(Diisopropylphosphoryl)serine Proteinases. Proton and Phosphorus-31 Nuclear Magnetic Resonance–pH Titration Studies†

Michael A. Porubcan,‡ William M. Westler, Ignacio B. Ibañez, and John L. Markley*

ABSTRACT: Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) peaks have been resolved and assigned to the modified serine-195 (chymotrypsinogen numbering system) of seven (diisopropylphosphoryl)serine (iPr_2P-serine) proteinases. In each derivative the ^{31}P NMR chemical shift of the predominant iPr_2P-serine-195 peak is pH dependent. Titration studies yielded the following pK' values at 30 °C in 0.5 M KCl (0.2 M KCl for iPr_2P-α-lytic proteinase): iPr_2P-α-lytic proteinase, 7.9 ± 0.2; iPr_2P-bovine chymotrypsin A_δ, 7.46 ± 0.07; iPr_2P-bovine chymotrypsinogen A, 7.5 ± 0.1; iPr_2P-bovine trypsin, 7.71 ± 0.08; iPr_2P-porcine trypsin, 7.31 ± 0.05; iPr_2P-bovine trypsinogen, 7.94 ± 0.07; iPr_2P-porcine trypsinogen, 7.42 ± 0.02. These pK′ values agree with pK' values derived from proton nuclear magnetic resonance (^{1}H NMR) data for peaks assigned to the C^{ϵ_1}-H of histidine-57 of each of these diisopropylphosphoryl derivatives. The ^{1}H NMR results were reported previously [Markley, J. L., & Ibañez, I. B. (1978) *Biochemistry 17*, 4627–4640; Porubcan, M. A., Neves, D. E., Rausch, S. K., & Markley, J. L. (1978) *Biochemistry 17*, 4640–4647], except for the single histidyl C^{ϵ_1}-H peak of iPr_2P-α-lytic proteinase which yields a pK' value of 8.16 ± 0.03 and the C^{ϵ_1}-H peak assigned to histidine-57 of iPr_2P-porcine trypsin which yields a pK' value of 7.36 ± 0.02. The ^{31}P NMR chemical shift perturbations are attributed to protonation of histidine-57, which is located close to the iPr_2P group of each derivative. The pK' of histidine-57 increases in all three enzymes when serine-195 is derivatized, and the range of pK' values found for histidine-57 is much narrower for the derivatives than for the native enzymes. In contrast, the pK' of histidine-57 in each zymogen is altered only slightly by the chemical modification. The phosphorus environment is more deshielded in each enzyme than in the corresponding zymogen. An additional ^{31}P NMR transition at low pH is observed with both iPr_2P-bovine chymotrypsinogen A and iPr_2P-bovine chymotrypsin A_δ, which correlates with the pH_{mid} for the second protonation of the catalytic triad of each respective species as determined by ^{1}H NMR.

Diisopropyl phosphorofluoridate (iPr_2P-F)[1] is a classic inhibitor of serine proteinases (Jansen et al., 1949a). The compound was used to establish the stoichiometry of chymotrypsin active sites (Jansen et al., 1949b) and the functional significance and identity of the catalytic seryl residue. The inhibited enzyme product, which contains a diisopropylphosphoryl (iPr_2P) group attached to the O^γ of Ser^{195}[2] (Schaffer et al., 1953; Cohen et al., 1955; Oosterbaan et al., 1955; Brown & Hartley, 1966), can undergo two reactions: "reactivation" by removal of the iPr_2P group (Cunningham & Neurath, 1953) or "aging" by conversion of the tertiary phosphate ester to a secondary phosphate ester through the removal of one alcohol group (Berends et al., 1959; Lee & Turnbull, 1961). Reactivation occurs only very slowly in water, but is catalyzed by nucleophilic reagents such as hydroxylamine (Cunningham & Neurath, 1954). The aging reaction, which is detected by the failure of nucleophilic agents to reactivate, takes place very slowly with iPr_2P-serine proteinases. Other tertiary phosphate ester derivatives of serine proteinases (Lee & Turnbull, 1958, 1961; Bender & Wedler, 1972) and the iPr_2P derivative of acetylcholinesterase (Jandorf et al., 1955; Davies & Green, 1956) age more rapidly. Blow (1969) reported that iPr_2P-Ctr is unsuitable for analysis by X-ray crystallography even at pH 4 because of hydrolysis of the iPr_2P group.

Recently, it was found that zymogens also react with iPr_2P-F in the same way as enzymes (Morgan et al., 1972; Robinson et al., 1973), leading to loss of their low level of catalytic activity (Kay & Kassell, 1971; Robinson et al., 1973). The hypothesis that inhibitors such as iPr_2P-F are transition-state analogues (Stroud et al., 1974; Kraut, 1977) has lent added interest to these derivatives. The X-ray structure of iPr_2P-F-inhibited bovine trypsin (Stroud et al., 1974) indicated that the free phosphate oxygen is located in the "oxyanion hole" (Robertus et al., 1972) and is hydrogen bonded to the peptide NH's of Gly^{193} and Ser^{195}.

Two independent ^{31}P NMR studies of diisopropylphosphoryl derivatives of serine proteinases have been published. Reeck et al. (1977) demonstrated that the ^{31}P NMR chemical shift of iPr_2P-bovine chymotrypsinogen A is 2 ppm upfield from that of iPr_2P-bovine chymotrypsin A_α. They attributed the chemical shift differences to changes in hydrogen bonding to the trialkyl phosphate which was assumed to occupy the

† From the Biochemistry Division, Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received March 23, 1979.* A preliminary account of this research has been presented (Markley et al., 1978). Supported by National Institutes of Health Grants GM 19907 to the Purdue Research Foundation and RR 01077 to the Purdue University Biochemical Magnetic Resonance Laboratory and by National Institutes of Health Research Career Development Award HL 00061 (J.L.M.).

‡ Present address: Squibb Institute for Medical Research, Princeton, NJ 08540.

[1] Abbreviations used: ATEE, *N*-acetyl-L-tyrosine ethyl ester; BCtr, bovine chymotrypsin; BCtg, bovine chymotrypsinogen; BTr, bovine trypsin; BTg, bovine trypsinogen; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (2,2-dimethyl-2-silapentane-5-sulfonate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; α-LPase, α-lytic proteinase; NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate; ppm, parts per million; PTg, porcine trypsinogen; PTr, porcine trypsin; Et_2P, diethylphosphoryl; iPrP, monoisopropylphosphoryl; iPr_2P, diisopropylphosphoryl; iPr_2P-F, diisopropyl phosphorofluoridate [phosphorofluoridic acid bis(1-methylethyl) ester]; Tris, 2-amino-2-(hydroxyethyl)-1,3-propanediol. The notation pH* is used to designate the uncorrected pH meter reading of ^2H_2O solutions measured with electrodes standardized in ^1H_2O buffers. Notation used follows the "Recommendations for the Presentation of NMR Data in Chemical Journals" (IUPAC, 1976). Atomic positions within residues are referenced according to crystallographic nomenclature.

[2] The chymotrypsinogen numbering system (Brown & Hartley, 1966) is used to designate residues in the serine proteinases investigated.

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oxyanion hole in both species. Previous comparison of the X-ray structures of chymotrypsinogen and chymotrypsin had indicated that the oxyanion hole of the zymogen contains one hydrogen-bond donor whereas that of the enzyme contains two hydrogen-bond donors (Robertus et al., 1972). In addition, iPr_2P-chymotrypsinogen and iPr_2P-neochymotrypsinogen can be distinguished on the basis of their ^{31}P NMR chemical shifts (Reeck et al., 1977). In contrast to Reeck et al. (1977), who observed single ^{31}P NMR peaks for each iPr_2P derivative, Gorenstein & Findlay (1976) reported that iPr_2P-bovine chymotrypsin A_α gives rise to two ^{31}P NMR peaks whose relative intensities change with pH. They assigned the peaks to two slowly interconverting isomers of the enzyme derivative.

Earlier investigations in our laboratory (Porubcan et al., 1978; Porubcan, 1978) had utilized iPr_2P derivatives of serine proteinases as a means for assigning the ^1H NMR peak corresponding to the C^{ϵ_1}-H of His^{57} at the active site. The ^1H NMR studies indicated that inactivation of serine proteinases by iPr_2P-F causes changes in the environment and pK' of His^{57}. The present report concerns parallel ^1H NMR and ^{31}P NMR investigations of iPr_2P derivatives of serine proteinases and their zymogen precursors. The new results are consistent with our previous NMR peak assignments (Markley & Porubcan, 1976; Porubcan et al., 1978; Westler & Markley, 1978) and indicate that the pK' values of catalytic groups in iPr_2P-serine proteinases can be derived from the pH dependence of the ^{31}P NMR chemical shift. Single peaks were obtained for each derivative studied, except for iPr_2P-F-inhibited chymotrypsin A_δ which underwent a slow irreversible reaction giving rise to a new ^{31}P peak and iPr_2P-F-inhibited α-lytic proteinase which also exhibited a second peak at low pH.

Experimental Section

Materials. Porcine trypsin was purchased from the Enzyme Development Corp. (Lot S-162); bovine trypsin (TRL 3.37C664), bovine trypsinogen (TG 35C823), and bovine chymotrypsinogen A (5× crystallized grade CGC) were from the Worthington Biochemical Corp. Porcine trypsinogen was isolated from fresh pig pancreas by the method of Charles et al. (1963). α-Lytic proteinase was isolated from *Sorangium* sp. (Myxobacter 495) according to the procedure of Hunkapiller et al. (1973). Special chemicals used and their sources were as follows: deuterium oxide (^2H_2O, 99.8 or 100.0% isotopically pure) and potassium deuteroxide (KO^2H), Bio-Rad Laboratories; deuterium chloride (^2HCl), Merck of Canada; Cbz-L-alanine *p*-nitrophenyl ester, Sigma; ATEE monohydrate, Aldrich; Hepes buffer and iPr_2P-F, Calbiochem; NPGB and NPA, Nutritional Biochemical Corp.; ultrapure KCl and $CaCl_2$, Alfa Chemical Co.; ultrapure Tris buffer, Schwartz/Mann Biochemicals. All other chemicals were reagent grade or better. Doubly deionized or distilled, deionized water was used for all solutions.

Assays of Enzyme Activity. Tryptic activity was measured by the active-site titration method of Chase & Shaw (1967) using NPGB. Trypsinogen samples were assayed after activation to trypsin by the addition of catalytic amounts of trypsin at pH 8.0 in 0.1 M Tris and 0.02 M $CaCl_2$ at room temperature. Chymotryptic activity was measured by the ATEE assay (Wilcox, 1970) using a Radiometer automatic titrator. Chymotrypsinogen was assayed after conversion to chymotrypsin A_δ by incubation for 30 min with 3% (w/w) trypsin at pH 7.5 and 25 °C (Garel & Labouesse, 1973). α-Lytic proteinase activity was measured spectrophotometrically at 410 nm by the hydrolysis of Cbz-L-alanine *p*-nitrophenyl ester; 50 μL of 2.3 mM substrate in acetonitrile was added to 1 mL of 0.05 M Hepes buffer, pH 8. Typically, 10 μL of a 0.1 mg/mL solution of α-lytic proteinase was used for the assay.

Preparation of iPr_2P-Inhibited Proteins. Trypsins were reacted with iPr_2P-F in 0.1 M Tris, pH 8.0, at a concentration of 5 mg/mL. Sufficient iPr_2P-F, 1 M, was added to bring the iPr_2P-F/trypsin ratio near 100. The reaction was allowed to proceed for 1 h, after which time no tryptic activity could be detected by NPGB analysis. The pH of the solution was lowered to around 3, and excess reactants and buffers were removed by dialysis against 0.001 M HCl. It was necessary to dialyze iPr_2P derivatives against a solution of high ionic strength in order to remove an extra peak in the ^{31}P NMR spectrum that arises from the hydrolysis product diisopropyl phosphate, which binds to the proteins. Trypsin samples to be used for ^1H NMR spectroscopy were preexchanged in ^2H_2O (Markley & Porubcan, 1976); the reaction with iPr_2P-F and subsequent purification of the derivative on a PD-10 column were carried out in ^2H_2O. The trypsin samples contained a mixture of α- and β-trypsins; only single ^{31}P NMR peaks were observed for these species, so no attempt was made to separate them. An adaptation of the procedure of Morgan et al. (1972) was used to prepare iPr_2P-trypsinogens. Bovine or porcine trypsinogen was reacted with a 100–200-fold excess of iPr_2P-F by the addition of the appropriate amount of a 1 M solution of iPr_2P-F in 2-propanol. The protein concentration was 3–5 mg/mL in 0.1 M Tris and 0.02 M $CaCl_2$ at pH 8.0. The zymogens were reacted for a period of 24 h, during which time the pH was kept between 7 and 9 by the addition of 1 M KOH. Reacted samples demonstrated little or no potential activity. At the end of the reaction, the pH was lowered to 3.0, and any insoluble protein present was removed by centrifugation. Buffer and excess reagents were removed by dialysis against 0.001 M HCl containing 0.5 M NaCl, followed by exhaustive dialysis against 0.001 M HCl.

iPr_2P-chymotrypsinogen was prepared according to a modification of the procedure of Gertler et al. (1974). Chymotrypsinogen A at a concentration of 5.4 mg/mL in 0.1 M Tris and 0.25 M NaCl buffer at pH 8.0 was reacted with sufficient 1 M iPr_2P-F in 2-propanol to bring the total concentration of iPr_2P-F to 2×10^{-2} M. After 20 h the pH was lowered to 3.0, and insoluble material was removed by centrifugation. Excess buffer and reagent were removed by dialysis against 0.25 M NaCl, followed by exhaustive dialysis against 0.001 M HCl. iPr_2P-chymotrypsin A_δ was prepared by using the same conditions as for the zymogen, except that the total reaction time was reduced to 1 h and the iPr_2P-F concentration in the reaction mixture was 5×10^{-3} M.

α-Lytic proteinase was reacted with iPr_2P-F in phosphate buffer (Whitaker & Roy, 1967). The use of phosphate buffer led to the presence of extra peaks in the ^{31}P NMR spectrum. These could be removed by extensive dialysis. For ^1H NMR spectroscopy, α-lytic proteinase was first preexchanged by successive lyophilizations from ^2H_2O at pH* 5.5 (twice) and at pH* 4.0 (twice). The protein was then dissolved in ^2H_2O at a concentration of 25 mg/mL, and the pH* was adjusted to 10.5. After 1 h at room temperature, the pH* was lowered to 4, and the solution was lyophilized. The preexchanged protein (25 mg) was then dissolved in 1 mL of 0.2 M KCl and 0.01 M Tris at pH* 8.0 in ^2H_2O. A total of 1.8 mL of 1 M iPr_2P-F in 2-propanol was added to the reaction mixture. The pH* was restored to 8.0, and the mixture was allowed to react for 30 min. The protein was purified by gel filtration on a PD-10 column preequilibrated with 29 mM KCl in ^2H_2O. The protein-containing eluant from the column (3.5 mL) was

Table I: Summary of NMR–pH Titration Data for Transitions Observed by ^1H NMR to Affect His-57 and Transitions Observed by ^{31}P NMR to Affect iPr_2-Ser-195 of iPr_2P-Ser Proteinases[a]

	high pH transition				low pH transition		
	Δδ (ppm)				Δδ (ppm)		
species	^1H	^{31}P	pH_{mid}	Hill coefficient (n)	^1H	^{31}P	pH_{mid}
iPr_2P-α-LPase	0.98		8.16 ± 0.03	0.9 ± 0.1	*f*		
		0.30	7.9 ± 0.2	0.7 ± 0.2		*f*	
iPr_2P-BCtr[b]		0.46	7.46 ± 0.07	1.0 ± 0.2		0.21	4.3 ± 0.2
iPr_2P-BCtg	0.62		7.63 ± 0.03	0.92 ± 0.03	−0.21		3.1 ± 0.1
		0.24	7.5 ± 0.1	0.9 ± 0.2		−0.8[c]	3.3 ± 0.1[c]
iPr_2P-BTr[d]		0.58	7.71 ± 0.08	0.7 ± 0.1		*f*	
iPr_2P-PTr	1.19		7.36 ± 0.02	1.00 ± 0.03	−0.18		3.0 ± 0.2
		0.89	7.31 ± 0.05	0.8 ± 0.1		*f*	
iPr_2P-BTg[e]	0.93		7.99 ± 0.02	0.99 ± 0.03	−0.10		4.3 ± 0.2
		0.24	7.94 ± 0.07	0.5 ± 0.1		*f*	
iPr_2P-PTg[e]	1.17		7.67 ± 0.02	0.97 ± 0.03	−0.17		4.3 ± 0.2
		0.48	7.42 ± 0.02	0.5 ± 0.1		*f*	

[a] Conditions: 30 °C; in ^2H_2O; all solutions contained 0.5 M KCl, except iPr_2P-α-LPase solutions which contained 0.2 M KCl. [b] Markley & Ibañez (1978); a ^1H NMR peak corresponding to the C^{ϵ_1}-H of histidine-57 has not been observed for this species (Markley & Ibañez, 1978). [c] Only an approximate value; see text. [d] A ^1H NMR peak corresponding to the C^{ϵ_1}-H of histidine-57 has not been observed for this species (Porubcan, 1978). [e] Porubcan et al. (1978). [f] Transition not observed; see text.

lyophilized and dissolved in 0.5 mL of ^2H_2O (100% isotopically pure) to produce a final KCl concentration of 0.2 M and a protein concentration of ~2 mM.

Solutions Used for NMR Spectroscopy. All solutions contained ^2H_2O to provide for the deuterium field/frequency lock and to permit comparison between the ^1H NMR and ^{31}P NMR results. Lyophilized protein samples (except α-lytic proteinase; see above) were dissolved in 0.5 M KCl in ^2H_2O at a concentration of 1 to 2 mM. Any insoluble material present was removed by centrifugation. The observation of ^{31}P NMR signals in iPr_2P-trypsinogens was dependent on the addition of $CaCl_2$ (0.02 M). Small amounts of soybean trypsin inhibitor (1 to 2% by weight) were also added to iPr_2P-trypsinogen solutions to prevent possible activation by trace amounts of trypsin or trypsinogen (Kay & Kassell, 1971) that may have been present.

Titration and pH Measurement. Methods were as described in Markley & Porubcan (1976).

NMR Spectroscopy. ^{31}P NMR spectra were obtained at 40.5 MHz on a modified XL-100-15 spectrometer equipped with a broad-band multinuclear receiver system (Santini & Grutzner, 1976a) employing a technique for total systematic noise reduction (Santini & Grutzner, 1975b). The spectrometer was operated in the pulse Fourier transform mode with the field modulation turned off. Pulse timing was achieved through a homemade pulsing unit (R. E. Santini, unpublished experiments). Spectra were coherently proton decoupled (Grutzner & Santini, 1975), and a directional wattmeter (Bird Electronics) was used to monitor the decoupler power (10 W). Each 8K spectrum was the result of 3–6 h of accumulation using a 1.2-s recycle time and a pulse width corresponding to ~45°. Data acquisition was accomplished with a Nicolet 1080 computer system with a Nicolet 293 I/O unit and a Diablo Model 33 disk drive. The free-induction decays were convoluted with an exponential decay function, yielding 1.0-Hz line broadening.

^1H NMR spectra at 360 MHz of α-lytic proteinase were obtained at the Purdue University Biological Magnetic Resonance Laboratory with a Nicolet NT-360 spectrometer system operating in the pulse Fourier transform mode with quadrature detection. Each 8K ^1H NMR spectrum was the average of 256 pulses obtained with a 3.0-s recycle time. The residual $^1HO^2H$ peak was minimized by continuous wave saturation using the decoupler. The spectral width was ±2500 Hz.

^1H NMR chemical shifts are given in parts per million downfield from DSS; ^{31}P NMR chemical shifts are in parts per million downfield from external 85% phosphoric acid in water. All spectra were obtained at 30 °C.

Results

iPr_2P-α-lytic Proteinase. ^1H NMR spectra of the histidyl C^{ϵ_1}-H region of iPr_2P-α-LPase are shown in Figure 1a. The single histidyl peak (H) shifts as a function of pH* with a pK' value of 8.16 ± 0.03 (Figure 2). At high pH, the peak broadens but still can be followed. ^{31}P NMR spectra of iPr_2P-α-LPase are shown in Figure 1b. Both of the peaks that are observed (P1 and P2) shift with pH. The major peak labeled P1 titrates with a pK' value of 7.9 ± 0.2 (Figure 2). The titration parameters for the ^1H and ^{31}P NMR experiments are summarized in Table I. The minor peak labeled P2 was observed only with certain samples; its origin has not been determined.

iPr_2P-bovine Chymotrypsin A_δ. ^{31}P NMR spectra of iPr_2P-BCtr A_δ are presented in Figure 3a. The single peak assigned to the modified Ser^{195} is affected by two pH*-dependent transitions occurring with pK' values of 4.3 ± 0.2 and 7.46 ± 0.07 (Table I). The chemical shift of this peak is plotted as a function of pH* in Figure 3b. Attempts to resolve the ^1H NMR peak of iPr_2P-BCtr A_δ which corresponds to the C^{ϵ_1}-H of His^{57} were unsuccessful (Markley & Ibañez, 1978).

iPr_2P-bovine Chymotrypsinogen A. Two peaks were observed in ^{31}P NMR spectra of iPr_2P-BCtg (Figure 4a). The chemical shift of the peak at lower field (−0.77 ppm) corresponds to diisopropyl phosphate. The peak at higher field is assigned to iPr_2P-Ser^{195}; it is affected by two transitions occurring with pK' values of 3.3 ± 0.1 and 7.5 ± 0.1 (Figure 4b, solid line). Also included for comparison in Figure 4b (dashed line) is the ^1H NMR titration curve for the peak assigned to the C^{ϵ_1}-H of His^{57} in the same derivative (Markley & Ibañez, 1978). The ^{31}P NMR peak of iPr_2P-BCtg broadens at low pH and could not be resolved below pH* 3. Hence, the pK' derived from the low pH transition is subject to large error. The curves shown in Figure 4b represent computer fits to two pK' values; the parameters obtained are given in Table I.

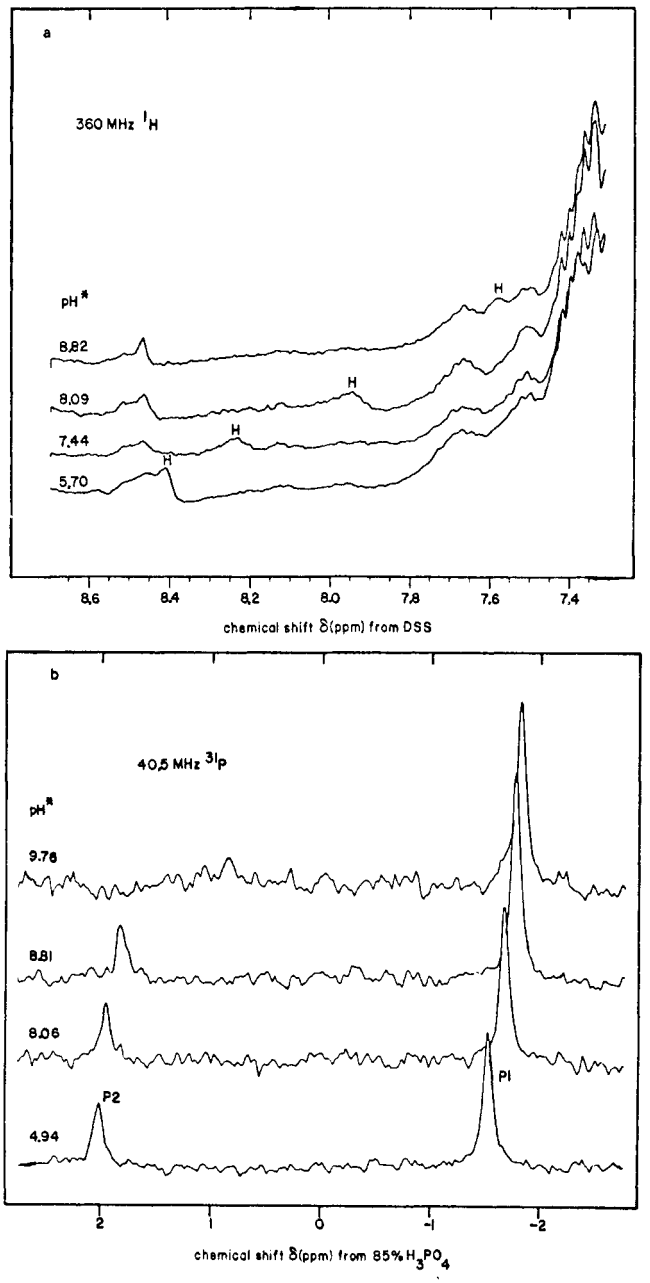

FIGURE 1: (a) 360-MHz ^{1}H NMR spectra, using a Carr–Purcell–Meiboom–Gill pulse sequence with a total sequence length of 6 ms to remove unexchanged broad N–H peaks (Campbell et al., 1975), and (b) 40.5-MHz ^{31}P NMR spectra of (diisopropylphosphoryl)-α-lytic proteinase at 30 °C. Samples contained 2–4 mM protein in 0.4 M KCl in ^2H_2O. Peak H in the ^{1}H NMR spectra is assigned to the C^{ϵ_1}-H of histidine-57. Peak P1 in the ^{31}P NMR spectra is assigned to (diisopropylphosphoryl)serine-195; P2 appears in aged samples and is unassigned.

iPr_2P Derivatives of Bovine and Porcine Trypsins. ^{31}P NMR spectra of iPr_2P-BTr and iPr_2P-PTr are shown in parts a and b of Figure 5, respectively. A single peak which shifts with pH was observed with each derivative. The ^{31}P NMR chemical shift data for iPr_2P-BTr were fitted to a single titration curve with a pK' of 7.71 ± 0.08 (Figure 6a). The ^{31}P NMR peak of iPr_2P-BTr broadens and disappears below pH* 4.0. Several unsuccessful attempts were made to resolve a ^{1}H NMR peak of iPr_2P-Btr corresponding to the C^{ϵ_1}-H of His^{57} (Porubcan, 1978). Porcine trypsin has proved to be a better candidate for ^{1}H NMR spectroscopy, and a peak in the ^{1}H NMR spectra of iPr_2P-PTr has been assigned to the C^{ϵ_1}-H of His^{57} (Porubcan, 1978). The ^{1}H NMR and ^{31}P NMR data

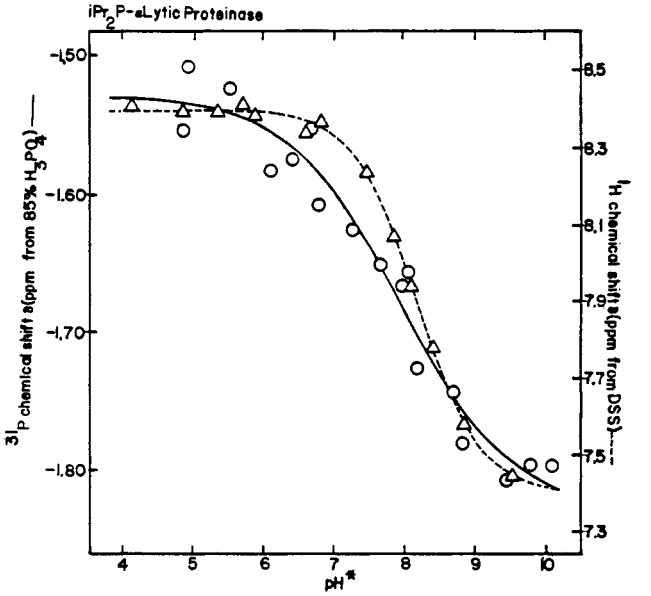

FIGURE 2: Comparison of the pH titration behavior of the ^{1}H NMR peak assigned to the C^{ϵ_1}-H of histidine-57 (△) and the ^{31}P NMR peak assigned to the (diisopropylphosphoryl)serine-195 (○) of iPr_2P-α-lytic proteinase. Sample spectra are shown in Figure 1. The computer-fitted titration curves yield pK' values of 8.16 ± 0.03 (---) and 7.9 ± 0.2 (—).

for iPr_2P-PTr are compared in Figure 6b. The pK' values obtained by fitting each set of data to a single titration curve agree within experimental error: 7.31 ± 0.05 for the ^{31}P NMR data (solid line, Figure 6b) and 7.36 ± 0.02 for the ^{1}H NMR data (dashed line, Figure 6b). The ^{1}H NMR peak is affected by a second transition with a pH_{mid} of 3.0 which is slow on the ^{1}H NMR time scale (discontinuous); the lifetime of the species involved must be in excess of 25 ms. The ^{31}P NMR peak of iPr_2P-PTr disappears below pH* 3.2; hence, the second transition could not be detected.

iPr_2P Derivatives of Bovine and Porcine Trypsinogens. ^{31}P NMR spectra of iPr_2P-BTg (Figure 7a) and iPr_2P-PTg (Figure 7b) revealed single peaks in the pH* range 6–10. The resonances were not followed below pH* 5.5–6.0 because of severe line broadening, which was not reversed by the addition of Ca^{2+} or EDTA. In Figure 8 the ^{31}P NMR data are compared with ^{1}H NMR data for the same derivatives (Porubcan et al., 1978). The titration parameters obtained by nonlinear least-squares analysis are summarized in Table I.

Discussion

Stroud and co-workers recently have refined the X-ray structure of iPr_2P-F-inhibited bovine trypsin and have solved the structure of iPr_2P-F-inhibited bovine trypsinogen (R. M. Stroud, personal communication). They have concluded that their original interpretation of the electron densities of iPr_2P-F-inhibited trypsin is in error. Although the unesterified phosphate oxygen was correctly placed in the oxyanion hole, the top isopropyl group (Stroud et al., 1974, Figure 12) is absent after refinement; and they have shown by chemical means that the crystals contain (monoisopropylphosphoryl)trypsin (iPrP-trypsin). The N^{ϵ_2} of His^{57} is hydrogen bonded to the iPr ester oxygen in iPrP-trypsin. On the other hand, whereas the crystals of iPr_2P-F-inhibited bovine trypsinogen contain (diisopropylphosphoryl)trypsinogen (iPr_2P-BTg), the phosphate oxygen is not located in the oxyanion hole; the imidazole of His^{57} has rotated out of the cleft so that it no longer is hydrogen bonded to the β-carboxylate of Asp^{102}. There are no hydrogen bonds to the phosphate oxygens in

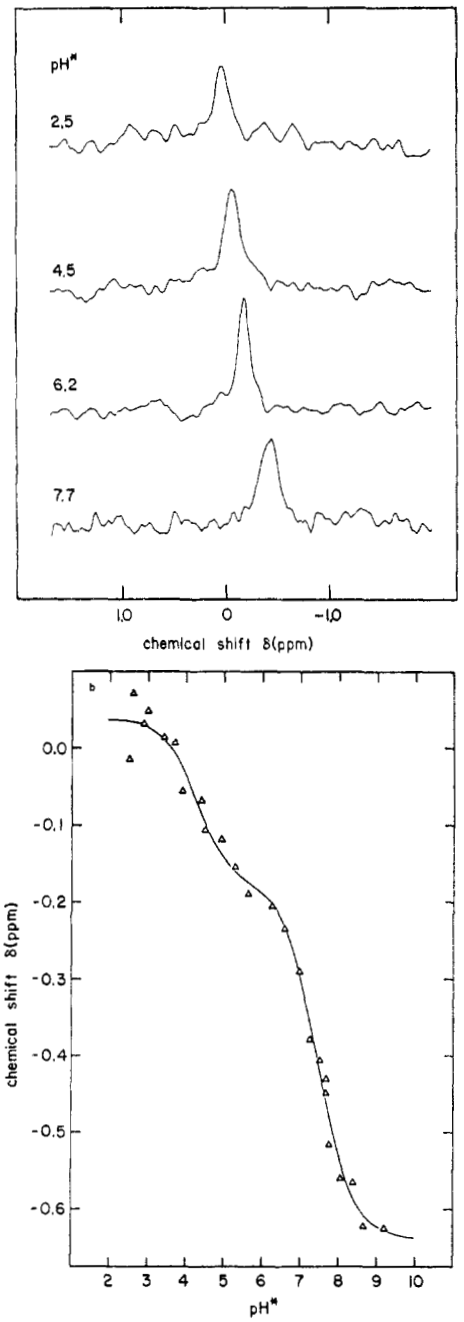

FIGURE 3: 40.5-MHz ^{31}P NMR data for 2 mM (diisopropylphosphoryl)bovine chymotrypsin A_δ in 0.5 M KCl in ^2H_2O, 30 °C. (a) Spectra at various pH* values. (b) The pH dependence of the ^{31}P chemical shift. The experimental peaks were fitted to a theoretical titration curve with pK' values of 4.3 ± 0.2 and 7.46 ± 0.07.

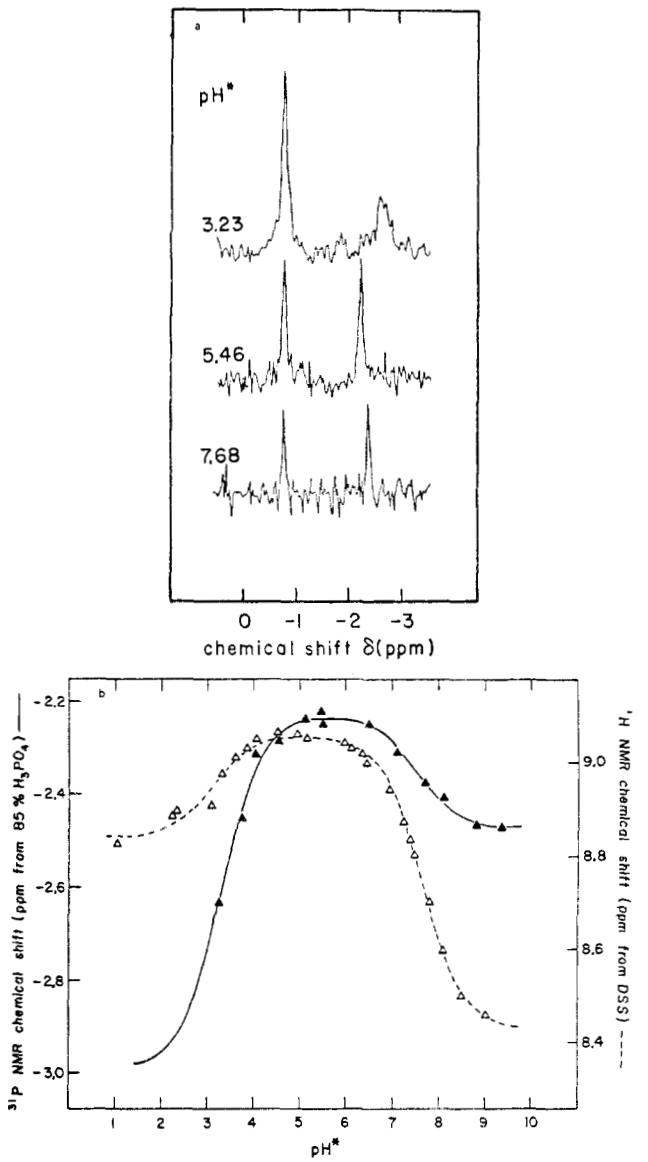

FIGURE 4: NMR data for 1 mM (diisopropylphosphoryl)chymotrypsinogen A in 0.5 M KCl in ^2H_2O, 30 °C. (a) 40.5-MHz ^{31}P NMR spectra at various pH* values. The low-field peak is assigned to the contaminant, diisopropyl phosphate, on the basis of its chemical shift; the high-field peak which moves with pH is assigned to the (diisopropylphosphoryl)serine-195. (b) The pH dependence of the ^{31}P NMR peak assigned to the inhibited enzyme (▲) was fitted by a titration curve (—) having pK' values of 3.3 ± 0.1 and 7.5 ± 0.1. The ^1H NMR data obtained at 250 MHz and assigned to the $C^{\epsilon 1}$-H of histidine-57 of the same derivative (Markley & Ibañez, 1978) are given for reference (△). These points were fitted by a theoretical titration curve (---) having pK' values of 3.1 ± 0.1 and 7.63 ± 0.03.

iPr_2P-BTg (R. M. Stroud, personal communication).

It is concluded that all the inhibited species listed in Table I contain (diisopropylphosphoryl)serine-195. The same ^{31}P NMR chemical shifts were obtained with freshly reacted enzyme samples, in which no attempt was made to purify the product, as with samples that had been dialyzed or chromatographed at low pH in order to remove diisopropyl phosphate or phosphate ions. A catalytic amount of PTr was added to a sample of iPr_2P-PTg in an NMR tube, and the activation was followed by ^{31}P NMR spectroscopy. The chemical shift of the activation product was identical with that of samples of iPr_2P-F-inhibited PTr. Hence, we have seen no evidence for conversion of iPr_2P-PTr (the presumed product of activation of iPr_2P-F-inhibited PTg) to iPrP-PTr during the time the samples were in solution (less than 1 week). An irreversible aging reaction did take place with samples of iPr_2P-BCtr$_\delta$. After 12–24 h in solution, the signal shown in Figure 3 gradually disappears and a new peak appears downfield. The chemical shift of the new peak has a much smaller pH dependence. The product of this aging reaction has not been characterized further. A minor ^{31}P NMR peak which grows with time appears in spectra of iPr_2P-F-inhibited α-lytic proteinase (peak P2 in Figure 1b). Its identity has not been determined. Several months were required to obtain crystals of the iPr_2P-F-inhibited trypsin used by Stroud et al. (1974) for X-ray analysis; during this time iPr_2P-BTr apparently aged to iPrP-BTr.

Gorenstein & Findlay (1976) reported doubling of the ^{31}P resonance of iPr_2P-Ctr. They speculated that one peak

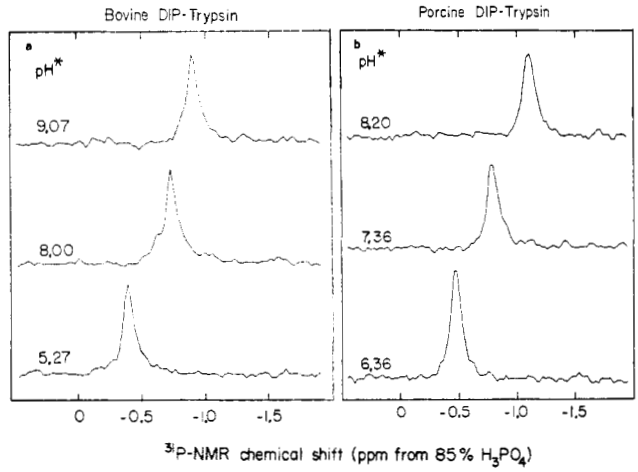

FIGURE 5: 40.5-MHz ^{31}P NMR spectra of 1 mM solutions of (diisopropylphosphoryl)trypsins in 0.5 M KCl in ^2H_2O, 30 °C. (a) iPr_2P-bovine trypsin; (b) iPr_2P-porcine trypsin.

corresponds to the iPr_2P-Ser^{195} derivative and the other to an iPr_2P-His^{57} derivative in which the substituent has been transferred to the N$^{\epsilon 2}$ of His^{57}. Such an equilibrium was originally proposed by Green & Nichols (1959) in order to explain the pH dependence of reactivation of alkyl phosphate inhibited serine proteinases. Since we have not observed doubling of the ^{1}H NMR peak assigned to the C$^{\epsilon 1}$-H of His^{57} in any of the seven iPr_2P derivatives investigated, we discount the importance of such a species.

The pH dependence of the ^{31}P NMR chemical shifts of iPr_2P-serine proteinases was not noted in previous publications (Reeck et al., 1977; Gorenstein & Findlay, 1976).[3] By reference to paralleled ^{1}H NMR studies, the transitions that affect the ^{31}P resonances may be assigned to specific proton equilibria. The transition at higher pH corresponds to the pK' assigned in the ^{1}H NMR studies to His^{57} and that at lower pH (in the single case where observed) agrees with the pH_{mid} attributed to Asp^{102} (Table I). The results suggest that the phosphate of iPr_2P-Ser^{195} is close enough to His^{57} in each derivative for its chemical shift to be affected by the change in protonation state of the imidazole. This simple model appears adequate for α-lytic proteinase and chymotrypsinogen derivatives where the Hill coefficient (n) is the same within experimental error and close to unity for the ^{1}H NMR and ^{31}P NMR data. In the cases of iPr_2P-BTg, iPr_2P-PTg, and perhaps iPr_2P-PTr, however, the Hill coefficient derived from ^{31}P NMR data is lower than that derived from ^{1}H NMR data. This result indicates that one or more additional groups with pK' values near the pH_{mid} affect the chemical shift of the phosphate as they titrate.

In all four enzymes studied, the pK' of His^{57} is raised substantially after reaction with iPr_2P-F. However, in all three zymogens the pK′ of His^{57} is affected only slightly by derivatization (Table II). The increase in pK' of His^{57} from 6.7 in BCtr$_\alpha$ to 7.5 in iPr_2P-BCtr$_\delta$ is consistent with previous observations concerning the relative pH dependence of photooxidation of the active-site histidine in BCtr and iPr_2P-BCtr (Jandorf et al., 1955). Studies of the pH dependence of proton release on reaction of chymotrypsin A$_\alpha$ with iPr_2P-F indicated that the pK' of one or more groups in the enzyme is higher in the iPr_2P derivative than in the native enzyme, and this hypothesis was confirmed by potentiometric titration experiments (Havsteen & Hess, 1964; Moon et al., 1965). The iPr_2P-induced perturbation of the titration curve of chymotrypsin extends from pH 6 to 10 (Moon et al., 1965). These potentiometric titration data require that the pK' of a second group with higher pK' be altered, possibly the α-amino group of Ile^{16} as suggested by Moon et al. (1965).

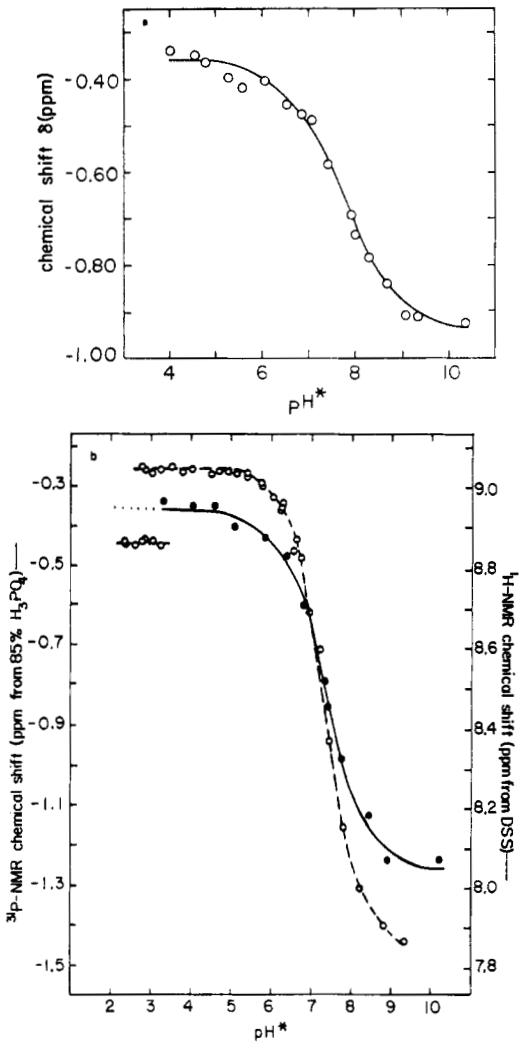

FIGURE 6: NMR–pH titration curves for 1 mM (diisopropylphosphoryl)trypsins in 0.5 M KCl in ^2H_2O, 30 °C. Sample ^{31}P NMR spectra are shown in Figure 5. (a) 40.5-MHz ^{31}P NMR data for iPr_2P-bovine trypsin fitted to a theoretical titration curve having a pK' of 7.71 ± 0.08. (b) 40.5-MHz ^{31}P NMR data for iPr_2P-porcine trypsin (●) fitted to a theoretical titration curve having a pK' of 7.31 ± 0.05, and 250-MHz ^{1}H NMR data for the same derivative (○) fitted to a theoretical titration curve having a pK' of 7.36 ± 0.02. The ^{1}H resonance, which is assigned to the C$^{\epsilon 1}$-H of histidine-57, exhibits a second transition with a pH_{mid} of 3.0 ± 0.2 (Porubcan, 1978).

In agreement with Reeck et al. (1977), we find that iPr_2P zymogens and iPr_2P enzymes have characteristic ranges of ^{31}P chemical shifts (Figure 9). The average enzyme–zymogen chemical shift difference of 1.7 ppm could be accounted for by a change in hydrogen bonding as postulated by Reeck et al. (1977), by a change in a phosphate ester O–P–O bond angle (Gorenstein, 1977), by a change in the dielectric environment of the tertiary phosphate ester (Jones & Katritzky, 1962), or by a combination of these effects. As noted above, the results of Stroud and co-workers (R. M. Stroud, personal communication) suggest that the position of the phosphate is very different in crystals of iPrP-trypsin and iPr_2P-trypsinogen. This structural difference probably is not simply a result of the fact that one derivative is monoisopropyl and the other diisopropyl since there is room for a second isopropyl group

[3] The pH dependence of the ^{31}P NMR peaks of iPr_2P-serine proteinases has also been observed by Dr. D. D. Mueller (personal communication).

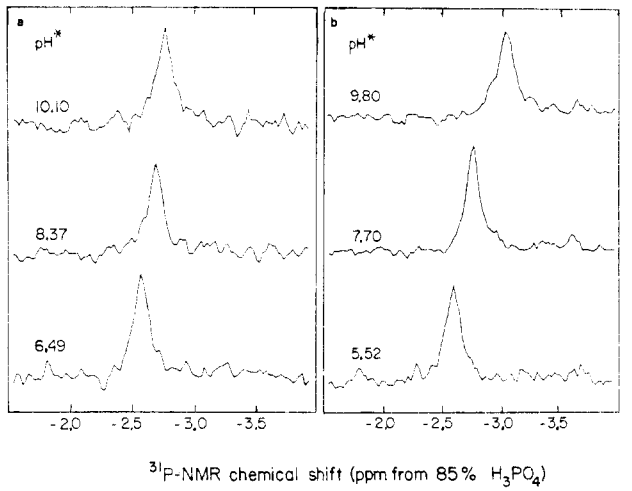

FIGURE 7: 40.5-MHz ^{31}P NMR spectra of 1 mM solutions of (diisopropylphosphoryl)trypsinogens in 0.5 M KCl and 0.02 M $CaCl_2$ in ^2H_2O, 30 °C. (a) iPr_2P-bovine trypsinogen; (b) iPr_2P-porcine trypsinogen.

in the structure proposed for iPr_2P-F-inhibited trypsin (Stroud et al., 1974). The X-ray results suggest that there are three hydrogen bonds donated to the phosphate in iPr_2P enzymes (the backbone NH's of Gly^{193} and Ser^{195} and the $N^{\epsilon 2}$-H of His^{57}) but no hydrogen bonds to the phosphate in iPr_2P zymogens (R. M. Stroud, personal communication). Since hydrogen bonds donated to the phosphate should result in a downfield shift, the data in Figure 9 are consistent with such a structural change. The ^{31}P NMR titration shift which accompanies the high pH transition in iPr_2P-BTr is 2.4 times as large as that in iPr_2P-BTg, suggesting that the phosphate is farther from the imidazole of His^{57} in iPr_2P-BTg than in iPr_2P-BTr. This result is consistent with the X-ray data which indicate a hydrogen bond between His^{57} and the phosphate in the enzyme derivative but not in the zymogen derivative (R. M. Stroud, personal communication).

The ^{1}H NMR results (Porubcan et al., 1978) also are in agreement with displacement of His^{57} away from Asp^{102} in iPr_2P-Btg (R. M. Stroud, personal communication) as compared with BTg (Fehlhammer et al., 1977; Bode et al., 1976; Kossiakoff et al., 1977). The ^{1}H NMR chemical shift of the $C^{\epsilon 1}$-H of His^{57} is altered substantially when BTg is converted to iPr_2P-BTg, and the magnitude of the low pH transition attributed to protonation of Asp^{102} is attenuated by a factor of almost 4 (Porubcan et al., 1978). In addition, the pK' attributed to Asp^{102} is more normal in iPr_2P-BTg (4.3) than in BTg (1.8), in agreement with the increased solvent accessibility of Asp^{102} in iPr_2P-BTg (R. M. Stroud, personal communication). Similar changes were observed with iPr_2P derivatives of porcine trypsinogen (Porubcan et al., 1978) and bovine chymotrypsinogen (Markley & Ibañez, 1978).

It would be most interesting to know the pK' of His^{57} in the transition states of the catalytic reaction. If iPr_2P enzymes are suitable models for the first transition state (Stroud et al., 1974; Kraut, 1977), the pK' values of His^{57} in these derivatives may represent an approximation of the transition-state pK' values. Following this assumption, we may speculate on the catalytic importance of the pK' values listed in Table II. The fact that His^{57} has a relatively low pK' in free enzymes ensures that the imidazole is in the unprotonated form at physiological pH. After formation of the Michaelis complex and as the transition state is approached, the pK' of His^{57} is raised. This makes the imidazole a more efficient base for accepting the hydroxyl proton of Ser^{195}. Zymogens are catalytically less

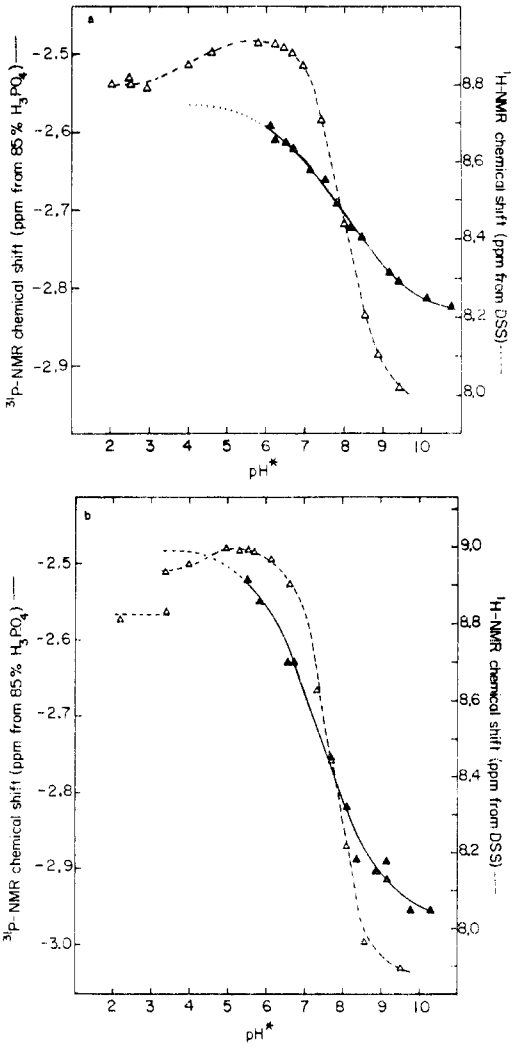

FIGURE 8: NMR–pH titration curves for 1 mM (diisopropylphosphoryl)trypsinogens in 0.5 M KCl and 0.02 M $CaCl_2$ in ^2H_2O, 30 °C. Sample ^{31}P NMR spectra are shown in Figure 7. (a) 40.5-MHz ^{31}P NMR data for iPr_2P-bovine trypsinogen (▲) fitted to a titration curve having a pK' of 7.94 ± 0.07, and 250-MHz ^{1}H NMR data for the same derivative (△) fitted to a theoretical titration curve having pK' values of 4.3 ± 0.2 and 7.99 ± 0.02 (Porubcan et al., 1978). (b) 40.5-MHz ^{31}P NMR data for iPr_2P-porcine trypsinogen (▲) fitted to a theoretical titration curve having a pK' of 7.42 ± 0.02, and 250-MHz ^{1}H NMR data for the same derivative (△) fitted to a theoretical titration curve having pK' values of 4.3 ± 0.2 and 7.67 ± 0.02 (Porubcan et al., 1978).

active than their respective enzymes because of lowered efficiency of the binding and catalytic apparatus (Freer et al., 1970; Robertus et al., 1972; Kossiakoff et al., 1977; Fehlhammer et al., 1977; Bode et al., 1976). Since the pK' of His^{57} is higher in zymogens than in enzymes (Table II), a part of this inefficiency at physiological pH values must be attributed to fractional protonation of His^{57}, which renders it catalytically inactive.

The reactivation of chymotrypsin inactivated by organic phosphates was investigated in detail several years ago. Reactivation of iPr_2P-chymotrypsin proceeds very slowly; only 5% reactivation was found after 50 h in 1 M hydroxylamine (Cunningham & Neurath, 1953). However, organic phosphate derivatives having less bulky substituents, such as Et_2P-chymotrypsin, are reactivated much more rapidly. Reactivation is catalyzed by nucleophiles such as hydroxylamine, oximes, and hydroxamic acid. From the pH dependence of the reactivation reaction, it was determined that Et_2P-chymotrypsin contains a group with a pK' of 8.0 (Cunningham,

Table II: Effect of Inhibition by iPr_2P-F on the pK' Value of His-57 in Various Enzymes and Zymogens[a]

species	pK' value of His-57	
	native	iPr_2P-F derivative[h]
α-lytic proteinase	5.7[b]	8.0
bovine chymotrypsin A	6.8[c]	7.5
bovine trypsin	*d*	7.7
porcine trypsin	5.0[e]	7.3
bovine chymotrypsinogen A	7.3[f]	7.6
bovine trypsinogen	7.7[g]	8.0
porcine trypsinogen	7.7[g]	7.5

[a] Conditions: 30 °C; in ^2H_2O; solutions contained 0.5 M KCl, except native and inhibited α-lytic proteinase solutions which contained 0.2 M KCl. [b] W. M. Westler and J. L. Markley, unpublished experiments. [c] I. B. Ibañez and J. L. Markley, unpublished experiments; recent results at 360 MHz indicate that the pK' is somewhat higher than that reported by Markley & Ibañez (1978). The native pK' is for chymotrypsin A_α; the iPr_2P derivative pK' is for chymotrypsin A_δ. [d] Not measured. [e] Markley & Porubcan (1976). [f] Markley & Ibañez (1978). [g] Porubcan et al. (1978). [h] Average of ^{31}P NMR and ^1H NMR data where available (see Table I).

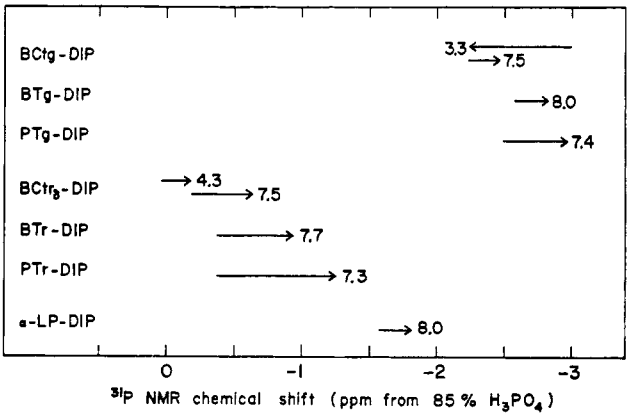

FIGURE 9: Summary of the ^{31}P NMR chemical shifts of iPr_2P-serine-195 in the (diisopropylphosphoryl)serine proteinases studied. The arrows indicate the chemical shift region covered in the high pH transition, and the numbers are the pK' values for each transition.

1954) or 7.0 (Green & Nichols, 1959; Cohen & Erlanger, 1960) which must be protonated for the reactivation to proceed. This group probably is His^{57}, which has a pK' of 7.5 in iPr_2P-BCtr$_\delta$ (Table I).

Acknowledgments

The authors thank Dr. R. M. Stroud for information about the X-ray structures of iPrP-BTr and iPr_2P-BTg prior to publication and Lydia Neely for assistance in preparing the manuscript.

References

Bender, M. L., & Wedler, F. C. (1972) *J. Am. Chem. Soc. 94*, 2101–2109.

Berends, F., Posthumus, C. H., Sluys, I. v. d., & Deierkauf, F. A. (1959) *Biochim. Biophys. Acta 34*, 576–578.

Blow, D. M. (1969) *Biochem. J. 112*, 261–268.

Bode, W., Fehlhammer, H., & Huber, R. (1976) *J. Mol. Biol. 106*, 325–335.

Brown, J. R., & Hartley, B. S. (1966) *Biochem. J. 101*, 214–228.

Campbell, I. D., Dobson, C. M., Williams, R. J. P., & Wright, P. E. (1975) *FEBS Lett. 57*, 96–99.

Charles, M., Rovery, M., Guidoni, A., & Desnuelle, P. (1963) *Biochim. Biophys. Acta 69*, 115–129.

Chase, T., Jr., & Shaw, E. (1967) *Biochem. Biophys. Res. Commun. 29*, 508–514.

Cohen, W., & Erlanger, B. F. (1960) *J. Am. Chem. Soc. 82*, 3928–3934.

Cohen, J. A., Oosterbaan, R. A., Warringa, M. G. P. J., & Jansz, H. S. (1955) *Discuss. Faraday Soc. 20*, 114–119.

Cunningham, L. W., Jr. (1954) *J. Biol. Chem. 207*, 443–458.

Cunningham, L. W., Jr., & Neurath, H. (1953) *Biochim. Biophys. Acta 11*, 310.

Davies, D. R., & Green, A. L. (1956) *Biochem. J. 63*, 529–535.

Fehlhammer, H., Bode, W., & Huber, R. (1977) *J. Mol. Biol. 111*, 415–438.

Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T., & Xuong, Ng. H. (1970) *Biochemistry 9*, 1997–2009.

Garel, J.-R., & Labouesse, B. (1973) *Eur. J. Biochem. 39*, 293–300.

Gertler, A., Walsh, K. A., & Neurath, H. (1974) *Biochemistry 13*, 1302–1310.

Gorenstein, D. G. (1977) *J. Am. Chem. Soc. 99*, 2254–2258.

Gorenstein, D. G., & Findlay, J. B. (1976) *Biochem. Biophys. Res. Commun. 72*, 640–645.

Green, A. L., & Nichols, J. D. (1959) *Biochem. J. 72*, 70–75.

Grutzner, J. B., & Santini, R. E. (1975) *J. Magn. Reson. 19*, 173–187.

Havsteen, B. H., & Hess, G. P. (1964) *Biochem. Biophys. Res. Commun. 14*, 313–317.

Hunkapiller, M. W., Smallcombe, S. H., Whitaker, D. R., & Richards, J. H. (1973) *Biochemistry 12*, 4732–4743.

IUPAC (1976) *Pure Appl. Chem. 45*, 219.

Jandorf, B. J., Michel, H. O., Schaffer, N. K., Egan, R., & Summerson, W. H. (1955) *Discuss. Faraday Soc. 20*, 134–142.

Jansen, E. F., Nutting, M. D. F., Jang, R., & Balls, A. K. (1949a) *J. Biol. Chem. 179*, 189–199.

Jansen, E. F., Nutting, M. D. F., & Balls, A. K. (1949b) *J. Biol. Chem. 179*, 201–204.

Jones, R. A. Y., & Katritzky, A. R. (1962) *Angew. Chem. 74*, 60–68.

Kay, J., & Kassell, B. (1971) *J. Biol. Chem. 246*, 6661–6665.

Kossiakoff, A. A., Chambers, J. L., Kay, L. M., & Stroud, R. M. (1977) *Biochemistry 16*, 654–664.

Kraut, J. (1977) *Annu. Rev. Biochem. 46*, 331–358.

Lee, W., & Turnbull, J. H. (1958) *Biochim. Biophys. Acta 30*, 655.

Lee, W., & Turnbull, J. H. (1961) *Experientia 17*, 360–361.

Markley, J. L., & Porubcan, M. A. (1976) *J. Mol. Biol. 102*, 487–509.

Markley, J. L., & Ibañez, I. B. (1978) *Biochemistry 17*, 4627–4640.

Markley, J. L., Porubcan, M. A., Ibañez, I. B., Westler, W. M., & Neves, D. E. (1978) *Int. Conf. Magn. Reson. Biol. Syst., 8th*, Abstract No. A-13.

Moon, A. Y., Sturtevant, J. M., & Hess, G. P. (1965) *J. Biol. Chem. 240*, 4204–4212.

Morgan, P. H., Robinson, N. C., Walsh, K. A., & Neurath, H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3312–3316.

Oosterbaan, R. A., Kunst, P., & Cohen, J. A. (1955) *Biochim. Biophys. Acta 16*, 299–300.

Porubcan, M. A. (1978) Ph.D. Thesis, Purdue University, West Lafayette, IN.

Porubcan, M. A., Neves, D. E., Rausch, S. K., & Markley, J. L. (1978) *Biochemistry 17*, 4640–4647.
Reeck, G. R., Nelson, T. B., Paukstelis, J. V., & Mueller, D. D. (1977) *Biochem. Biophys. Res. Commun. 74*, 643–649.
Robertus, J. D., Kraut, J., Alden, R. A., & Birktoft, J. J. (1972) *Biochemistry 11*, 4293–4303.
Robinson, N. C., Neurath, H., & Walsh, K. A. (1973) *Biochemistry 12*, 420–426.
Santini, R. E., & Grutzner, J. B. (1976a) *Anal. Chem. 48*, 941–943.
Santini, R. E., & Grutzner, J. B. (1976b) *J. Magn. Reson. 22*, 155–160.
Schaffer, N. K., May, S. C., Jr., & Summerson, W. H. (1953) *J. Biol. Chem. 202*, 67–76.
Stroud, R. M., Kay, L. M., & Dickerson, R. E. (1974) *J. Mol. Biol. 83*, 185–208.
Westler, W. M., & Markley, J. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 1795.
Whitaker, D. R., & Roy, C. (1967) *Can. J. Biochem. 45*, 911–916.
Wilcox, P. E. (1970) *Methods Enzymol. 19*, 64–108.

Mechanistic Studies on Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase from *Escherichia coli* Using Phosphorothioate Analogues. 1. Initiation and Pyrophosphate Exchange Reactions†

David Yee, Victor William Armstrong, and Fritz Eckstein*

ABSTRACT: The diastereomers of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) and adenosine 5′-*O*-(2-thiotriphosphate) (ATPβS) can replace adenosine triphosphate (ATP) in the initiation reaction catalyzed by deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase from *Escherichia coli*. In both cases, the S_p diastereomer is a better initiator than the R_p isomer. The diasteromers of 3′-uridyl 5′-adenosyl *O,O*-phosphorothioate [Up(S)A] can replace UpA in the primed initiation reaction catalyzed by RNA polymerase; however, the R_p diastereomer is a better initiator than the S_p isomer. By using ATP or CpA as initiator and UTPαS, isomer A, as substrate, we determined the stereochemical courses of both the initiation and primed initiation reactions, respectively, with T7 DNA template and found them to proceed with inversion of configuration. Determination of the stereochemical course of the pyrophosphate exchange reaction catalyzed by RNA polymerase provides evidence that this reaction is the reverse of the phosphodiester bond-forming reaction.

DNA-dependent RNA polymerase from *Escherichia coli* mediates the transcription of DNA to RNA by catalyzing the polymerization of ribonucleoside triphosphates in the presence of DNA template (Chamberlin, 1976). The DNA-directed synthesis of RNA by RNA polymerase may be considered to involve two kinds of phosphodiester bond-forming steps (Chamberlin, 1976; Krakow et al., 1976): (1) an initiation step, wherein a purine ribonucleoside triphosphate and another ribonucleoside triphosphate are coupled to give a dinucleoside tetraphosphate, and (2) an elongation step, wherein a ribonucleoside triphosphate is added to the 3′-OH terminus of the growing RNA chain. RNA polymerase can also catalyze a primed initiation reaction involving the addition of a ribonucleoside triphosphate to a dinucleoside monophosphate "primer" (So & Downey, 1970). Furthermore, RNA polymerase catalyzes a template-dependent pyrophosphate exchange into ribonucleoside triphosphates in the presence of an initiating ribonucleoside triphosphate or primer (Furth et al., 1962; Krakow & Fronk, 1969; So & Downey, 1970).

Phosphorothioate analogues of nucleoside triphosphates have proved to be useful in the study of enzyme mechanisms (Eckstein, 1975, 1979); e.g., the stereochemistry of the elongation step catalyzed by RNA polymerase has been determined by the use of ATPαS[1] (Eckstein et al., 1976; Burgers & Eckstein, 1978).

This paper reports on the substrate specificity and stereochemistry of the initiation, primed initiation, and pyrophosphate exchange reactions catalyzed by RNA polymerase using phosphorothioate analogues. The following paper (Armstrong et al., 1979) deals with the substrate specificity of the elongation step.

Experimental Procedure

Materials. ATP, UTP, UpA, and CpA were purchased from Pharma-Waldorf; poly[d(A-T)] was obtained from Miles Laboratories; ^{32}P-labeled Na_4PP_i was supplied by Amersham Buchler. Bacteriophage T7 DNA was prepared according to the procedure of Thomas & Abelson (1966), and T4 DNA was a generous gift from Professor W. Zillig. The molarities

† From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, Federal Republic of Germany. *Received February 22, 1979.* This research was supported in part by a grant from the Deutsche Forschungsgemeinschaft.

[1] Abbreviations used: ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); UTPαS, uridine 5′-*O*-(1-thiotriphosphate); ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); UDPαS, uridine 5′-*O*-(1-thiodiphosphate; Up(S)A, 3′-uridyl 5′-adenosyl *O,O*-phosphorothioate; poly-[d(A-T)], copolymer of alternating deoxyadenylate and thymidylate; LC, high-pressure liquid chromatography.

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