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ELECTROPHORETIC AND ^{31}P NUCLEAR MAGNETIC RESONANCE EVIDENCE FOR ALTERATIONS IN CONFORMATION AND NET CHARGE ON PHOSPHORYLATION AND "AGING" OF α-CHYMOTRYPSIN

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Abstract—Native α-chymotrypsin and its phosphoenzyme and "aged" phosphoenzyme derivatives are conveniently differentiated by polyacrylamide gradient gel electrophoresis in a pH 4.5 buffer. The changes in relative mobilities most likely reflect alterations in conformation induced by phosphorylation and, for the aged product, also the formation of the negatively charged substituent. Similar electrophoretic separations are achieved for the corresponding derivatives of γ-chymotrypsin and α-chymotrypsinogen A, but not for those of trypsin. Aged α-chymotrypsins give rise to two ^{31}P resonances, also suggesting conformational isomerization of the enzyme, and the chemical shifts exhibit a small, reversible, pH dependence. A third phosphoenzyme arises on longer term incubations at pH 7.6. Under the conditions examined, ^{31}P n.m.r. spectroscopy does not allow differentiation of the aged phosphoenzymes, intermediates in their formation, or the phosphorylated but non-aged analogs.

Many esterases, including cholinesterase, chymotrypsin and trypsin, react with organophosphorus (OP) compounds to yield esteratically inactive phosphoenzymes. In some cases, loss of a second substituent from the OP moiety ensues, and the resultant "aged" esterase is characterized by an ionizable phosphorus group bound to the active site [1].

$$\underset{\text{esterase}}{EH} + \underset{\text{OP inhibitor}}{XP(O)R(OR')} \xrightarrow{\text{phosphorylation}} \underset{\text{phosphoesterase}}{EP(O)R(OR')} \xrightarrow{\text{aging}} \underset{\text{aged phosphoesterase}}{EP(O)R(O^-)}$$

Aging of a phosphoesterase is generally recognized by its conversion to a form refractory to regeneration of activity by oximes or other nucleophiles [2], or by monitoring the release of substituent groups from the initial phosphoenzyme. Aging of α-chymotrypsin inhibited by saligenin cyclic phosphorus esters is evidenced by the formation of saligenin (either free or enzyme-bound) and of what appears to be *N*-(*o*-hydroxybenzyl)-derivatized protein [3]. α-Chymotrypsin aging following inhibition by diphenyl phosphorochloridate, tris-*p*-nitrophenyl phosphate and tris-*p*-acetylphenyl phosphate is conveniently monitored by the release of 1.0 molar equivalent of the appropriate phenol [4, 5]. Following phosphorylation, the saligenin derivatives age by C—O bond cleavage in an acid-catalyzed type mechanism, while the diarylphosphoryl-enzymes undergo the alternate base-catalyzed displacement with P—O bond breakage [1].

Two other methods are also useful in examining the phosphorylation and aging of esterases. The aging results in an enzyme with a modified net charge which alters the mobility on paper [6] and disk [7] electrophoresis as shown in studies with α-chymotrypsin and its aged derivatives from reaction with saligenin cyclic phosphorus esters. ^{31}P nuclear magnetic resonance (n.m.r.) spectroscopy is applicable to investigations of phosphoproteins since the chemical shift of the phosphorus atom is sensitive to the nature of the substituent groups as well as to the conformational changes between them resulting from variations in the surrounding environment. For example, ^{31}P n.m.r. examination of diisopropylphosphoryl-α-chymotrypsin reveals two readily interconvertible conformational phosphoenzyme isomers [8] and examinations of diisopropylphosphoryl-α-chymotrypson and diisopropylphosphoryl-chymotrypsinogen A allow monitoring of the activation process of the latter [9].

The present study compares various phosphorylated and aged chymotrypsins, chymotrypsinogen, and trypsin by electrophoresis, and aged α-chymotrypsins by ^{31}P n.m.r.

MATERIALS AND METHODS

Chemicals

α-Chymotrypsin (type II), diisopropylphosphoryl-α-chymotrypsin (type V), γ-chymotrypsin (type II), α-chymotrypsinogen A (type II) and trypsin (type III) were obtained from the Sigma Chemical Co. (St. Louis, MO). Sources for the saligenin cyclic phosphorus esters were described previously [3]. Haloxon and the

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dichlorvos analogs were gifts from the Burroughs Wellcome Co., Research Triangle Park, NC, and the Shell Development Co., Modesto, CA, respectively. The other OP esters and chemicals were available either in this laboratory or from commercial sources. Electrophoretic gels were obtained from Isolab Inc., Akron, OH.

Preparation of phosphoesterases

For electrophoresis. Enzyme–inhibitor incubations at 25° were generally carried out with a protein concentration of 2 mg ml^{-1} in Na phosphate buffer (0.01 M, pH 7.6) or in distilled water. The inhibitor, dissolved in methoxytriglycol (MTG), was added to the enzyme to achieve the desired molar ratio. Esterase assays were performed with *p*-nitrophenyl acetate as the substrate. These procedures are described elsewhere in detail [3]. The OP compound:enzyme molar ratios were selected to achieve rapid inhibition of esterase activity (<2 hr) and thereby minimize autolysis. For α-chymotrypsinogen-A, phosphorylation was slower and only achieved by using a large excess of inhibitor.

For ^{31}P n.m.r. analysis. Two methods were employed to prepare the aged phosphoenzyme samples. The α-chymotrypsin used for method A was essentially homogeneous, as determined by electrophoresis, while that for method B had two distinct esteratically active bands.

Method A: α-Chymotrypsin (400 mg) was dissolved in distilled water (20 ml, resulting pH ~ 4.4) or in Na phosphate buffer (20 ml, 0.01 M, adjusted to pH 7.6) at 25°, and the OP inhibitor was added (2:1 molar ratio of inhibitor:enzyme) using MTG (750 μl) as carrier solvent. After 30 min, complete enzyme inhibition and aging were confirmed using assays as detailed previously [3]. The enzyme solution was cooled (ice bath) and ice-cold acetone (10 vol.) was added dropwise with gentle stirring. The precipitated protein was collected by centrifugation, redissolved in distilled water (20 ml), and the precipitation and collection procedure repeated. The precipitate was lyophilized, the product redissolved in distilled water (~150 mg ml^{-1}) and any insoluble material removed by centrifugation. The supernatant fraction was adjusted to the desired pH using dilute NaOH or HCl and the ^{31}P n.m.r. spectrum was recorded (see below). As a control, a sample of uninhibited enzyme was subjected to the same procedure; when assayed for activity, no alteration was evident.

Method B: α-Chymotrypsin (400 mg, unless otherwise specified) was dissolved in distilled water (2 ml) and the pH adjusted as desired. The solution was transferred to a n.m.r. tube and the spectrum taken immediately after addition of the OP inhibitor (1.2:1 molar ratio of inhibitor:enzyme) in MTG (30 μl). Changes in pH were made as desired for subsequent spectra, or the sample was subjected to the acetone precipitation and washing procedure.

For non-enzymic hydrolysis, the OP compound was placed in water at pH 7.6 (adjusted with NaOH) or in 2 N NaOH with MTG carrier as required, and the spectra were taken at various time intervals.

Electrophoresis procedures

Standard and preferred method. Electrophoresis utilized a Gradipore model 342–500 polyacrylamide slab gel apparatus with a Heathkit regulated high voltage supply. Isophore polyacrylamide gels (5–20% linear gradient, 8 cm length) were pre-run (400 V, initial current ~50 mA) for 15 min to equilibrate with the electrophoresis buffer of β-alanine (3.1 g l^{-1})–acetic acid (to pH 4.5 at 25°). Each protein solution (see above) was diluted to 1 mg ml^{-1} with 40% sucrose and aliquots (2–15 μl) were then placed in alternate sections of a 14-slot spacer. Separations were achieved with a run time of 90 min (400 V). All the electrophoresis was performed in a cold room with a buffer temperature of 4°.

To stain for both protein and esterase activity, each gel was sliced lengthwise into two pieces. One piece was treated with 12.5% trichloroacetic acid (37°, 40 min), and then the protein bands were visualized by the addition of Coomassie Brilliant Blue G (2 ml of 0.25% aqueous solution) [10]. The other piece was equilibrated with tris (5.2 g l^{-1})–HCl (to pH 6.6) buffer (2 × 100 ml, 15 min each, 37°), then placed in the same buffer (100 ml) containing Fast Blue RR salt (100 mg) and α-naphthyl acetate (4 ml of 1% acetone solution; see Ref. 11), and shaken for 60 min. The solution was decanted, and the gel washed with a 95% ethanol (20 ml)–10% acetic acid (80 ml) solution for 15 min. Gel storage in both cases was effected in 5% acetic acid.

Less suitable or unsatisfactory methods. The following variations in the electrophoretic method were found less suitable. Linear 7.5% polyacrylamide gels at pH 4.5 failed to separate native α-chymotrypsin from its phosphoenzyme and aged phosphorylated forms. With buffers of higher pH, the longer electrophoresis times necessary resulted in severe swelling and distortion of the gel and diffuse protein bands, thereby destroying the analytical value of the technique. The buffers tested were: glycine (2.9 g l^{-1})–tris (0.6 g l^{-1}), pH 8.3; β,β-dimethylglutaric acid (8 g l^{-1})–NaOH (as required), pH 7.2; and diethylbarbituric acid (5.5 g l^{-1})–tris (1.0 g l^{-1}), pH 7.0. Concave gradient gels achieved separation of the various enzymes and phosphoenzymes, but resolution was not enhanced with respect to the linear gradient system. Moreover, the higher final percentage of polacrylamide present results in a more brittle gel which is less convenient to handle.

Spectroscopy

Most of the ^{31}P phosphoenzyme n.m.r. spectra were acquired on a multinuclear spectrometer built at the Laboratory of Chemical Biodynamics (Berkeley, CA), based on a Bruker Instruments 63 Kgauss superconducting solenoid operating at 109.37 MHz for phosphorus. Experiment control and data acquisition were handled by a Nicolet Instrument NIC-1180 data processor. Spectra were taken at 22° with a 25 μsec (90°) pulse at a 5-sec repetition rate using a ±5000 Hz spectral width and quadrature detection. Adequate signals were generally obtained after 254 acquisitions for a phosphoenzyme sample concentration of 200 mg ml^{-1} in a 10 mm diameter tube, and with the instrument run in an unlocked mode. Long-term drift was estimated to be less than 1 Hz/day and, although some difficulties were encountered with field homogeneity, resolution was judged to be better than 10 Hz. For longer term acquisitions, deuterium was used as a lock signal. Decoupling experiments utilized a decoupling field of 6 W, applied during the full data acquisition period.

Table 1. Relative mobilities on electrophoresis of chymotrypsins, chymotrypsinogen, trypsin and their phosphorylated, and phosphorylated and aged derivatives

Enzyme	R_m, cm from base of gel			
		Phosphoenzyme*		
	Native enzyme	A EP(O)R(OR′)	B EP(O)R(OR′) + EP(O)R(O⁻)	C EP(O)R(O⁻)
α- or γ-				
Chymotrypsin (homogeneous)	5.70	5.65	5.65 5.30	5.30
α-Chymotrypsin (heterogeneous)	5.85 5.70	5.89 5.65	5.80 5.65 5.45 5.30	5.45 5.30
Chymotrypsinogen A	5.90	5.85		5.50
Trypsin	6.20	6.20	6.20	6.20

* See Table 2 for the nature of the R and R′ substituents.

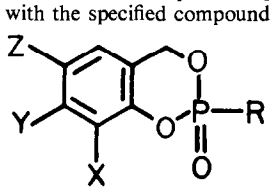
Table 2. Electrophoretic patterns obtained with α-chymotrypsin reacted with various organophosphorus esters

No.	Compound* Structure	Ratio, I/E†	Gel pattern‡
	Saligenin cyclic phosphorus esters §		
	X Y Z R		
1‖	H H H C_6H_5	1–100	C
2	H H H Cyclohexyl	100	C
3	H H H OC_6H_5	1–100	C¶
4	H H H OCH_3	100	C
5	Cl H H $NHCH_3$	100	C
6	H CH_3 H $NHCH_3$	100	C
7	H H Cl $NHCH_3$	100	C
	Triaryl phosphates and diaryl phosphorochloridate		
8	$(4\text{-}NO_2\text{-}C_6H_4O)_3P(O)$	60	B** or C
9	$[4\text{-}CH_3C(O)\text{-}C_6H_4O]_3P(O)$	60	C
10	$(C_6H_5O)_2P(O)Cl$	100	B or C
	Dialkyl aryl phosphates, dialkyl phosphorofluoridate and dialkyl 2,2-dichlorovinyl phosphates		
11	$(C_2H_5O)_2P(O)OC_6H_4\text{-}NO_2\text{-}4$	90	A
12	$(ClCH_2CH_2O)_2P(O)O\text{-coumarin-7-yl-3-Cl-4-}CH_3$	60	A
13‖	$(i\text{-}C_3H_7O)_2P(O)F$	20–100	A
14–20	$(RO)_2P(O)OCH{=}CCl_2$††	100	A

* Common names for some of the chemicals are as follows: 4, salioxon; 11, paraoxon; 12, haloxon; 13, DFP; and 14 (R = CH_3), dichlorvos.

† Molar ratio, inhibitor:enzyme.

‡ Letters refer to electrophoretic gel patterns described in Table 1 for α-chymotrypsin inhibited with the specified compound at pH 7.6.

§ 

‖ Examined also with γ-chymotrypsin, chymotrypsinogen A and trypsin.

¶ Gives additional minor bands at R_m 5.26 and 5.22 when reacted with the homogeneous preparation. In addition to saligenin, a small amount of phenol is also liberated, suggesting that this moiety competes as a leaving group.

** pH ~ 4.4 when enzyme was dissolved in distilled water.

†† R = CH_3, $n\text{-}C_3H_7$, $n\text{-}C_4H_9$, $n\text{-}C_5H_{11}$, $n\text{-}C_6H_{13}$, $n\text{-}C_7H_{15}$ and $n\text{-}C_8H_{17}$.

Some chemical shift data were also obtained on a Varian CFT-20 spectrometer, operating at 32.19 MHz for phosphorus, by courtesy of the Stauffer Chemical Co. (Richmond, CA). Chemical shifts in parts per million are denoted as positive values when downfield from 85% H_3PO_4.

RESULTS

Electrophoresis

Relative mobility (R_m) values of the protein bands under the standard polyacrylamide gradient gel electrophoretic conditions are given in Tables 1 and 2 and are discussed below by type of enzyme and phosphoenzyme derivative. Enzyme inhibition was verified by determining esterase activity of the bands. The R_m of any component is a reflection not only of its net charge but also of its effective size [12] as clearly seen for the different chymotrypsin preparations, since the variations in homogeneity are evidenced only on gradient gels. This, in turn, suggests that the pore sizes in the linear 7.5% gels are insufficient to alter the migration rates of the conformers.

Native enzymes

Some α-chymotrypsin preparations are essentially homogeneous (R_m 5.70) and others are heterogeneous, yielding two bands (R_m 5.85 and 5.70) of which the R_m 5.70 band is major and the second varies in amount (as judged by staining intensity) from trace quantities to ~80 per cent of the other component. Traces of esteratically inactive proteins are also present at both higher and lower mobilities than the native enzyme, and are considered to be autolysis products. Both bands in the heterogeneous samples are esteratically active, and the specific activities of the homogeneous and heterogeneous preparations, when analyzed in solution, are similar. γ-Chymotrypsin, chymotrypsinogen A and trypsin each gave a single band at R_m 5.70, 5.90 and 6.20 respectively.

Phosphorylated and phosphorylated and aged α-chymotrypsin

Three general types of electrophoretic patterns are obtained on inhibition of α-chymotrypsin with different classes of OP esters. DFP forms a stable diisopropylphosphoryl-enzyme [13] and, by analogy, the other dialkyl OP compounds examined should simply phosphorylate the enzyme without subsequent aging. They all generate the same electrophoretic pattern (A in table 1 and compounds 11–20 in Table 2) characterized by a slight retardation in mobility from R_m 5.70 to 5.65 or, in the case of heterogeneous α-chymotrypsin, from 5.85 and 5.70 to 5.80 and 5.65 respectively. This effect is not restricted to OP inhibitors, since a similar result is observed for α-chymotrypsin carbamoylated with $(n\text{-}C_3H_7)_2NC(O)S(O)C_2H_5$ or the corresponding sulfone.

OP esters that yield phosphoenzymes that age slowly (i.e. compound 8 at pH 4.4 [5] and compound 10 at pH 8.0 [4]) give a pattern for phosphorylated enzyme as above plus for phosphorylated and aged enzyme (R_m 5.65 and 5.30 for homogeneous preparations plus additional bands at 5.80 and 5.45 for heterogeneous preparations; B, Table 1). Phosphoenzymes that age very rapidly (e.g. those derived from saligenin cyclic phosphorus esters 1,2 and 4–7 [3] and the triaryl phosphates at pH 7.6) give only the band for aged phosphoenzyme (R_m 5.30 or 5.45 and 5.30 for homogeneous or heterogeneous preparations, respectively; C, Table 1). When partially inhibited with compound 1, the gel pattern is consistent with the percentage inhibition achieved (i.e. R_m 5.70 and 5.30 for homogeneous α-chymotrypsin). For all saligenin cyclic phorus esters 1,2 and 4–7 [3] and the triaryl phosnor the pH of the solution during incubation with the inhibitor has any effect on the gel pattern observed. Further, once complete inactivation is achieved, there is no change in the electrophoretic properties of the protein with time (up to 24 hr).

Table 3. ^{31}P n.m.r. chemical shifts of three saligenin cyclic phosphorus esters, diphenyl phosphorochloridate, their respective aged phospho-α-chymotrypsin derivatives, and two phosphorus acid derivatives

	Inhibitor resonances†		Aged phosphoenzyme resonances‡		
			Chemical shift (ppm)		
Compound*	Chemical shift (ppm)	Solvent (pH)	Major	Minor	Solvent (pH)
1	13.0	$CHCl_3$			
	16.9	H_2O (7.6)	0.3	19.6	H_2O (4.4)
	16.2	H_2O (2 N NaOH)	3.0	19.3, 13.4	H_2O (7.6)
2	27.9	$CDCl_3$	1.1	20.1	H_2O (4.4)
3	15.6	$CDCl_3$	0.7§	−3.7§	D_2O (4.4)
10	−6.8	$(CH_3)_2CO$	3.0§	−1.3§	H_2O (7.6)
	−5.2	H_2O			
$PhP(O)(OH)_2$	12.2	H_2O (7.6)			
$(PhO)_2P(O)OH$	−8.4	H_2O (7.6)			

* See Table 2 for structures of inhibitors.

† Positive chemical shifts indicate resonances downfield from 85% H_3PO_4. The chemical shifts of $PhP(O)(OH)_2$ and $(PhO)_2P(O)OH$ are measured in the presence of enzyme (~200 mg ml^{-1}). The chemical shift of compound 1 is 16.8 ppm when measured in the presence of inhibited enzyme (~200 mg ml^{-1}) at pH 7.6.

‡ Aged phosphoenzymes were prepared by method A described in Materials and Methods. The ratios of the major and minor phosphoenzyme resonances vary as noted in Results. The 13.4 ppm resonance for aged phosphoenzyme prepared with compound 1 is only evident with long incubation times.

§ Compounds 3 and 10 give the same aged phosphoenzymes so differences in the tabulated chemical shifts are probably associated with pH or solvent effects.

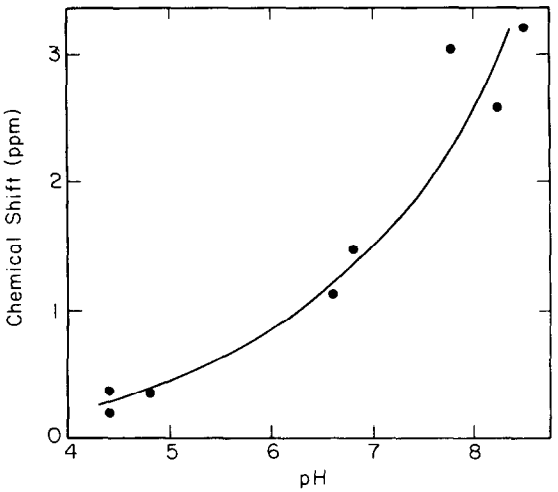


Fig. 1. Effect of pH on the chemical shift of the major phosphoenzyme resonance of phenylphosphonyl-α-chymotrypsin.

Phosphorylated and phosphorylated and aged γ-chymotrypsin, chymotrypsinogen and trypsin

γ-Chymotrypsin elicits electrophoretic behaviour identical to α-chymotrypsin, both as the native enzyme and its phosphorylated derivatives. α-Chymotrypsinogen A undergoes a similar electrophoretic alteration, but with different relative mobilities for the various forms (Table 1). Trypsin and its phosphorylated and aged derivatives [3] are not resolved by the standard method used.

^{31}P *n.m.r. spectra*

Table 3 gives the chemical shift data for three saligenin cyclic phosphorus esters (1–3) and their respective aged phosphoenzymes prepared by method A with the pH maintained as specified. Each inhibitor exhibits a sharp singlet resonance with W/2 (width at half the height) of 4 Hz (proton decoupled) in contrast to the phosphoenzymes which each show two broad singlet peaks with W/2 of 40 Hz in a ratio of ~ 20:1, and which are not affected by an applied proton decoupling field. The phosphoenzyme chemical shifts also show a reversible pH dependence, with the major resonance being the more sensitive (Table 3 and Fig. 1).

The ^{31}P chemical shifts do not vary significantly within the range of protein concentrations at which phosphoenzyme spectra were taken, i.e. from 20 mg ml^{-1} (12,500 acquisitions) to 200 mg ml^{-1}. Moreover, the chemical shift of the inhibitor in water at pH 7.6 is only altered by 0.1 ppm in the presence of phosphorylated chymotrypsin at 200 mg ml^{-1}, a result that most likely reflects the difficulty of accurate pH adjustment of the viscous protein solution.

Spectra recorded following direct addition of compound 1 to the enzyme solution as per method B reveal some interesting relationships. At pH 7.6, there are two phosphoenzyme resonances (19.3 and 3.0 ppm) of relative intensity ~1:2 with other signals for unreacted inhibitor (16.8 ppm) and its hydrolysis product (12.2 ppm, Fig. 2A); the latter two assignments were verified in a separate experiment by further additions of the inhibitor. A spectrum taken 4 hr subsequent to that

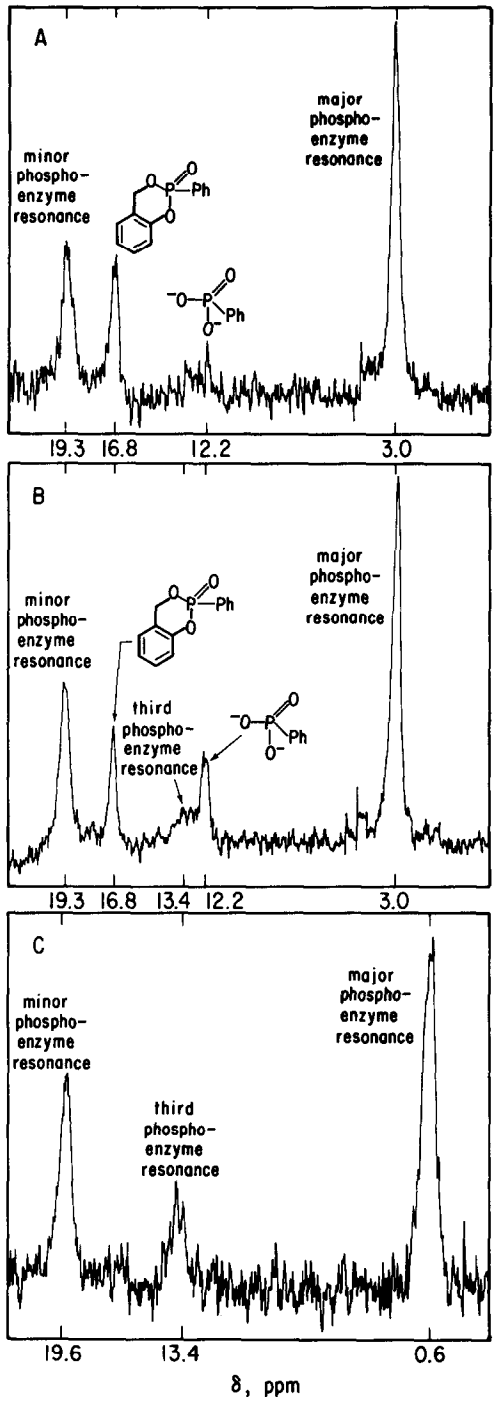


Fig. 2. ^{31}P n.m.r. spectra of phenylphosphonyl-α-chymotrypsin. Panel A: derivative prepared by method B described in Materials and Methods and measured at pH 7.6. Panel B: same as panel A at pH 7.6 but spectrum was measured 4 hr later. Panel C: same as panel B but spectrum was measured after acetone precipitation and washing, redissolving the enzyme in water, and adjustment to pH 4.4

of Fig. 2A shows an increase in the hydrolysis product at the expense of the inhibitor and a new third phosphoenzyme signal at 13.4 ppm (Fig. 2B). Only the phosphoenzyme signals are retained after acetone precipitation and washing, and pH adjustment to 4.4 gives the anticipated changes in chemical shifts (Fig. 2C).

A similar relationship is evident when the reaction is initiated at pH 4.4 except for the 13.4 ppm phosphoenzyme resonance which is absent. Alteration of the pH to 7.6 gives the expected chemical shift changes, and a further spectrum taken 3 hr later shows the appearance of the third phosphoenzyme resonance. Neither the appearance of the third phosphoenzyme resonance nor the variations in relative intensity of the other phosphoenzyme resonances is reflected in the electrophoretic patterns of the inhibited enzymes. Furthermore, the acetone precipitation and washing procedure does not cause any alteration in the electrophoretic properties of native α-chymotrypsin or its various phosphorylated forms.

Incubating chymotrypsin for 2 hr with a 15:1 molar ratio of ethoxy formic anhydride (EFA):enzyme prior to adding compound 1 (method B, pH 7.6) resulted in a reduction in total phosphorylation, with the minor phosphoenzyme resonance being more greatly affected than the major one. Addition of diphenyl phosphorochloridate (compound 10) to chymotrypsin at pH 7.6 gives a result similar to that obtained with compound 1 for the major phosphoenzyme resonance (3.0 ppm) but the minor phosphoenzyme resonance appears at a significantly different chemical shift (−1.3 ppm) (Table 3). A third resonance at −8.4 ppm was identified as the diphenylphosphoric acid formed on hydrolysis of unreacted inhibitor with the long incubation time involved. The W/2 values of ~36, 42 and 12 Hz, respectively, support these assignments with verification by monitoring the non-enzymic hydrolysis of the OP compound.

DISCUSSION

Non-aged phospho-α-chymotrypsin is not separated from native enzyme by disk electrophoresis [7] but is distinguished by gradient gel electrophoresis. Since no alteration in net charge of the enzyme is involved, the separation is only achieved by virtue of a conformational change leading to an altered effective size of the phosphoenzyme. Both disk and gradient gel electrophoresis give altered mobilities for the aged phosphoenzyme as a result of the introduced, negatively charged substituent. Additional minor bands from aging of enzyme inhibited by saligenin cyclic phenyl phosphate (compound 3) may be due either to a doubly ionized phosphorus group or to an *o*-hydroxymethylphenyl phosphoryl residue at the active site. With aged α-chymotrypsin prepared from various saligenin cyclic phosphorus esters, it is interesting to note that, although the physical separation of the released saligenin is not complete [3], neither this form of the enzyme nor the *N*-alkylation product [3] is evidenced by the electrophoretic pattern.

Gradient gel electrophoresis differentiates the native enzyme from its phosphorylated, and its phosphorylated and aged, derivatives with α- and γ-chymotrypsin and α-chymotrypsinogen, but not with trypsin. Electrophoretic behavior of γ-chymotrypsin and its phosphoenzyme derivatives identical to that of the homogeneous α-chymotrypsin and its phosphoenzymes is expected since the only difference ascribed to the native enzymes is a minor variation in their crystal structures [14].

When examined by ^{31}P n.m.r., each aged phosphoenzyme gives rise to a major resonance at ~2 ppm and a minor one of variable intensity whose chemical shift more closely reflects the chemical nature of the residual enzyme-bound phosphoryl moiety. (Chemical shifts for the saligenin cyclic phosphonates [compounds 1 and 2] are in accord with those generally found for compounds containing a P–C bond and, although phosphates resonate over a wide range, the deshielding seen for compound 3 is consistent for other cyclic phosphates [15].) The variation in the relative intensities of the major and minor phosphoenzyme reflects the differences in the homogeneity of the α-chymotrypsin, as defined by electrophoresis, used to prepare the samples by methods A and B. Moreover, in agreement with the n.m.r. result, the acetone precipitation and washing procedure caused no electrophoretic alteration of the phosphoenzyme.

Inhibition of chymotrypsin by compound 1 at either pH 4.4 or 7.6 gives the same initial spectrum, small pH-dependent shift differences excepted. At pH 7.6, after standing for 4 hr, or in the pH 4.4 sample after adjustment to pH 7.6, a third, minor phosphoenzyme appears. This phenomenon involves more than enzyme aging, since with the saligenin compounds this aging process is rapid and pH independent [3]. Thus, this third phosphoenzyme resonance probably reflects a further conformational isomer. Based on the observed pH independence and irreversibility, it is not attributable to a change in the hydrogen bonding configuration [16] within the phosphorylated active site, and the alternate possibility involving P group transfer to another amino acid residue [17] is unlikely [8].

EFA pretreatment of chymotrypsin reduces the amount of phosphorylation at serine-195 [3] and decreases the intensity of the minor relative to the major phosphoenzyme resonance. Acylation of the serine-195 residue by EFA [18] accounts for the decreased phosphorylation, and the change in relative signal intensities suggests differences in accessibility of the EFA reagent to the active site of the different conformers present.

Diphenylphosphoryl-chymotrypsin at pH 7.6 undergoes slower aging [4] than the analogs based on the saligenin OP inhibitors [3]. However, no differences are noted between concurrent spectra taken within the time limits to accumulate the data, indicating that the aging is sufficiently fast to preclude observation of shift differences, if any, between the phosphorylated and phosphorylated and aged forms. Thus, although the ^{31}P n.m.r. study supports the suggestion of conformational isomerism of the enzyme [8], it does not allow specific characterization of an aged product or further delineation of the aging mechanism.

The findings may have relevance to the reactions involved in producing the lesion leading to OP delayed neurotoxicity. The OP inhibitor specificity for chymotrypsin is similar to that for "neurotoxic esterase" [19], and aging of this esterase, as distinct from its prolonged inhibition, is implicated as fundamental for onset of the neuropathy [20]. In the present study, although there is no correlation between compounds that induce delayed neurotoxicity [Table 2, compounds 1, 3, 9, 12, 13 and 14 (higher alkyl derivatives)] [21, 22] and those that readily age with α-chymotrypsin (Table 2, compounds

1–10) it is clear that aging induces an alteration in the physical properties of α-chymotrypsin, as characterized by its electrophoretic behavior and ^{31}P n.m.r. analysis, other than just the simple change in net charge. Similar changes in the physical properties of the aged neurotoxic esterase may disrupt its normal physiological functions [20].

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