless indicated otherwise. Uncorrected values since differences between the corrections for magnetic susceptibility were negligible. Standard error of the mean < 0.1 ppm. Upon denaturation the line widths of DP enzymes (10–20 Hz) were reduced to values about equal to that of the model compounds under the same conditions (Table II). [b] Limiting values at 4 °C obtained from Figure 2 by extrapolation; estimated standard errors < 0.05 ppm. [c] At pH >5.5 there is always a second line upfield due to nondenatured DP-Sub–SDS complex. The titration curve of this line is identical with that of DP-Sub (Figure 2) but shifted to higher pH over about 0.5 pH unit ($pK_a \sim 7.9$). [d] With 10 mM 1,4-dithiothreitol. [e] A value of −3.2 ppm is observed at lower concentrations (~4 M) and exposures shorter than 36 h [cf. Bock (1976)].

Table II: ^{31}P Chemical Shifts (δ) and Mean Line Widths at Half-Height ($\bar{W}_{1/2}$) of Model Compounds in Different Aqueous Systems at Room Temperature

system	δ (ppm)[a] I	II	III
buffer			
pH 3.2	−3.0	−3.2	−0.8
pH 9.3	−2.9	−3.1	−0.8
buffer with denaturant			
pH 3.2			
0.2 M SDS	−3.2	−2.6	−0.8
8.0 M urea	−3.6	−3.6	−1.4
8.0 M urea + 0.2 M SDS	−3.5	−3.0	−1.4
6 M Gdn-HCl[b]	−3.2	−3.2	−1.0
pH 9.3			
0.2 M SDS	−2.9	−2.7; −3.1[c]	−0.8
8.0 M urea	−2.9	−3.1	−0.9
8.0 M urea + 0.2 M SDS	−2.9	−2.9	−0.9
6 M Gdn-HCl[b]	−3.9	−4.3	−1.8

	$\bar{W}_{1/2}$ (Hz)[d] I	II	III
all systems			
pH 3.2	5	5	4
pH 9.3	5	6	4

[a] Model compounds: I = Gly-Ser(DP); II = Gly-Glu-Ser(DP)-Gly-OEt; III = diisopropyl hydrogen phosphate. Uncorrected values of δ (see legend to Table I). Concentrations were 1–10 mM. Standard error in δ < 0.1 ppm. [b] With 10 mM 1,4-dithiothreitol. [c] Chemical shift of a small second peak, which disappears at higher SDS concentrations and is, therefore, ascribed to free compound. [d] Mean value of the different systems with and without denaturant at the pH indicated; standard error < 1 Hz.

DFP-treated Sub at pH below 4.5 could not be obtained because the enzyme was rapidly denatured as appeared from a substantial increase in the viscosity of the solution (gelation) and the concomitant loss of the capacity to age (see below). Because of denaturation, no measurements were performed with DFP-inhibited AtrE at pH <5.

Differences in chemical shift may be due to differences in substitution at the phosphorus nucleus (attachment to different functional groups in the protein, loss of isopropyl groups) or to different interactions of the phosphoryl group with its environment. Since phosphate monoesters [pK_a = 6–7; cf. Sober (1970)] show an upfield shift with decreasing pH (Bock, 1976), the curves in Figure 2 do not reflect a primary phosphate. Table I shows that after denaturation the differences between the enzymes and the pH dependence of the chemical shift have disappeared, which means that they result from interactions of the label with the environment and not from differences in substitution.

To establish whether the signal of the denatured enzymes originated from a tertiary or a secondary phosphate ester, the two model peptides Gly-Ser(DP) and Gly-Glu-Ser(DP)-Gly-OEt, both phosphorylated at the hydroxyl group of the serine side chain, as well as diisopropyl hydrogen phosphate, have been investigated under comparable conditions. The results for pH 3.2 and 9.3 are compiled in Table II. Neither the chemical shift nor $W_{1/2}$ depended on concentration. The results at pH 7.5 were similar to those at pH 9.3, except that for Gdn-HCl the upfield shift was 0.1–0.2 ppm smaller. The results at pH 3.5 were similar to those at pH 3.2. After denaturation, the chemical shifts of the phosphorylated proteins approximate those of the model DP compounds. Consequently, the signal of the DFP-treated proteins is due to a tertiary phosphate ester, i.e., to a DP–O–seryl structure.

Native and Denatured MP Enzymes. Upon prolonged exposure to 36 °C at pH values within the titration range, another line appeared in the spectra of DP-Chymo, DP-Sub, and DP-AtrE with a position downfield relative to the first one and different for the three enzymes (Figure 3). Its intensity gradually increased at the expense of the original line, which finally disappeared, and in contrast to the original line, its position did not show any significant pH dependence (Table III). This phenomenon did not occur with denatured DP enzymes nor with DP-Chymogen.

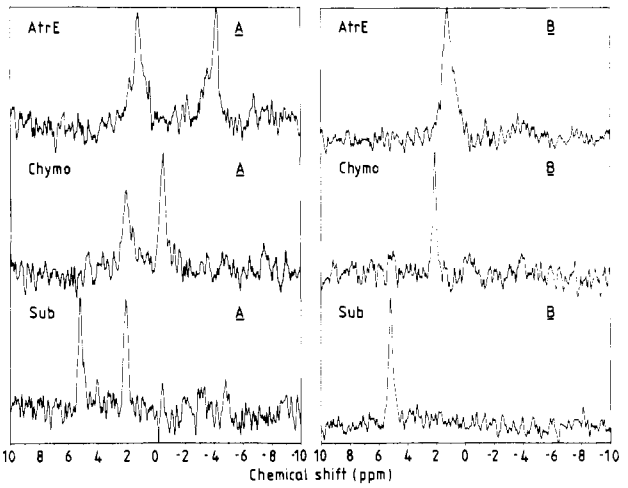

FIGURE 3: ^{31}P NMR spectra of partially (A) and completely aged (B) DP-serine enzymes in buffered aqueous solution at 4 °C. AtrE = atropinesterase (pH 7.95), Chymo = α-chymotrypsin (pH 7.80), and Sub = subtilisin A (pH 8.20).

Denaturation caused an upfield shift that yielded the same line for the three enzymes (Table III), but about 2.7 ppm downfield with respect to that of the denatured, freshly DFP-inhibited enzymes (Table I). This difference in chemical shift under the same denaturing conditions indicates that the second line must be ascribed to a ^{31}P nucleus with a different substitution and not to a conformational isomer as suggested

Table III: ^{31}P Chemical Shifts (δ) of Aged DP Enzymes in Different Aqueous Systems and Mean Line Widths at Half-Height ($\bar{W}_{1/2}$) in Buffer[a]

system	δ (ppm) chymotrypsin[b]	subtilisin[c]	atropinesterase
buffer (pH 3.5–9.5)[d]	2.1	5.2	1.3
buffer with denaturant			
pH 3.5			
0.2 M SDS	−0.5	−0.4	−0.4
8.0 M urea	−0.8	−0.8	−0.8
8.0 M urea + 0.2 M SDS	−0.6	−0.6	−0.7
pH 7.5			
0.2 M SDS	−0.4	−0.4 (5.3)	−0.4
8.0 M urea	−0.4	−0.4 (5.0)	−0.4
8.0 M urea + 0.2 M SDS	−0.4	−0.3 (5.1)	−0.4
6.0 M Gdn-HCl[e]	−1.2[f]	−1.2 (4.9)	−1.2

	$\bar{W}_{1/2}$ (Hz)[g] chymotrypsin	subtilisin	atropinesterase
buffer (pH 3.5–9.5)[h]	13	11	15

[a] At room temperature, unless stated otherwise. Uncorrected values of δ (see legend to Table I). [b] Only after denaturation of DP-chymotrypsinogen subjected to ageing conditions some aged zymogen may be observed. The values of δ and $\bar{W}_{1/2}$ for aged zymogen are not significantly different from those given for the enzyme. [c] Within parentheses are values for nondenatured aged subtilisin at the same conditions. [d] Mean value of δ for aged enzyme at 4 °C in the pH range indicated. [e] With 10 mM 1,4-dithiothreitol. [f] A value of −0.6 ppm is observed at lower concentrations (~4 M) and exposures shorter than 36 h. [g] The values of $W_{1/2}$ of the denatured aged enzymes are not significantly different from those of the DP enzymes under denaturing conditions. [h] Mean value of $W_{1/2}$ for aged enzyme at 4 °C in the pH range indicated.

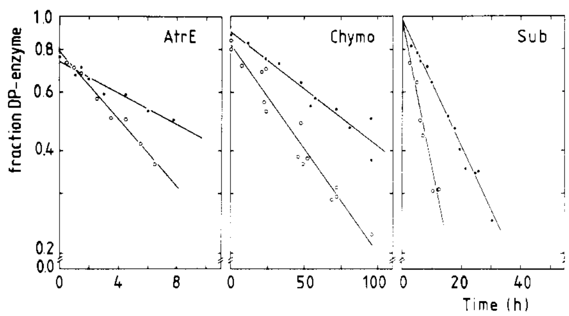

FIGURE 4: Semilogarithmic plot of the decrease in the fraction of DP enzyme with time, at 36 °C and different pH. The fractions of DP enzyme were obtained from the NMR spectra. AtrE = atropinesterase at pH 7.6 (●) and pH 6.7 (○), Chymo = α-chymotrypsin at pH 7.6 (●) and pH 3.5 (○), and Sub = subtilisin A at pH 7.6 (●) and pH 5.5 (○). The straight lines were calculated by the method of least squares.

by Gorenstein & Findlay (1976).[3] Comparison of Tables III and Table II shows that the second line should be attributed to a secondary phosphate group, viz., to an MP–O–seryl structure. The phenomenon noted above, therefore, results from the conversion of a DP group into an MP group, i.e., from ageing.

Model Compounds in Nonaqueous Solvents. The data above indicate that under nondenaturing conditions differences in chemical shift between the DP enzymes as well as between the MP enzymes result from different environments. To analyze the environmental contribution, the influence of various types of organic solvents on the chemical shift of the model compounds was investigated (Table IV). Phenol was added to the solvents as it forms strong hydrogen bonds with the phosphoryl oxygen [cf. Joesten & Schaad (1974)]. A limited number of data has been obtained for the tetrapeptide since it is only soluble in a few solvents.

Kinetics of Ageing. From NMR spectra of DP enzymes subjected to ageing for some time, the fraction of remaining DP enzyme was determined. In some cases (low protein concentrations), the aged samples were denatured before the NMR experiments to improve the signal-to-noise ratio. Figure 4 shows the decrease in the fraction of DP enzyme with time,

Table IV: Corrected ^{31}P Chemical Shifts (δ) and Mean Line Widths at Half-Height ($\bar{W}_{1/2}$) of Model Compounds in Solvents of Different Polarity and Hydrogen-Bonding Capacity at Room Temperature

solvent	ϵ_0[a]	type[b]	δ (ppm)[c] I	II	III
ethyl acetate	6.0 (25 °C)	b			−1.1
+1 M phenol		a, b			−2.4
+5 M phenol		a, b	−4.8	−4.5	−3.6
acetic acid	6.2	a, b	−4.6		−1.9
aniline	6.9	a	−3.7		−2.6
pyridine	12.3 (25 °C)	b	−3.5		−1.7
ethanol	25.1	a	−4.3		−3.6
methanol	33.6	a	−4.2		−2.9
dimethylformamide	38.7	b	−3.6	−3.1	−2.7
+1 M phenol		a, b	−3.8		−3.1
+5 M phenol		a, b	−4.5	−4.1	−3.4
dimethyl sulfoxide	46.7 (25 °C)	b	−3.7		−3.2
+5 M phenol		a, b	−4.4		−3.8
formic acid	58.5 (16 °C)	a, b	−5.2		−3.2
water					
pH 9.3	80.4	a, b	−3.5	−3.7	−1.4
pH 3.2	80.4	a, b	−3.6	−3.8	−1.4
pH 0.5	80.4	a, b	−4.0	−3.9	−2.7
formamide	109	a, b	−3.8	−4.0	−1.9
+1 M phenol		a, b	−4.0		−2.2
+5 M phenol		a, b	−4.4	−4.3	−2.6

	$\bar{W}_{1/2}$ (Hz)[d] I	II	III
all solvents	7	6	5

[a] ϵ_0 = static dielectric constant at 20 °C (unless stated otherwise) obtained from Riddick & Bunger (1970), Steffen (1970), and West (1974). [b] Solvents have been classified according to acids (a) and bases (b) in hydrogen bonding. [c] Model compounds: I = Gly-Ser(DP), II = Gly-Glu-Ser(DP)-Gly-OEt, and III = diisopropyl hydrogen phosphate. Concentrations were 1–10 mM. Experimental values of δ have been corrected in the usual way for the difference in magnetic susceptibility between the reference and the sample (Crutchfield et al., 1967). Inaccuracy in δ < 0.1 ppm. [d] Average of $W_{1/2}$ over all solvents, except for compound I where formic acid and acetic acid have been excluded, since for these media $W_{1/2}$ = 40 ± 2 Hz. In general, compound I shows a tendency of increasing $W_{1/2}$ with strength of hydrogen bonding. Estimated error in $\bar{W}_{1/2}$ is about 1 Hz.

Table V: Rate Constants of Ageing (k_a) for Different pHs at 36.0 °C

enzyme	pH	k_a (×10^{-6} s^{-1}) NMR data[a]	lit. data[b]
chymotrypsin	8.7	0.8 ± 0.1	
	7.6	2.1 ± 0.2	
	7.5		1.7; 1.9[c]
	3.5	3.9 ± 0.3	4.4
subtilisin	8.7	2.0 ± 0.1	
	7.6	11.9 ± 0.4	
	5.5	27.2 ± 1.9	
atropinesterase	7.6	14.7 ± 1.5	
	6.7	31.6 ± 1.7	

[a] With standard error. [b] Berends (1964). [c] At 37 °C.

[3] Since the occurrence of conformational isomers has been questioned (Bock, 1976; Cohn & Rao, 1979; Porubcan et al., 1979), it should be mentioned that with DP-Chymo and DP-Sub, kept for some time at a pH around 5 at 4 °C, a second line was observed close to that of the aged enzyme. Upon denaturation this second line coincided completely with the DP line for Chymo or, in the case of Sub, sometimes partly with the aged and partly with the nonaged enzyme. This points to the occurrence of slowly converting conformational isomers (Gorenstein & Findlay, 1976).

Table VI: Results of Mass Spectrometric Analysis of Products Formed upon Ageing of DP Enzymes in Aqueous Solution at 36.0 °C

	chymotrypsin	subtilisin	atropinesterase
2-propanol (%)[a]	97 ± 13	42 ± 6	18 ± 14
propene (%)[a]	1.2 ± 0.2	3.8 ± 0.6	11.3 ± 5.5
$^{18}O/^{16}O$ ratio[b]	0.99 ± 0.03	1.01 ± 0.01	0.98 ± 0.05

[a] The amount measured after complete ageing at 36.0 °C (±SD, n = 4–6) expressed as percentage of the amount expected on the basis of the concentration of phosphorylated enzyme. Ageing was completed at pH 5.7 for chymotrypsin and subtilisin and at pH 6.7 for atropinesterase in either 0.1 M citric acid–sodium citrate buffer or 0.05 M sodium sulfate. Since the results in these media did not differ significantly, the averaged values of both media are given. In some experiments also a small amount of acetone was found, presumably due to contamination. [b] The ratio (±SD, n = 5–6) of $H_2{}^{18}O/H_2{}^{16}O$ and [^{18}O]-2-propanol-/[^{16}O]-2-propanol.

at two pH values. Obviously, the conversion of DP enzyme into MP enzyme occurs according to first-order kinetics. First-order rate constants (k_a) calculated from such linear plots by the method of least squares are compiled in Table V. Results for Chymo agreed very well with those obtained by chemical analysis (Berends, 1964).

Mass Spectrometric Characterization of Products. In view of results obtained with low molecular weight phosphorus compounds [cf. Hudson (1965) and Kirby & Warren (1967)], ageing of the DP enzymes may be envisaged to occur via C–O or P–O fission (Aldridge, 1975) by a nucleophilic substitution reaction in which either a unimolecular (S_N1) or a bimolecular (S_N2) character may dominate (Ingold, 1969; Badea, 1977). To determine the scissile bond involved in the loss of the isopropyl group and the mechanism of this release, the ageing reaction was run to completion at pH ≤6.7 in the presence of $H_2{}^{18}O$ in either a citric acid–sodium citrate buffer (0.1 M) or 0.05 M sodium sulfate and the products formed were analyzed by mass spectrometry. The results (Table VI) show that invariably 2-propanol with an $^{18}O/^{16}O$ ratio equal to that of the solvent and propene were formed; no other products were found. The amount of propene increased and that of 2-propanol decreased in the order Chymo to Sub to AtrE. In the same order, an increase is seen in the difference between the total amount of products actually found and that expected upon complete ageing. Since at low pH the presence of the dialysis tube led to 10–20% exchange of ^{18}O between $H_2{}^{18}O$ and 2-propanol, ageing experiments in dialysis tubes were performed at pH >5.5, where exchange was negligible.

Discussion

DP and MP enzymes belong to the class of phosphate esters of the type RO(R'O)P(O)OR'' where R is a proton, an alkyl group, or an aromatic group. Due to appreciable differences in bonding to phosphorus, depending on the substituents and their inter- and intramolecular interactions (Ionin, 1968; Mavel, 1973), the ^{31}P chemical shifts of these compounds cover a range of roughly 30 ppm (Mark et al., 1967; Bock, 1976). However, the complex interplay between steric and electronic factors that determines the principal values and intramolecular orientation of the nuclear shielding tensor (Blackburn et al., 1971; Gorenstein, 1975; Kohler & Klein, 1977) is still poorly understood. An interpretation of the chemical shifts of the phosphorylated enzymes can, therefore, only be qualitative and empirically based on results obtained with appropriate model systems.

Chemical Shifts of Model Systems. Various sets of experimental data indicate that an increase in (smallest) bond angle between alkoxy substituents in di- and trialkyl phosphates is accompanied with an upfield shift (Blackburn et al., 1964, 1971; Mark et al., 1967) and a somewhat stronger hydrogen bonding at the phosphoryl oxygen [cf. Joesten & Schaad (1974)]. The apparently increased negative charge on this oxygen points to a weakened phosphoryl bond due to an increased electron release from other substituents toward phosphorus (Bell et al., 1954; Halpern et al., 1955; Wagner, 1963; Hudson, 1965). Conversely, if there is a one-to-one correspondence between chemical shift and O–P–O bond angle [cf. Gorenstein (1975) and Gorenstein & Kar (1975)], changes in the electronic charge distribution of alkyl phosphates, which cause an upfield shift, may be related to a larger bond angle. The valence shell electron pair repulsion (VSEPR) theory provides a generally applicable conceptual framework for the investigation of such a relationship in a qualitative way (Gillespie, 1972).

Hydrogen bonding (and maybe protonation in organic acids) at the phosphoryl oxygen of the model peptides causes a small but significant upfield shift (Table IV), similar to that for protonation of triisopropyl and analogous trialkyl phosphates [cf. Olah & McFarland (1971)]. The upfield shifts at low and high pH caused by urea and Gdn-HCl, respectively (Table II), are, therefore, probably due to hydrogen bonding at the phosphoryl oxygen. According to the VSEPR theory, the resulting decreased charge density of the phosphoryl bond and the slight increase in charge density of the P–O bonds (Bell et al., 1954; Halpern et al., 1955; Wagner, 1963; Olah & McFarland, 1971) yield a somewhat larger bond angle between the alkoxy groups. Similarly, the upfield shift due to protonation of the diisopropyl phosphate anion in aqueous solution (Table IV) will be accompanied by an increase in this bond angle because of a smaller orbital overlap between the phosphorus and the unsubstituted oxygen involved, as indicated by the increased bond length [cf. Cruickshank (1961)]. Since the effect of urea and Gdn-HCl on the chemical shift is about the same for both types of esters (Table II), conversion of a tertiary phosphate ester into a secondary one apparently does not enhance overall hydrogen bonding at the unsubstituted oxygens.

The data for diisopropyl hydrogen phosphate in organic solvents (Table IV) show that hydrogen bonding with the phosphoryl compound as proton donor also leads to an upfield shift. In this case, electron density will be displaced from the proton toward the oxygen and the phosphorus (Kollman, 1977). Stronger proton acceptors or replacement of the hydrogen by an alkyl group, e.g., a serine side chain (Table IV) or an isopropyl group (Olah & McFarland, 1971), enhances this effect. The resulting smaller fractional positive charge on phosphorus will decrease the π-bond character of the phosphoryl bond (Kirby & Warren, 1967; Letcher & Van Wazer, 1967) and thus its electron density, which according to VSEPR leads to an increase in the O–P–O bond angle.

The above analysis in terms of VSEPR suggests that variations in the electronic charge distributions of di- and trialkyl phosphate esters that lead to an upfield shift can be related to an increase in O–P–O bond angle, i.e., a decrease in crowding of the alkoxy groups. The electronic charge density of the phosphoryl bond seems to be a main factor underlying the apparent one-to-one relation between chemical shift and O–P–O bond angle. Accordingly, the downfield shift observed for the tetrapeptide but not for the smaller model compounds upon addition of SDS (Table II) indicates that perturbation of the charge distribution around phosphorus by nonbonding interactions at one of the substituents may lead to a smaller O–P–O bond angle.

Chemical Shifts of Phosphorylated Enzymes. The simple titration behavior of the chemical shift in the pH range 9–5 (Figure 2) and its abolishment by denaturation indicate that protonation of one particular group, which in the native tertiary structure resides in the vicinity of the DP group, leads to increased crowding of the substituents at the phosphorus. A pK_a between 7.2 and 7.6 (Figure 2) is too high for a carboxylic acid and points to a histidine of a special kind, since it lies above the range 5.5–7.0 characteristic for most histidines (Edsall & Wyman, 1959; Meadows, 1972). Such a special histidine is known to be present in Chymo and its zymogen (His-57) and in Sub (His-64), where it is part of the so-called charge-relay system, an essential element of their catalytic site (Kraut, 1977). In unperturbed native serine proteases, this histidine has $pK_a < 7.0$ [cf. Cunningham & Brown (1956), Glazer (1967), and Steitz & Shulman (1982)], but a DP group at the active serine leads to $pK_a \geq 7.2$ [cf. Markley (1979)]. The indication of a similar histidine at the active site of AtrE with pK_a 7.6 in the DP enzyme but $pK_a < 7.0$ in the native enzyme [cf. Berends et al. (1967) and Stevens (1969)] is corroborated by fluorescence studies (Van der Drift, 1983).

Since protonation of the active site histidine does not show an appreciable structural change of the active center [Mavridis et al., 1974; cf. Huber & Bode (1977) and Matthews et al. (1977)], increased crowding in Chymo, Chymogen, and Sub points to a common direct interaction between the protonated histidine and the DP group. According to X-ray data, such an interaction may involve hydrogen-bond formation between the protonated $N^{\epsilon 2}$ of the histidine and the oxygen of the P–O–serine link or one of the isopropoxy groups [Matthews et al., 1977; Chambers & Stroud, 1979; R. M. Stroud quoted in Porubcan et al. (1979)]. The increase in the pK_a of the histidine upon phosphorylation of the active serine is consistent with hydrogen bonding [cf. Jaffé et al. (1954)]. The above analysis of the model systems shows that the increased fractional positive charge on phosphorus resulting from electron withdrawal by hydrogen bonding at an ester-type oxygen will lead to increased crowding. Since the occurrence of ageing via intramolecular general-acid catalysis indicates that only the oxygen of the scissile P–O–C link is involved in interaction with a proton donor (see below), it is concluded that the increased crowding depends, at least partly, on hydrogen bonding between the protonated histidine and the oxygen of an isopropoxy group.

The close similarity of AtrE and the proteases as to pH dependence of the chemical shift (Figure 2) and ageing (see below) indicates that in DP-AtrE there is also a direct interaction between the protonated active site histidine and a nearby isopropoxy group that involves hydrogen bonding. At high pH, when this hydrogen bonding is absent, native DP-AtrE (Table I) shows an upfield shift with respect to the DP model peptides in buffer (Table II), whereas the other DP proteins (Table I) show a downfield shift. Comparison with the DP model compounds (Tables II and IV) and the denatured DP enzymes (Table I) shows that hydrogen bonding at the phosphoryl oxygen can sufficiently account for the upfield shift of DP-AtrE, with nonbonding interactions between the DP group and the protein being of minor influence. The chemical shift at low pH (Table I) indicates that this interaction is not disrupted by the interaction of the isopropoxy group with the protonated histidine. Such a specific hydrogen bonding between the phosphoryl oxygen and the protein points to an oxyanion hole in AtrE, comparable to that in the proteases [cf. Kraut (1977)]. The downfield shift at high pH of the other three proteins can be accounted for by nonbonding interactions between protein and DP group, which increase crowding of the phosphorus substituents in the order Chymogen < Chymo < Sub. Apparently, in proteases the relative contribution of the existent hydrogen bonding between phosphoryl oxygen and oxyanion hole (Kraut, 1977) has a smaller influence than that in AtrE.

In DP- and MP-serine proteases investigated so far, one isopropyl group interacts with the entrance of the primary substrate binding pocket and is more or less excluded from the solvent and out of reach of the histidine of the charge-relay system (Drenth et al., 1971; Stroud et al., 1974; Chambers & Stroud, 1979; Kossiakoff & Spencer, 1981). In phosphorylated Chymogen and trypsinogen, such a nonbonding interaction will be much weaker because a fully developed and rigid binding pocket is lacking (Freer et al., 1970; Wright, 1973; Fehlhammer et al., 1977; Kossiakoff et al., 1977). Besides, in the native enzymes a stronger hydrogen bonding of the phosphoryl oxygen in the oxyanion hole is expected than in the zymogens because of a more favorably oriented –NH of Gly-193 (Freer et al., 1970; Henderson, 1970; Wright, 1973; Stroud et al., 1974; Kossiakoff et al., 1977), which will lead to an upfield shift. The downfield shift of about 2 ppm of the titration curve of Chymo relative to that of Chymogen (Figure 2) will therefore reflect nonbonding interactions of an isopropyl group with the primary substrate binding pocket [cf. Reeck et al. (1977)]. For DP-trypsinogen and DP-trypsin a similar phenomenon has been observed (Porubcan et al., 1979). Since these interactions also dominate the chemical shifts of the aged proteases (see below), increased crowding by nonbonding interactions in DP- and MP-serine enzymes may result from a decrease in the O–P–O bond angle between the active serine and the nonhydrolyzable isopropyl group due to binding of the latter nonpolar substituent in a crevice-like structure, e.g., the primary substrate binding pocket. According to Figure 2, enhanced binding of the DP group due to this interaction with an adjacent crevice makes a larger contribution to the decrease in O–P–O bond angle of DP enzymes in the order AtrE < Chymo < Sub than that resulting from the hydrogen bonding with the active site histidine.

The insensitiveness of the chemical shift to pH, the negligible effect of hydrogen bond disrupting compounds on the chemical shift of nondenatured MP-Sub (Table III), and the lack of enhanced hydrogen bonding at unsubstituted oxygens by urea and Gdn-HCl in the presence of O^- suggest that in MP enzymes neither appreciable hydrogen bonding [cf. Robillard & Shulman (1974)] nor significant Coulombic interaction [cf. Kossiakoff & Spencer (1981)] will occur between the active site histidine and the phosphoryl group. Accordingly, results obtained with Sub and Chymogen (unpublished experiments) indicate that displacement of the imidazole ring of the histidine residue into the solvent by specific binding of Ag^+ [cf. Chambers et al. (1974)] has no effect on the chemical shift of MP enzymes whereas in DP proteins it causes an upfield shift. The downfield shifts of the MP enzymes (Table III) with respect to diisopropyl hydrogen phosphate (Table II), which largely disappear upon denaturation, thus point to increased crowding by mainly nonbonding interactions in the order AtrE < Chymo < Sub. The obvious increase in nonbonding interaction upon ageing of DP-AtrE and the difference in the chemical shift between MP-AtrE and MP-Chymo of 0.8 ppm and between MP-AtrE and MP-Sub of 3.9 ppm (Table III) suggest a rather nonpolar pocket-like structure nearby the active serine that binds the remaining isopropyl group similarly to Chymo rather than to Sub. Other investigations by means of fluorescence and ESR spectrometry as

well as kinetic methods (Van der Drift, 1983; Van der Drift et al., 1985) also pointed to the presence of a nonpolar crevice adjacent to the active serine in AtrE.

For Chymo, Sub, and AtrE the differences between the chemical shifts of the DP species at high pH (Table I) and the corresponding aged species (Table III) are 0.0, 0.7, and 2.9 ppm larger than 2.7 ppm, respectively. A maximum value of about 2.7 ppm is expected (on the basis of Tables I and III and the data of the model compounds in Tables II and IV) when one isopropyl group is lost without affecting interactions with the environment that contribute to the chemical shift. These extra downfield shifts suggest conformational changes upon ageing that lead to enhanced binding of the phosphoryl substituent in the order Chymo < Sub < AtrE due to an increase in the nonbonding interaction between the remaining isopropyl group and the protein, while interaction with the histidine disappears. This antagonism of interactions may play an important role in ageing (see below).

Ageing of DP Enzymes. In neutral and acidic solutions, hydrolysis of noncyclic trialkyl phosphates to secondary phosphate esters proceeds according to various mechanisms (Cox & Ramsay, 1964; Kirby & Warren, 1967; Ingold, 1969). However, under the conditions employed in this investigation, the (unimolecular) rate constants will be significantly smaller than those in Table V (Barnard et al., 1961; Cox & Ramsay, 1964; Keyer, 1971). This and the absence of any noticeable conversion in denatured enzymes indicate that ageing is a catalytic process that depends on the native protein structure (Berends, 1964; Keyer, 1971). The increase in k_a with decreasing pH (Table V) points, therefore, to an intramolecular general acid catalyzed reaction requiring a protonated group in the protein close to the phosphoryl substituent analogous to the ageing of phosphonylated cholinesterases (Keyer, 1971; Keyer et al., 1974).

In the absence of particular metal ions or special groups that may act as Lewis acids, intramolecular hydrogen bonding with a neighboring proton donor, and perhaps a subsequent proton transfer, will underlie the internal general acid catalyzed hydrolysis of phosphate esters [cf. Hudson (1965) Kirby & Warren (1967), and Singleton (1973)]. Ageing of the DP enzymes will, therefore, require hydrogen bonding between the oxygen of the isopropoxy group to be released and an adjacent proton donor, which according to the NMR data might be a protonated histidine. Results obtained with DP-Sub (unpublished experiments) indicate that hampering interaction with the histidine by specific binding of Ag^+ indeed leads to a drastic reduction in k_a.

If protonation of the active site histidine is essential for ageing, a plot of the chemical shift (at 4 °C, Figure 2) vs. k_a (at 36 °C, Table V) for pH values in the titration range will be linear (Figure 5), as the pK_a of such a histidine does not differ appreciably for these temperatures [cf. Robillard & Shulman (1974), Markley (1978), Porubcan et al. (1979), and Steitz & Shulman (1982)]. From these plots, k_a^m, the maximum value of k_a expected for a fully protonated histidine in the otherwise unperturbed active site, can be estimated by using the values of the chemical shift at pH 5.0 (Figure 2). The k_a^m values (Table VII) increase in the order Chymo < Sub < AtrE and suggest that the active site region in AtrE involved in ageing resembles that in Sub more closely than that in Chymo. For Chymo, k_a at pH 3.5 (Table V) is smaller than k_a^m, in agreement with other data that indicate a decrease in k_a below pH 4–5 [Berends, 1964; cf. Keyer (1971)]. This may result from distortion of the active site structure caused by protonation of particular groups at low pH. The value of

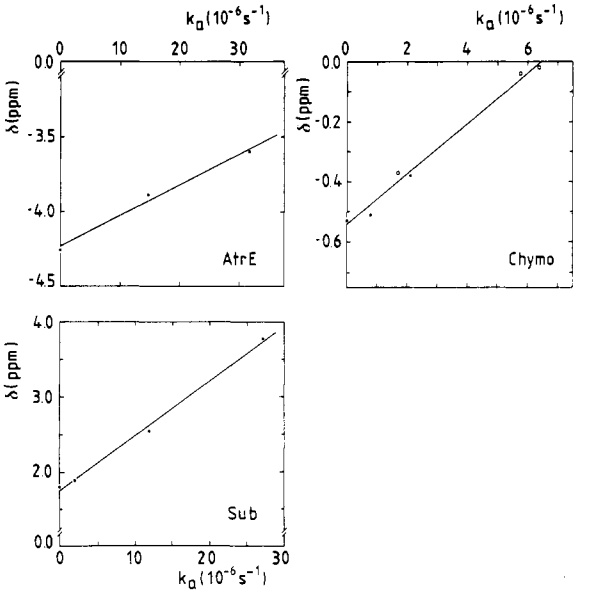

FIGURE 5: Correlation between the chemical shift (δ) and the rate constant of ageing (k_a) in their dependence on pH. Values of δ (Figure 2) were plotted vs. the corresponding k_a values for different pH. (●) k_a determined by ^{31}P NMR (Table V); (○) k_a obtained from Berends (1964). The straight lines were calculated by the method of least squares, including the limiting value of δ at high pH where $k_a = 0$. For abbreviations, see legend to Figure 3. For Chymo, only data for pH ≥4.5 have been used (see Discussion).

Table VII: Some Characteristic Quantities Related to the Ageing of DP Enzymes in Aqueous Solution at 36.0 °C

quantity[a]	chymotrypsin	subtilisin	atropinesterase
k_a^m ($\times 10^{-6}$ s^{-1})	6.2	28.0	34.5
pK_a[b]	7.2	7.4	7.6
$\delta_{MP} - \delta_{DP}$ (ppm)	2.7	3.4	5.6

[a] k_a^m = maximum value of the rate constant of ageing obtained from the plots in Figure 5 for a fully protonated histidine at pH 5.0. $\delta_{MP} - \delta_{DP}$ = difference in chemical shift between MP enzyme (Table III) and the corresponding DP species at high pH (Table I). [b] Based on Figures 2 and 5.

k_a^m for AtrE approaches that of DP-acetylcholinesterase [cf. Berends (1964)].

Ageing of the DP enzymes proceeds exclusively by C–O fission since the $^{18}O/^{16}O$ ratio of the 2-propanol formed is equal to that of the solution (Table VI). Fission of the alkyl–oxygen bond according to an S_N1 type reaction will lead to formation of an isopropyl carbenium ion, which may react with water to yield 2-propanol, may be captured by other nucleophilic reactants present in the protein or solution, or may lose a proton to give propene (Badea, 1977). Dealkylation according to an S_N2 type reaction yields only 2-propanol. Thus, the formation of propene (Table VI) unambiguously proves that ageing in all three enzymes has at least some S_N1 character.

Since the solubility of propene in aqueous solution is low and there are no indications for high-affinity binding sites for 2-propanol in serine enzymes (Glazer, 1966), the difference between the total amount of low molecular weight products expected and that actually found for Sub and AtrE (Table VI) points to capture of carbenium ions by nucleophilic centers other than water (Badea, 1977). Since the yields in citrate buffer and sodium sulfate, which can be regarded as chemically almost inert with respect to carbenium ions [cf. Badea (1977)], were nearly the same, the loss in isopropyl groups very likely results from reaction of carbenium ions with the protein. Consequently, the S_N1 contribution to ageing of Sub and AtrE will be larger than is indicated by the recovery of propene. For

DFP-inhibited neurotoxic esterase, a similar loss of released groups due to binding at the protein upon ageing has been observed (Clothier & Johnson, 1979).

The effect of temperature on the rate of ageing of DP-Chymo (Berends, 1964) gives an enthalpy and entropy of activation of 18.5 kcal/mol and −25.4 cal mol^{-1} K^{-1}, respectively. The enthalpy is characteristic for S_N2 displacement by water at a saturated carbon atom (Hudson & Keay, 1956; Barnard et al., 1961); the entropy is too large for a unimolecular reaction (Schaleger & Long, 1963) and corresponds with that for immobilization of a water molecule (Barnard et al., 1961; Kirby & Warren, 1967). Together with the results in Table VI, this suggests that in DP-Chymo the 2-propanol is formed by a mainly S_N2-type reaction. Therefore, the data (Table VI) point to a shift in the ageing from a mainly S_N2-type reaction in DP-Chymo to one with a considerable S_N1 contribution in DP-Sub and DP-AtrE. This implies a larger ionizing power of the immediate environment of the scissile C–O bond in Sub and AtrE than in Chymo, which apparently leads to a substantial increase in k_a^m (Table VII).

The stronger hydrogen bonding with the active site histidine as reflected by the larger pK_a (Table VII) will increase the polarity of the scissile C–O bond and accordingly promote the S_N1 character of ageing in DP-Sub and DP-AtrE to a larger extent than in DP-Chymo. Since hydrolysis of phosphate esters by acid-catalyzed unimolecular C–O fission proceeds through the ionic conjugate acid of the ester (Ingold, 1969), a larger S_N1 contribution via hydrogen bonding indicates that disruption of this interaction by proton transfer from the histidine to the ester oxygen at some stage of the reaction is more likely in Sub and AtrE than in Chymo. However, in all three MP enzymes the interaction of the histidine with the phosphoryl group is lost, indicating the involvement of at least one other interaction, which shows an increase upon ageing but to a different extent for Chymo and the other enzymes. Probably, nonbonding interactions between the remaining isopropyl group and the protein, which show a larger increase upon ageing in Sub and AtrE than in Chymo, will play an important role in determining the S_N1 character of this process. Accordingly, it is conceivable that the larger S_N1 contribution to ageing of DFP-inhibited Sub and AtrE results from a particular interplay of hydrogen bonding and nonbonding interactions between the phosphoryl group and the protein, which leads to preferential stabilization of the anion in the transition state.

Comparison of Atropinesterase with Serine Proteases. The direct interaction with the DP group and its crucial role in ageing similar to that in DP-Chymo and DP-Sub indicate that the particular active site histidine in AtrE has a position and proton-donating properties characteristic for a histidine in a so-called charge-relay system. Together with the indications for an oxyanion hole and a nonpolar crevice-like structure, both with a similar position relative to the active serine as in Chymo and Sub, this points to a close structural resemblance of the active sites of the three enzymes in agreement with other investigations (Van der Drift, 1983). This structural similarity suggests that esterolysis by AtrE will proceed according to the same mechanism as proteolysis by Chymo and Sub. Moreover, since each of the proteases belongs to a different superfamily and does not show sequence homology with AtrE either (Hessing, 1983), it strongly supports the view that the functional structure of serine hydrolases results from a convergent molecular evolution.

ACKNOWLEDGMENTS

The technical assistance of E. Mobach and N. Kramer is gratefully acknowledged. Special thanks are due to Drs. H. P. Benschop, F. Berends, and L. P. A. de Jong and to Ir. J. F. Bleichrodt for many stimulating discussions and helpful comments during preparation of the manuscript. The critical reading of the manuscript by Profs. G. H. de Haas, J. Smidt, and C. Veeger has been greatly appreciated.

Registry No. AtrE, 59536-71-9; Chymo, 9004-07-3; Sub, 9014-01-1; Chymogen, 9035-75-0; Gly-Ser(DP), 98395-60-9; Gly-Glu-Ser(DP)-Gly-OEt, 98420-39-4; Ser, 56-45-1; HOP(O)(OPr-*i*)$_2$, 1611-31-0.

REFERENCES

Aldridge, W. N. (1975) *Croat. Chem. Acta 47*, 225.
Badea, F. (1977) *Reaction Mechanisms in Organic Chemistry*, Abacus Press, Tunbridge Wells, Kent, England.
Barnard, P. W. C., Bunton, C. A., Llewellyn, D. R., Vernon, C. A., & Welch, V. A. (1961) *J. Chem. Soc.*, 2670.
Bell, J. V., Heisler, J., Tannenbaum, H., & Goldenson, J. (1954) *J. Am. Chem. Soc. 76*, 5185.
Berends, F. (1964) Ph.D. Thesis, University of Leiden.
Berends, F., Rörsch, A., & Stevens, W. F. (1967) in *Proceedings of the Conference on Structure and Reactions of DFP Sensitive Enzymes* (Heilbronn, E., Ed.) p 45, Försvarets Forskningsanstalt, Stockholm.
Blackburn, G. M., Cohen, J. S., & Lord Todd (1964) *Tetrahedron Lett.*, 2873.
Blackburn, G. M., Cohen, J. S., & Weatherall, I. (1971) *Tetrahedron 27*, 2903.
Bock, J. L. (1976) Ph.D. Thesis, Yeshiva University, New York.
Chambers, J. L., & Stroud, R. M. (1979) *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem. 26*, 1861.
Chambers, J. L., Cristoph, G. G., Krieger, M., Kay, L., & Stroud, R. M. (1974) *Biochem. Biophys. Res. Commun. 59*, 70.
Clothier, B., & Johnson, M. K. (1979) *Biochem. J. 177*, 549.
Cohen, J. A., Oosterbaan, R. A., Warringa, M. G. P. J., & Jansz, H. S. (1955) *Discuss. Faraday Soc. 20*, 114.
Cohn, M., & Rao, B. D. N. (1979) *Bull. Magn. Reson. 1*, 38.
Cox, J. R., Jr., & Ramsay, O. B. (1964) *Chem. Rev. 64*, 317.
Cruickshank, D. W. J. (1961) *J. Chem. Soc.*, 5486.
Crutchfield, M. M., Dungan, C. H., & Van Wazer, J. R. (1967) *Top. Phosphorus Chem. 5*, 1.
Cunningham, L. W., & Brown, C. S. (1956) *J. Biol. Chem. 221*, 433.
Drenth, J., Hol, W. G. J., Jansonius, J. N., & Koekoek, R. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 107.
Edsall, J. T., & Wyman, J. (1958) *Biophysical Chemistry*, Vol. 1, Academic Press, New York.
Ernst, R. (1966) *Adv. Magn. Reson. 2*, 1.
Fehlhammer, H., Bode, W., & Huber, R. (1977) *J. Mol. Biol. 111*, 415.
Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T., & Xuong, Ng. H. (1970) *Biochemistry 9*, 1997.
Gerlach, E., & Deuticke, B. (1963) *Biochem. Z. 337*, 477.
Gillespie, R. J. (1972) *Molecular Geometry*, Van Nostrand-Reinhold, London.
Glazer, A. N. (1966) *J. Biol. Chem. 241*, 635.
Glazer, A. N. (1967) *J. Biol. Chem. 242*, 433.
Gorenstein, D. G. (1975) *J. Am. Chem. Soc. 97*, 898.
Gorenstein, D. G., & Kar, D. (1975) *Biochem. Biophys. Res. Commun. 65*, 1073.
Gorenstein, D. G., & Findlay, J. B. (1976) *Biochem. Biophys. Res. Commun. 72*, 640.
Halpern, E., Bouck, J., Finegold, H., & Goldenson, J. (1955) *J. Am. Chem. Soc. 77*, 4472.
Hartley, B. S. (1960) *Annu. Rev. Biochem. 29*, 45.

Henderson, R. (1970) *J. Mol. Biol. 54*, 341.
Hessing, J. G. M. (1983) Ph.D. Thesis, University of Leiden.
Huber, R., & Bode, W. (1977) in *NMR in Biology* (Dwek, R. A., Campbell, I. D., Richards, R. E., & Williams, R. J. P., Eds.) p 1, Academic Press, New York.
Hudson, R. F. (1965) *Structure and Mechanism in Organo-Phosphorus Chemistry*, Academic Press, New York.
Hudson, R. F., & Keay, L. (1956) *J. Chem. Soc.*, 2463.
Ingold, C. K. (1969) *Structure and Mechanism in Organic Chemistry*, 2nd ed., Cornell University Press, Ithaca, NY.
Ionin, B. (1968) *J. Gen. Chem. USSR (Engl. Transl.) 38*, 1618.
Jaffé, H. H., Freedman, L. D., & Doak, G. O. (1954) *J. Am. Chem. Soc. 76*, 1548.
Joesten, M. D., & Schaad, L. J. (1974) *Hydrogen Bonding*, Marcel Dekker, New York.
Keyer, J. H. (1971) Ph.D. Thesis, University of Leiden.
Keyer, J. H., Wolring, G. Z., & De Jong, L. P. A. (1974) *Biochim. Biophys. Acta 334*, 146.
Kienhuis, H., & Baar, J. A. J. (1964) Internal Report, Prins Maurits Laboratory TNO, Rijswijk, The Netherlands.
Kirby, A. J., & Warren, S. G. (1967) *Reaction Mechanisms in Organic Chemistry*, Vol. 5, Elsevier, Amsterdam.
Kohler, S. J., & Klein, M. P. (1977) *J. Am. Chem. Soc. 99*, 8290.
Kollman, P. A. (1977) *Mod. Theor. Chem. 4*, 109.
Kossiakoff, A. A., & Spencer, S. A. (1981) *Biochemistry 20*, 6462.
Kossiakoff, A. A., Chambers, J. L., Kay, L. M., & Stroud, R. M. (1977) *Biochemistry 16*, 654.
Kraut, J. (1977) *Annu. Rev. Biochem. 46*, 331.
Letcher, J. H., & Van Wazer, J. R. (1967) *Top. Phosphorus Chem. 5*, 75.
Mark, V., Dungan, C. H., Crutchfield, M. M., & Van Wazer, J. R. (1967) *Top. Phosphorus Chem. 5*, 227.
Markley, J. L. (1978) *Biochemistry 17*, 4648.
Markley, J. L. (1979) in *Biological Applications of Magnetic Resonance* (Shulman, R. G., Ed.) p 397, Academic Press, New York.
Matthews, D. A., Alden, R. A., Birktoft, J. J., Freer, S. T., & Kraut, J. (1977) *J. Biol. Chem. 252*, 8875.
Mavel, G. (1973) *Annu. Rep. NMR Spectrosc. 5B*, 1.
Mavridis, A., Tulinkski, A., & Liebman, M. N. (1974) *Biochemistry 13*, 3661.
Meadows, D. H. (1972) *Methods Enzymol. 26*, 638.
Olah, G. A., & McFarland, C. W. (1971) *J. Org. Chem. 36*, 1374.
Oosterbaan, R. A., Kunst, P., & Cohen, J. A. (1955) *Biochim. Biophys. Acta 16*, 299.
Porubcan, M. A., Westler, W. M., Ibañez, I. B., & Markley, J. L. (1979) *Biochemistry 18*, 4108.
Reeck, G. R., Nelson, T. B., Paukstelis, J. V., & Mueller, D. D. (1977) *Biochem. Biophys. Res. Commun. 74*, 643.
Riddick, J. A., & Bunger, W. B. (1970) *Tech. Chem. (N.Y.) 2*.
Robillard, G., & Shulman, R. G. (1974) *J. Mol. Biol. 86*, 541.
Rörsch, A., Berends, F., Bartlema, H. C., & Stevens, W. F. (1971) *Proc. K. Ned. Akad. Wet., Ser. C 74*, 132.
Schaleger, L. L., & Long, F. A. (1963) *Adv. Phys. Org. Chem. 1*, 1.
Singleton, R., Jr. (1973) *J. Chem. Ed. 50*, 538.
Sober, H. A., Ed. (1970) *Handbook of Biochemistry*, 2nd ed., The Chemical Rubber Co., Cleveland, OH.
Steffen, M. (1970) *Ber. Bunsen-Ges. Phys. Chem. 74*, 505.
Steitz, T. A., & Shulman, R. G. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 419.
Stevens, W. F. (1969) Ph.D. Thesis, University of Leiden.
Stroud, R. M. (1979) quoted in Porubcan et al. (1979).
Stroud, R. M., Kay, L. M., & Dickerson, R. E. (1974) *J. Mol. Biol. 83*, 185, 209.
Usdin, E. (1970) *Int. Encycl. Pharmacol. Ther. 1*, 249.
Van der Drift, A. C. M. (1983) Ph.D. Thesis, University of Utrecht.
Van der Drift, A. C. M., Rousseeuw, B. A. C., & Van der Drift, E. (1981) *Bull. Magn. Reson. 2*, 208.
Van der Drift, A. C. M., Moes, G. W. H., Van der Drift, E., & Rousseeuw, B. A. C., (1985) *Biochemistry 24*, 5333–5342.
Wagner, E. L. (1963) *J. Am. Chem. Soc. 85*, 161.
West, R. C., Ed. (1976) *Handbook of Chemistry and Physics*, 55th ed., CRC Press, Cleveland, OH.
Wright, H. T. (1973) *J. Mol. Biol. 79*, 1,13.