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^{31}P NMR of Covalent Phosphorylated Derivatives of α-Chymotrypsin†

David G. Gorenstein,*,‡ Dinesh Shah,§ Rouhlwai Chen,§ and Deborah Kallick§

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, and Department of Chemistry, University of Illinois at Chicago, Chicago, Illinois 60680

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ABSTRACT: The structures of various covalent phosphorylated derivatives of α-chymotrypsin (α-CT) have been studied by ^{31}P NMR spectroscopy. Diisopropylphosphoryl-α-chymotrypsin (α-DIPCT) shows a single ^{31}P signal at ca. 0.0 ppm (pH 4). At low pH, the ^{31}P NMR spectrum of α-DIPCT gradually changed with the appearance of one or two additional peaks. The ratio of the peaks varied with pH, time, and concentration. One of these two new downfield peaks (both at ca. 2.0 ppm) has been previously identified by Markley and co-workers (Markley, 1979; Porubcan et al., 1979) and van der Drift et al. (1985) as an aged monoisopropylphosphoryl-α-chymotrypsin (α-MIPCT) and is confirmed by our studies. A new additional downfield signal, separate from the α-MIPCT signal, is attributed to a dimer of the phosphorylated α-DIPCT. Phosphorylation of the enzyme with diphenyl chlorophosphate yields a monophenylphosphoryl-α-chymotrypsin (α-MPPCT) that also showed a single ^{31}P signal at −2.1 ppm (pH 7). However, the spectrum did not change as a function of pH, incubation time, or concentration. Comparison of the ^{31}P chemical shifts of the native and denatured phosphorylated derivatives of α-chymotrypsin suggests changes in the conformation about the P–O ester bonds are at least partially responsible for the various ^{31}P chemical shift differences.

Many of the serine proteinases and serine esterases are irreversibly inhibited by phosphorylating agents such as diisopropyl fluorophosphate (DFP) (Hartley, 1960). This inhibition is a result of the covalent binding of the diisopropylphosphoryl (DP) group to the γ-oxygen of the active-site serine residue (Osterbaan et al., 1955; Schaffer et al., 1953, 1954). The phosphorylated enzyme forms a stable, tetrahedral adduct (Figure 1) that is considered to be a good transition-state analogue (Lienhard, 1973; Stroud et al., 1974; Wolfenden, 1972; Kossiakoff & Spencer, 1981).

The structure of these irreversibly inhibited phosphorylated derivatives has been studied by ^{31}P NMR (Gorenstein & Findlay, 1976; Kallick et al., 1983; Markley, 1979; Porubcan et al., 1979; Reeck et al., 1977; Shah et al., 1983; van der Drift et al., 1985). ^{31}P chemical shifts are sensitive to both bonding and nonbonding interactions with the environment as well as to subtle geometric alterations about the phosphate tetrahedron

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‡ Purdue University.
§ University of Illinois at Chicago.

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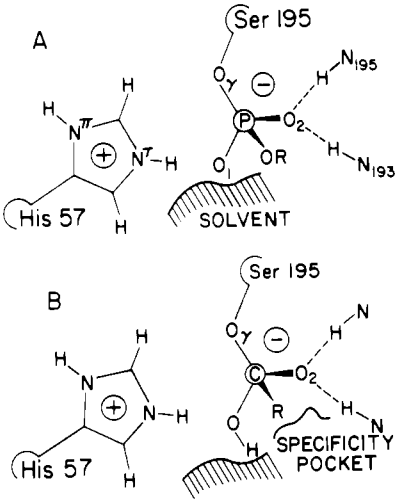


FIGURE 1: Comparison of (A) the monoisopropylphosphoryl–serine proteinase complex with (B) a model of the tetrahedral intermediate–serine proteinase complex. In both complexes, the O_2 oxygen bears a negative charge and is positioned in the oxyanion hole and hydrogen bonds to NH peptide backbone of residues 193 and 195. In (B), group R is the amino acid side chain that binds in the specificity pocket and in (A) is an isopropyl group. A diisopropylphosphoryl–enzyme complex (not shown) would presumably have an additional isopropyl group bonded to the O_1 oxygen of the phosphate and would be located toward the solvent-accessible side of the active site. The O_2 oxygen in the DIP complex would bear a partial negative charge because of the polarity of the phosphoryl bond. Derived from Kossiakoff and Spencer (1981).

(Gorenstein, 1984, 1987; Gorenstein & Post, 1989), and thus the ^{31}P nucleus of the covalently bound phosphate ester is a suitable "NMR reporter group" that provides structural and mechanistic information on the active site of the serine proteinases.

Findlay and Gorenstein initiated the first ^{31}P NMR study of a covalently phosphorylated derivative of the serine proteinases, diisopropylphosphoryl-α-chymotrypsin (α-DIPCT) (Gorenstein & Findlay, 1976). Two ^{31}P NMR signals, at 2 and 0 ppm, were observed. These signals were shown to chemically exchange as a function of pH, which led to the tentative assignment of the peaks to either two slowly interconverting structural or conformational isomers or possibly a monomer/dimer equilibration. The former interpretation has been questioned by Cohn and Rao (1976) and Porubcan et al. (1979). Reeck et al. (1977) observed a DIP-chymotrypsinogen (DIPCT-gen) ^{31}P signal at −2.5 ppm and an α-DIPCT signal at −0.5 ppm. They proposed that the 2 ppm downfield shift of α-DIPCT was due to various environmental changes upon activation of the zymogen in the active site.

Markley and co-workers (Markley, 1979; Porubcan et al., 1979) studied seven DIP-labeled serine proteinases, with DIPCT-gen and δ-DIPCT among them. While confirming the 2 ppm downfield shift from DIPCT-gen to chymotrypsin, they noted that the upfield peak of native DIP-trypsin and DIP-α-lytic proteinase gradually decreased in intensity and a new downfield ^{31}P signal grew with time. They suggested that this new downfield peak was associated with an aging process that is commonly observed in many phosphorylated serine hydrolases. Aging is defined as the loss of the ability to undergo reactivation of an inhibited enzyme. The aging process for the DIP enzymes involves conversion of the tertiary phosphate ester into the secondary ester; that is, the DIP group loses one isopropyl group and becomes a monoisopropyl phosphoryl (MIP) group (Lee & Turnbull, 1961; Toia & Casida, 1979) (Figure 2). More recently, van der Drift et al. (1985) have given additional evidence for aging as a low

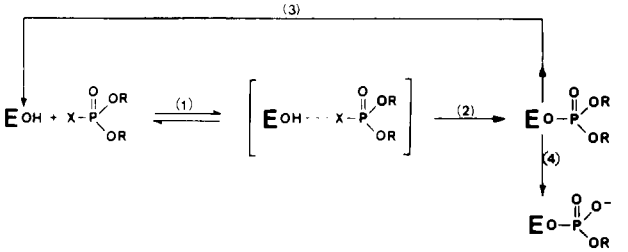


FIGURE 2: Mechanism of reaction of a serine proteinase of esterase with an organophosphorus inhibitor such as diisopropyl fluorophosphate (X = F, R = isopropyl): (1) Formation of the Michaelis complex; (2) covalent phosphorylation of the enzyme; (3) reactivation of the enzyme through hydrolysis of the P–$OSer_{195}$ bond; (4) aging of the triester complex (such as α-DIPCT) to a diester complex (such as α-MIPCT) with hydrolysis of the C–O bond.

pH, temperature-dependent phenomenon for several serine proteinases and esterases.

Because of its negative charge and its geometry, the MIP group may be regarded as an even better transition-state analogue than the DIP tetrahedral adduct as shown in Figure 1 (Stroud et al., 1974; Kossiakoff & Spencer, 1981). In fact, after further refinement of the original X-ray structure (Stroud et al., 1974) of the DFP-inhibited trypsin [R. M. Stroud, personal communication in Porubcan et al. (1979); Chambers & Stroud, 1979], it was concluded that the DIP-trypsin sample had aged to monoisopropylphosphoryltrypsin. The electron density for one of the isopropyl groups [Figure 12 of Stround et al. (1974)] is missing after refinement. As depicted in Figure 1, the phosphoryl oxygen is located in the oxyanion hole, which is believed to contribute to the catalytic activity of the native serine proteinases. Importantly, in the X-ray structure of the phosphorylated zymogen DIP-trypsinogen [R. M. Stroud, personal communication in Porubcan et al. (1979)], the phosphoryl oxygen is not located in the oxyanion hole and the imidazole of histidine-57 is rotated so as to no longer hydrogen bond to the active-site carboxylate of aspartate-102, the third component of the "catalytic triad" (Blow, 1976). Apparently, there are no hydrogen bonds to any of the phosphate oxygens in the phosphorylated zymogen.

Although the ^{31}P NMR signal observed by Markley and co-workers at ~2.0 ppm was clearly established as the aged, monoisopropylphosphoryl-α-chymotrypsin (α-MIPCT) species, we continued to observe two ^{31}P NMR signals, even when care was taken to avoid aging of the α-DIPCT sample. As described in this paper, we show that the time dependence of the ^{31}P NMR spectral changes in these complexes is quite complex and that both aging and a second process are occurring simultaneously. We describe evidence that this second process represents a reversible dimerization of the phosphorylated chymotrypsin.

Although inactive, the α-DIPCT has many similarities to native α-CT. Of particular relevance, is that, like α-CT, α-DIPT dimerizes as well. This dimerization process is facilitated at low pH and higher concentrations of enzyme (Gladner & Neurath, 1954; Egan et al., 1957; Gorbunoof et al. 1978; Chen, 1982; Horbett & Teller, 1973). The dimerization is functionally interesting because α-CT is a proteolytic enzyme whose biological role must involve formation of supramolecular complexes with other proteins. The quaternary interactions involved in dimerization may be quite similar to those interactions involved in the proteolysis of other molecules, specifically since the formation of α-CT itself involves limited autolysis. The chemical interactions responsible for dimerization appear to be the partly hydrophobic, partly ionic interaction between the aromatic ring and the carboxylate ion

of tyrosine-146 (the site of limited proteolysis) in each monomer with the protonated imidazole ring of histidine-57 of the other monomer (Horbett & Teller, 1973; Blevins & Tulinsky, 1985).

Materials and Methods

^{31}P (80-MHz) NMR spectra were obtained on an IBM WP 200 SY series Fourier transform spectrometer equipped with an Aspect 2000 computer. We usually employed a 67° pulse. The spectra were broad-band decoupled with a power of $^1/_2$ W. Acquisition was with 2K data points in the quadrature detection mode at 27 °C. Generally 5000–20 000 transients were acquired. Chemical shifts are in parts per million for ^{31}P NMR spectra and are referenced to 85% H_3PO_4. pHs were adjusted on a Radiometer Model PHM 64 research meter and in D_2O represent the pH meter reading without correction for the isotope effect. They were measured before and after each NMR run and were accepted only if there was less than 0.1 pH difference. Infrared spectra were obtained on a Perkin-Elmer 727B spectrometer. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected.

Chemicals were generally of the highest purity. Baker analyzed 60–200 mesh silica gel was used for column chromatography after being activated at 130° overnight. Pyridine was refluxed over and then distilled from calcium hydride.

α-Chymotrypsin (bovine pancreatic) was purchased from Sigma as a three times crystallized and lyophilized salt-free type II powder. Active-site titration of the enzyme followed the method of Schonbaum et al. (1961) and routinely yielded 80–90% active sites. Pronase type XIV (Pronase E) was purchased from Sigma.

α-DIPCT (bovine pancreatic) was purchased from Sigma. Sigma's preparation involves the following procedures: (i) the α-CT enzyme solution is prepared at 10 mg/mL. (ii) The diisopropyl fluorophosphate (DFP) solution is prepared in 2-propanol at 5 mM. (iii) The DFP solution is added dropwise to the α-CT solution at a 1:1 ratio. (iv) The deactivated enzyme is stirred for 2–4 h at 4 °C. (v) The solution is then dialyzed under cold running water for at least 16 h. (vi) Any precipitate is discarded, and the clear solution is lyophilized.

With this preparation, we usually received α-DIPCT that had a large proportion of the aged, monoisopropylphosphoryl-α-chymotrypsin (α-MIPCT). On the basis of our findings we recommended to Sigma that the dialysis period should be shortened to no more than 8 h and that the temperature during inactivation and workup should be kept close to 4 °C. After Sigma modified their preparation, we subsequently began receiving 100% nonaged α-DIPCT. Active-site titration of the inhibited enzyme preparations always showed 100% inactivation. Sephadex G-200 column chromatography (1.8 × 50 cm) of the Sigma α-DIPCT preparation (2 mg applied in 0.2 mL of buffer), eluted at 12 mL/h (pH 6.10, 0.01 M sodium phosphate, 0.005 M Tris), showed only a single sharp peak.

Dimer Preparation. Partially dimerized samples of α-DIPCT were prepared as follows. Usually 200 mg of redialyzed α-DIPCT was dissolved in 10 mL of 0.01 M sodium acetate containing 10 mM EDTA, 0.2 M NaCl, and 20–25% D_2O at pH 7.6 (uncorrected pH meter reading). The solution was stirred until all of the enzyme appeared to go into solution and then for a few additional minutes, the total time being about 20 min. The solution was then ultrafiltered at 4 °C through an Amicon UM10 membrane (M_r cutoff 10 000) at 80 psi of nitrogen over a period of ~2.5 h. Filtration proceeded until there was about 0.5–0.8 mL remaining in the filtration unit. This was collected and either adjusted to pH 3–4 and the NMR spectrum recorded or frozen without pH adjustment. Immediately after the pH was lowered, prior to running the NMR spectrum, the solution was stirred for a few minutes with Chelex 100 ion-exchange resin to remove any paramagnetic metal ion impurities and centrifuged for 2 min in an Eppendorf microcentrifuge. The clear supernatant was transferred to a 5-mm NMR tube. All glassware had been previously soaked in concentrated nitric acid and rigorously washed with deionized, distilled water.

O-Phenylphospho-α-chymotrypsin. A solution of diphenyl phosphorochloridate (Aldrich, 0.2 mL, 2% in 2-propanol) was added to 200 mg of three times recrystallized α-chymotrypsin in 200 mL of aqueous Tris (0.05 M) buffer, pH 8.0, with stirring at room temperature. After an hour of stirring, an additional 0.1 mL of 2% diphenyl phosphorochloridate solution was added and left for an additional 2 h with stirring at 25 °C. Essentially no enzymatic activity remained [activity was monitored by the method of Elmore and Smyth (1968)]. The contents were adjusted to pH 3 and dialyzed against 0.05 M EDTA at 4 °C for 8 h and then against 3.5 L of pH 3 (1 mM HCl) buffer for 48 h. During this time the buffer solution (1 mM HCl) was changed twice. The sample was lyophilized: yield 170 mg; $^{31}P\{^1H\}$ NMR, 0.01 M Tris, 0.25 M KCl, and 1 mM EDTA buffer, pH 7.2, D_2O external lock δ −2.1; $^{31}P\{^1H\}$ NMR under denaturing conditions, after 7 days at 4 °C, 8 M urea and buffer [Tris (0.01 M), KCl (0.25 M), and EDTA (1 mM)], pH 7.0, D_2O external lock δ −4.51.

N-Carbobenzoxy(O-phenylphospho)-L-serine methyl ester was prepared as follows: To a stirred mixture of 1.0 g (6.4 mmol) of L-serine methyl ester hydrochloride and 17.5 mL of saturated sodium bicarbonate solution was added 1.12 g (6.5 mmol) of carbobenzoxy chloride at 15 °C. After 2 h, the ester was extracted with ether, yielding 1.13 g (70%) of *N*-carbobenzoxy-L-serine methyl ester. This was recrystallized from chloroform–petroleum ether to yield fine needles: mp 38 °C; IR (KBr) 3360 (m), 2960 (m), 2380 (w), 1740 (s), 1710 (s), 1480 (m), 1440 (m), 1340 (m), 1220 (m), 1060 (s); NMR ($CDCl_3$) δ 2.5 (broad s, 1 H, OH), 3.75 (s, 3 H, $COCH_3$), 3.85–3.9 (d, 2 H, CH_2CH), 4.25–4.55 (m, 1 H, CH_2CH), 5.05 (s, 2 H, CH_2Ph), 5.6–6.15 (broad, 1 H, NH), 7.3 (s, 5 H, aromatic).

To a stirred solution of 1.1 g (5 mmol, 95% pure) of phenyl dichlorophosphate (Aldrich) in 5 mL of dry pyridine was added dropwise 0.09 mL (5 mmol) of double-distilled H_2O under an argon atmosphere over 10 min at 0 °C. The contents were stirred further at 0 °C for an additional 30 min, and then to the same contents was added dropwise a solution of *N*-carbobenzoxy-L-serine methyl ester (1.3 g, 5.1 mmol) in 6 mL of dry pyridine at 0–5 °C over 15 min under an argon atmosphere. The contents were stirred further at 0 °C for an additional 24 h, and then the mixture was diluted with 100 mL of chloroform and washed with approximately 200 mL of 1 M HCl and finally with water. The separated organic solvent was dried over sodium sulfate and evaporation of the solvent left a solid product. The crude product was purified on a silica gel column using chloroform–methanol (90:10) as eluent in about 65% yield. This was recrystallized from a chloroform–methanol–*n*-hexane mixture to yield thin needles: mp 203–205 °C; NMR ($CDCl_3$) δ 3.7 (s, 3 H, $COOCH_3$), 4.0 (s broad, OH), 4.2–4.6 (m, 3 H, CH_2CH), 5.2 (s, 2 H, CH_2 aromatic), 7.2–7.5 (m, 10 H, aromatic); $^{31}P\{^1H\}$ NMR (10% methanol, 0.01 M Tris, 0.25 M KCl, and 1 mM EDTA buffer, pH 7.5), D_2O external lock δ −4.9 ppm; $^{31}P\{^1H\}$ NMR under denaturing conditions, 8 M urea and buffer [Tris (0.01 M), KCl (0.25 M), and EDTA (1 mM)], pH 7.0, D_2O ex-

ternal lock δ −4.81 ppm. Calcd for $C_{18}H_{20}NO_8P\ H_2O$: C, 50.58; H, 5.15; N, 3.27; P, 7.25. Found: C, 50.45; H, 4.75; N, 3.23; P, 6.96.

N-Carbobenzoxy(di-O-phenylphospho)-L-serine Methyl Ester. To a stirred solution of *N*-carbobenzoxy-L-serine methyl ester (1.25 g, 4.9 mmol) in 6 mL of dry pyridine was added 1.27 g (4.7 mmol) of diphenyl phosphorochloridate in 6 mL of dry pyridine at 0 °C. After stirring for an additional $2^1/_2$ h, the mixture was diluted with 25 mL of chloroform and washed with dilute HCl and finally with water. The separated organic layer was dried over sodium sulfate, and evaporation of the solvent left a solid product. It was recrystallized from ether–petroleum ether (35–60 °C) in fine colorless needles: mp 50–51 °C; yield 1.72 gm (90%); ^{1}H NMR ($CDCl_3$) δ 3.7 (s, 3 H, $COOCH_3$), 4.45–4.75 (m, 3 H, CH_2CH), 5.15 (s, 2 H, CH_2 aromatic), 5.45–5.85 (broad, 1 H, NH), 7.2–7.4 (db, 15 H, aromatic); ^{31}P NMR ($CDCl_3$) δ −12.22; ^{31}P NMR (under denaturing conditions, 8 M urea in 20% D_2O, 1 mM EDTA, pH 7.0, δ −12.40 ppm.

Hydrolysis of N-Carbobenzoxy(di-O-phenylphospho)-L-serine Methyl Ester with 0.5 M NaOD. Approximately 3.0 mg of *N*-carbobenzoxy(di-*O*-phenylphospho)-L-serine methyl ester was dissolved in 0.2 mL of dioxane and diluted to 0.5 mL with 0.5 M NaOD and left for 7 days at room temperature: ^{31}P NMR of reaction mixture, δ −4.63 and −9.99 in the ratio 1:3.4.

NMR Spin–Lattice Relaxation Times. The samples for the spin–lattice relaxation time (T_1) measurements had nitrogen passed over them for several minutes while in a microcentrifuge tube. The T_1 relaxation time measurements were performed by the inversion recovery method (Harris, 1983). A 180°–τ–90° pulse sequence was employed with delay list cycling to allow for field drift and other perturbations. This tended to minimize the effect of aging over the course of an experiment. Data were analyzed on the Aspect 2000 computer interface to the spectrometer (IBM WP 200 SY) by a nonlinear least-squares fit to

$$\ln (A_\infty - A_\tau) = \ln 2\ A_\tau - \tau/T_1 \qquad (1)$$

where A_τ is the intensity of the peak after some delay, A_∞ is the intensity of the totally relaxed peak at thermal equilibrium, and τ is the variable delay time. We employed seven delay times ranging from 0.0001 to 4 s. An additional 10-s relaxation delay was introduced prior to each inversion recovery pulse sequence to ensure return of equilibrium magnetization.

Denaturation of the samples was effected by addition of urea to a concentration of 8 M. The sample was heated to 50 °C for 30 min. In some instances dithiothreitol (Sigma) was also added.

Results

Dimerization. At low α-DIPCT concentrations only one peak is observed in the ^{31}P NMR spectrum at ~0 ppm in freshly prepared samples (cf. Figure 3A). When the freshly prepared α-DIPCT sample is concentrated (by ultrafiltration) at low pH, an additional peak in the ^{31}P NMR spectrum appears at 1.97 ppm (Figure 3B). As described in the following, we do not believe this downfield peak can be solely ascribed to the aged α-MIPCT form of the phosphorylated enzyme (Porubcan et al., 1979; van der Drift et al., 1985), and we have assigned the downfield peak in the ultrafiltered, low pH, freshly prepared samples to a dimer of α-DIPCT. The upfield signal at ~0 ppm is acribed to the monomer α-DIPCT. Ultrafiltration and concentration of the NMR sample at low pH always resulted in an increase in the relative amount of the downfield (dimer) peak, although, as described below,

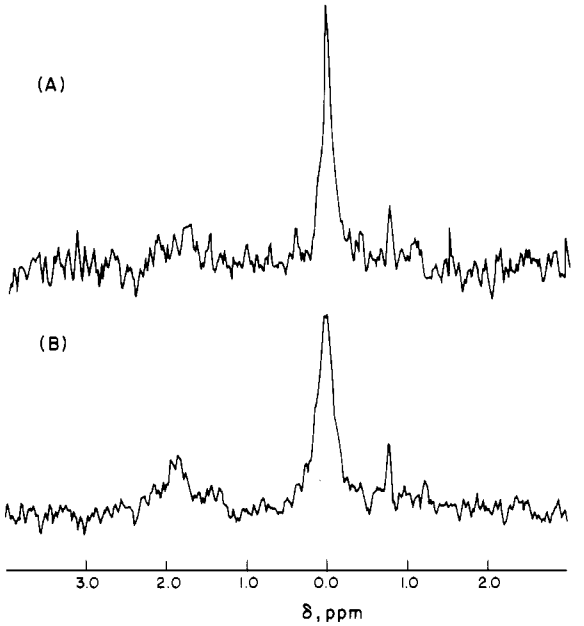


FIGURE 3: ^{31}P NMR spectra (80 MHz) of diisopropylphosphoryl-α-CT at pH 4.0 (uncorrected pH meter reading): (A) (α-DIPCT) = 0.77 mM, 0.01 M sodium acetate, 0.2 M NaCl, 10 mM EDTA, ambient temperature, showing only monomer; (B) (α-DIPCT) = 3.8 mM, showing both monomer and dimer (downfield) peaks.

simultaneous appearance of an additional peak, at times superimposable on the dimer peak, which we attribute to the aged α-MIPCT form of the enzyme, makes quantification of these changes difficult.

The pH dependence on the ratio of these two peaks supports the dimer/monomer assignments. Thus, as our laboratory had previously shown (Gorenstein & Findlay, 1976), at high pH in a partially aged sample, the proportion of the two ^{31}P signals, dimer plus aged peak (at 1.97 ppm) increases relative to the monomer peak (0 ppm) as the pH is lowered to 4.0. When the pH is raised again on the same sample, the signal at 0 ppm increases. However, this phenomenon is not completely reversible since over time the downfield peak also increases, even at 4 °C (Gorenstein & Findlay, 1976; Porubcan et al., 1979; van der Drift et al., 1985; Chen, 1982).

Aging. Partial reversibility of the spectral changes described above is attributed to the appearance of another species with time. Thus, at higher field, incubation at low pH of a sample that shows ^{31}P signals associated with both monomer and dimer α-DIPCT results in the time-dependent appearance of a third peak at 2.01 ppm. Markley and co-workers (Markley, 1979; Porubcan et al., 1979) and van der Drift et al. (1985) have shown that this new peak represents the aged α-MIPCT species. As shown in Figure 4 the two peaks in the vicinity of 2.0 ppm are much better resolved at a ^{31}P frequency of 80 MHz than at the lower field strength (32.4 MHz) of the spectra shown in Gorenstein and Findlay (1976).

The partial reversibility of the spectral changes of α-DIPCT is consistent with the simultaneous dimerization and aging of the α-DIPCT into the monoisopropylphosphoryl-α-CT (α-MIPCT) (Figure 2). As noted above, the aged α-MIPCT resonates at ~2.01 ppm, which is very close to the dimer peak (1.97 ppm; Figure 4). In many ^{31}P NMR spectra such as those of Gorenstein and Findlay (1976), the aged α-MIPCT peak lies under the dimer peak. The simultaneous aging of α-DIPCT into α-MIPCT and the similar ^{31}P chemical shifts of the aged and dimer signals very much complicates ^{31}P spectral analysis of the dimerization process. At lower field the downfield generally represents both aged and dimer species.

Table I: ^{31}P Chemical Shifts of Various Phosphorylated Derivatives of α-CT and Related Model Compounds

structure	^{31}P chemical shifts (ppm) monomer enzyme complex[a] or model compound[a]	aged enzyme complex[a]	dimer enzyme complex[a]
α-DIPCT (native)	−0.6 (0.0)[b]	+2.01	+1.97
α-DIPCT (denatured)[c]	−3.2 (0.0)[b]	−0.6	-
Gly-Glu-Ser(DIP)-Gly-OEt[c]	−3.2[d]		
α-MPPCT (native)		−2.1 (−1.3)[e]	-
α-MPPCT (denatured)[c]		−4.5	-
$HOP(O_2^-)OCH_2C(H)(CO_2)NH_2$[a,f] (*O*-phospho-L-serine)	+3.16		
$PhOP(O_2^-)OCH_2C(H)(CO_2Me)NHCBz$[c,f]	−4.81		
$(PhO)_2P(O)OCH_2C(H)(CO_2Me)NHCBz$[c,f]	−12.4		

[a] pH 7–8 (uncorrected pH meter reading), D_2O unless otherwise specified, internal lock. [b] pH 4. [c] 8 M urea, pH 7, 0.01 M Tris, 0.25 M KCl, 1 mM EDTA. [d] van der Drift et al. (1985). [e] Toia and Casida (1979), pH 7.6. [f] Locked on an internal capillary of D_2O.

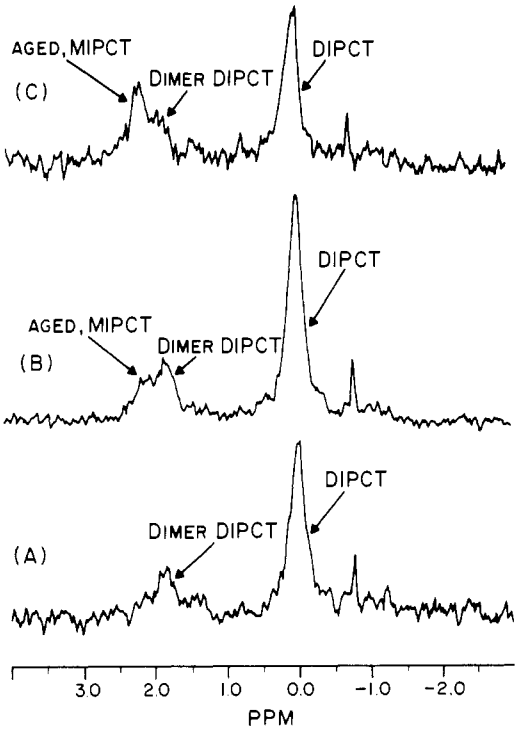


FIGURE 4: ^{31}P NMR spectra (80 MHz) of diisopropylphosphoryl-α-chymotrypsin (α-DIPCT), 3.8 mM, pH 4.0: (A) Freshly prepared, unaged sample; (B) 48-h incubation at 4 °C; (C) 2-week incubation, showing both monomer (DIPCT) aged (MIPCT) and dimer (DIPDT).

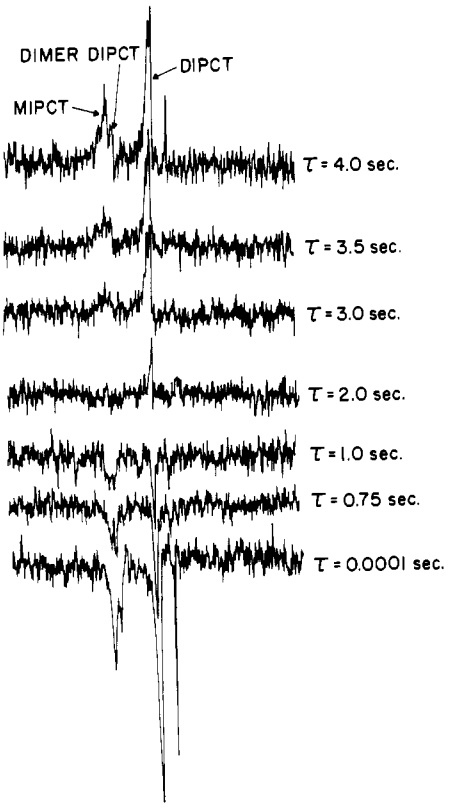


FIGURE 5: Inversion recovery ^{31}P NMR spectra (80 MHz) of α-DIPCT (3.8 mM) in 20% D_2O, pH 4.5. Indicated τ value is the delay between the 180° and 90° pulses. An additional relaxation delay of 10 s was included in the pulse sequence.

This explains the inability to observe complete reversibility of the ratio of the ^{31}P signals upon pH titration. The NMR samples initially prepared from Sigma's α-DIPCT nearly always resulted in our obtaining a partially aged sample. If care is taken to prepare an α-DIPCT sample to prevent aging (see Materials and Methods), only one ^{31}P peak is observed at 0 ppm when the sample is freshly dissolved.

Relaxation Times. Additional support for the dimer assignment was provided by ^{31}P NMR spin–lattice relaxation time measurements on a sample in which it was possible to resolve in the spectrum all three species: α-DIPCT dimer, aged α-MIPCT, and monomer α-DIPCT. The series of inversion recovery ^{31}P NMR spectra used to measure the T_1 spin–lattice relaxation time are shown in Figure 5. The T_1 relaxation time of 2.65 ± 0.10 s for the dimer peak at 1.97 ppm was found to be longer than that of the native α-DIPCT peak (T_1 = 2.06 ± 0.14 s; sample pH 4.5). The T_1 for the α-MIPCT peak at 2.01 ppm relaxation time is 2.35 ± 0.21 s. The longer T_1 for the dimer may be justified for a molecule that is twice the molecular weight of the normal α-DIPCT. Thus, assuming that the line widths at half-height ($\Delta\nu_{1/2} \sim 15$ Hz) of the peaks represent intrinsic spin–spin relaxation rates, $1/T_2$ ($=\pi\Delta\nu_{1/2}$), then the T_1's of all of the species are much greater than the T_2's (~ 0.02 s). For the T_1's to be greater than the T_2's the overall rotational correlation time of the enzymes must be longer than the inverse of the Larmor frequency. In this motional limit, the T_1 of the dimer should be longer than the T_1 of the monomer because the dimer should have a longer overall rotational correlation time than the monomer α-DIPCT. Finally, the T_1's for both α-DIPCT and α-MIPCT peaks are comparable, as expected for monomeric forms of the enzyme.

Denaturation. Denaturation in 8 M urea or 12 M guanidinium hydrochloride of the unaged α-DIPCT yields a ^{31}P NMR spectrum with only one ^{31}P signal at $\sim$−3.2 ppm (van der Drift et al., 1985; see Table I). However, if a partially aged α-DIPCT sample is denatured, two ^{31}P peaks are observed, a downfield peak at $\sim$−0.5 ppm (denatured α-MIPCT) and another peak at $\sim$−3.2 ppm (denatured α-DIPCT; van

der Drift et al., 1985). We never observe a third signal in denatured samples originally containing dimer as well as aged phosphorylated enzyme. The denaturation experiment shows that the two ^{31}P signals arise from two chemically distinct species. The downfield peak at −0.5 ppm has been assigned (van der Drift et al., 1985) to the aged α-MIPCT and the upfield peak at −3.2 ppm to the nonaged α-DIPCT (presumably derived from both monomer and dimer).

pH Dependence. The pH dependence of the ^{31}P chemical shift of α-DIPCT follows a simple titration curve with p$K \sim$ 7.0, confirming previous results (Markley, 1979; Porubcan et al., 1979; van der Drift et al., 1985) (plot not shown). Titration of α-DIPCT from high pH (>8) to low pH (<4) shifts the ^{31}P signal from −0.6 to 0.0 ppm (Table I). In contrast, both aged α-MIPCT and dimer α-DIPCT show little ^{31}P chemical shift change between pH 3 and 8.5. A slight titration of the ^{31}P signals can just be resolved with an apparent inflection occurring between pH 4 and 5 (spectra not shown; Chen, 1982).

Monophenylphosphoryl-α-chymotrypsin (α-MPPCT). Phosphorylation of α-CT with diphenyl chlorophosphate yields a monophenylphosphoryl-α-chymotrypsin (α-MPPCT) that shows a single ^{31}P signal at −2.1 ppm (pH 7). Assignment of this ^{31}P signal to the *aged* diester species is provided by comparison of the ^{31}P chemical shift with that of various model compounds under denaturing conditions, shown in Table I. Denaturation shifts the ^{31}P signal of α-MPPCT from −2.1 to −4.5 ppm. Clearly, the only model compound with similar ^{31}P chemical shift is that of the monophenylphosphorylserine derivative (Table I). As is expected for a much more reactive aryl phosphate triester, no evidence is found for the initially formed diphenylphosphoryl-α-chymotrypsin, which must rapidly hydrolyze to the α-MPPCT (Lee & Turnbull, 1961; Toia & Casida, 1979). In contrast to the lability and dimerization of the α-DIPCT, the α-MPPCT shows no additional ^{31}P NMR signals.

Discussion

Dimerization vs Aging. Gorenstein and Findlay (1976) had noted that the ^{31}P NMR spectra of α-DIPCT showed two signals at ~0 and 2 ppm. The ratio of the two peaks varied with pH, and various suggestions, including a dimer/monomer equilibration, for the origin of these two peaks were offered. Markley and co-workers (Markley, 1979; Porubcan et al., 1979) and later van der Drift et al. (1985) questioned the origin of the downfield peak at 2 ppm and showed that freshly prepared samples of α-DIPCT showed only one signal and that a new downfield signal appeared in a time-dependent fashion. This new ^{31}P NMR signal observed by Markley and coworkers and van der Drift et al. at ~2.0 ppm was clearly established as an aged monoisopropylphosphorylated enzyme species (Figure 2).

The results of Markley and co-workers (Markley, 1979; Porubcan et al., 1979) and later van der Drift et al. (1985) were inconsistent with Gorenstein and Findlay's (1976) results, who showed that the pH dependence on the ratio of the two ^{31}P signals was at least partially reversible. However, aging of the α-DIPCT must be an irreversible process. Furthermore, under certain conditions our laboratory (Chen, 1982; Kallick, 1986) continued to observe two ^{31}P NMR signals, even when care was taken to avoid aging of the α-DIPCT sample. The time, concentration, and pH dependence of the ^{31}P NMR spectral changes are quite complex and cannot be entirely explained by aging of the sample alone. Thus, under favorable conditions, a different downfield signal from that of the aged sample, also at ~2.0 ppm, can be resolved. Van der Drift et al. (1985) also mentioned in a footnote the appearance of a new peak near the α-MIPCT peak when the sample was allowed to incubate for a period of time at pH 5 and 4 °C. Toia and Casida (1979) also report additional ^{31}P NMR signals upon incubation of various phosphorylated saligenin derivatives of α-CT. As shown in Figure 4, a third signal separate from the aged signal can be reproducibly observed. The additional signal in Figure 4 has been assigned to the *dimer* of α-DIPCT.

Both α-CT and α-DIPCT have been shown to dimerize at lower pH (Gladner & Neurath, 1954; Horbett & Teller, 1973). The chemical interactions responsible for dimerization appear to be the partly hydrophobic, partly ionic interaction between the aromatic ring and the carboxylate ion of tyrosine-146 (the C terminal) in each monomer with the imidazolium ion of histidine-57 of the other monomer (Vandlen & Tulinsky, 1973; Horbett & Teller, 1973; Sigler et al., 1968). The dimerization is abolished by removing tyrosine-146 by limited carboxypeptidase A treatment (Gladner & Neurath, 1954; Horbett & Teller, 1973). Chymotrypsinogen and δ-chymotrypsin, which both lack a C-terminal tyrosine-146, do not dimerize (Miller et al., 1971). Tyrosine analogues such as *N*-acetyl-L-tyrosine bind to chymotrypsin at low pH and are competitive inhibitors of the dimerization of α-chymotrypsin, giving evidence that the tyrosine-146 could bind in the active site at low pH (Horbett & Teller 1973).

Horbett and Teller (1973) also showed by sedimentation equilibrium measurements that a number of acylated and phosphorylated α-CT derivatives dimerize, including α-DIPCT. The dimerization constant at pH 4.4, 0.2 M KCl, 0.01 M acetate, 20 °C, is 1.7×10^4 and 3.9×10^3 M^{-1} for α-CT and α-DIPCT, respectively. Thus, in the concentration range that we typically run the ^{31}P NMR spectra (1–4 mM) significant amounts of the α-DIPCT will be associated and the observed percentage of dimer (~20%) at 30 °C is in qualitative agreement with the measured dimerization constant. Actually, the downfield dimer signal may represent only one of the two phosphate esters in the dimer. The dimer is asymmetric (Vandlen & Tulinsky, 1973; Blevins & Tulinsky, 1985), and both dimer signals need not have the same chemical shift. Indeed, one of the dimer phosphates could give rise to a signal that could actually be superimposed under the normal monomer ^{31}P signal. However, we found no evidence supporting this alternative assignment.

The crystal structure of the native α-CT dimer at pH 4.2 (Vandlen & Tulinsky, 1973; Blevins & Tulinsky, 1985) explains how it is possible for both native α-CT and α-DIPCT to dimerize. In the native α-chymotrypsin dimer a bridging sulfate anion is located between O_γ of Ser_{195} and the hydroxyl group of Tyr_{146} located on the second protein molecule. Clearly, a tetrahedral phosphoryl group can replace the tetrahedral sulfate ion. The X-ray structure of tosyl-α-chymotrypsin dimer shows similar interactions (Matthews et al., 1967; Birktoft & Blow, 1972). However, large bulky groups (such as the isopropyl group of DIP or the tosyl group) significantly interfere with dimerization, and it is believed that rotation of these groups is required to allow proper access of the Tyr_{146} to the active site (Horbett & Teller, 1973).

Aging. Van der Drift et al. (1985) have given conclusive evidence for aging as a low pH, temperature-dependent phenomenon for several serine proteases. Previous difficulty in separately observing the dimer and aged species is attributable to the near superposition of their ^{31}P signals: the aged monoisopropylphosphorylchymotrypsin resonates at 2.01 ppm (Markley, 1979; Porubcan et al., 1979; van der Drift et al., 1985; Figure 4), which places the α-MIPCT peak quite close

to the dimer peak (1.97 ppm; Figure 4). At lower field (32 MHz) it is not possible to resolve dimer from aged α-MIPCT.

Denaturation. Denaturation in 8 M urea of the unaged α-DIPCT yields a ^{31}P NMR spectrum with only one ^{31}P signal at ~−3.2 ppm (Chen, 1982; Kallick, 1986; van der Drift et al., 1985) (Table I). In contrast, if a largely aged α-DIPCT sample is denatured, two ^{31}P peaks are observed at ~−0.6 and ~−3.2 ppm (Chen, 1982; Kallick, 1986; van der Drift et al., 1985). Van der Drift et al. (1985) have also shown that the model compound Gly-Glu-Ser(DIP)-Gly–OEt has a ^{31}P signal at −3.2 ppm under the same denaturing conditions in 8 M urea. The downfield peak at −0.5 ppm is thus assigned to the aged α-MIPCT and the upfield peak at −3.2 ppm to the nonaged α-DIPCT (Chen, 1982; Kallick, 1986).

Significantly, as shown in Table I of van der Drift et al. (1985), there is a difference of as much as 7.3 ppm between the ^{31}P chemical shift of various DIP phosphorylated enzymes under native conditions (at low pH the ^{31}P chemical shift of α-DIPCT, DIP-subtilisin, DIP-atropinesterase, and DIP-chymotrypsinogen is 0.0, 3.8, −3.5, and −1.8 ppm, respectively). In 8 M urea, pH 7.5, the ^{31}P chemical shift of all of these DIP-phosphorylated enzymes is the same as the model compound, ca. −3.2 ppm. This provides strong evidence that the differences in the native ^{31}P chemical shifts result from interactions of the phosphorus with the enzyme environment and not from differences in substitution in these four enzymes.

Similarly, while the aged MIP-serine proteases and esterases (van der Drift et al., 1985; Chen, 1982; Kallick, 1986) show ^{31}P signals at quite different ^{31}P chemical shifts (with a spread of 3.9 ppm; van der Drift et al., 1985), upon denaturation of the aged species all have the same chemical shift, −0.6 ppm. Importantly, the ^{31}P signal of the aged α-MIPCT, the chemically quite unrelated α-MPPCT *and* α-DIPCT all shift 2.4–3.0 ppm upfield upon denaturation (Table I). The ^{31}P signal of DIP-chymotrypsinogen also shifts 0.6–1.8 ppm upfield (depending upon pH) as well (van der Drift et al., 1985). As discussed below, whatever factors are responsible for these ^{31}P chemical shift differences, they are not greatly affected by the chemical nature of the phosphate ester (aryl and alkyl esters or monoanionic diesters and neutral triesters).

pH Dependence. The pH dependence of the ^{31}P chemical shifts of the DIP-serine enzymes has been examined (Chen, 1982; Kallick, 1986; Markley, 1979; Porubcan et al., 1979; van der Drift et al., 1985). At pH's >4 the ^{31}P signals of the DIP-serine proteinases/esterases all shift upfield with increasing pH. The data fit a simple titration curve of a group with pK between 7.0 and 7.6. Markley and co-workers have attributed this upfield shift to H-bonding from histidine-57 to one of the phosphoryl oxygens (Markley, 1979; Porubcan et al., 1979) and have associated the observed pK to titration of the active-site histidine. The X-ray results of Stroud and co-workers [Stroud, personal communication in Porubcan et al. (1979)] indicate that there are three hydrogen bonds to the phosphate group (the backbone NH's of glycine-193 and serine-195 and the N_2^{ϵ}–H of histidine-57). As pointed out by Reeck et al. and Markley et al., consistent with this interpretation is the 1.7–2 ppm upfield shift of the ^{31}P resonance of the DIP zymogens relative to the DIP enzymes (Porubcan et al., 1979; Reeck et al., 1977). The X-ray structure of the DIP zymogen shows no hydrogen bonds to the phosphate [Stroud, personal communication in Porubcan et al. (1979)], possibly explaining the upfield shift for the zymogens.

However, hydrogen-bonding effects are generally small (Gorenstein, 1984; Gorenstein et al., 1976; Gorenstein & Post, 1989). Thus, the ^{31}P signal of a 3′-CMP·RNase A complex is shifted *upfield* less than 0.3 ppm from the free 3′-CMP around neutral pH (Gorenstein et al., 1976). In fact, protonation of an active-site histidine and hydrogen bonding to the dianionic mononucleotide phosphates bound to RNase A produces an *upfield* shift of ~2 ppm. Van der Drift et al. (1985) studied the effect of various acids in organic solvents on the ^{31}P chemical shift of model diisopropylphosphorylserine peptides. Thus, phenol in ethyl acetate or formamide can hydrogen-bond to the phosphoryl oxygen (Joesten & Schaad, 1974), and van der Drift et al. (1985) observed that these H-bonding donors produced ca. 0.3–2.5 ppm (1.0–5 M phenol) *upfield* shifts. This is similar to the effect on the ^{31}P chemical shifts resulting from the protonation of trialkyl phosphates (Olah & McFarland, 1971). These RNase A and model compound ^{31}P chemical shift perturbations are thus in the *opposite* direction to those observed for hydrogen bonding to the neutral phosphate triester enzyme complex. It is likely that the change in chemical shift associated with ionization of the active-site histidine is not directly attributable to H-bonding, but to some other factor(s) as well (Gorenstein & Post, 1989), such as a change in phosphate ester O–P–O bond or torsional angles (Gorenstein, 1981, 1984; Gorenstein & Kar, 1975, 1977), solvation (Lerner & Kearns, 1980), or dielectric environment (Costello et al., 1976).

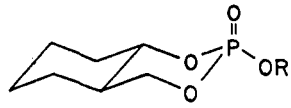
It is quite unusual that, unlike the neutral DIP enzymes, the monoanionic aged MIP enzymes and the monophenylphosphoryl-α-chymotrypsin show little ^{31}P chemical shift dependence with pH (van der Drift et al., 1985), although there appears to be a slight titration of a group, possibly Asp_{102} with pK 4–5 (Chen, 1982). Either the active-site histidine is not titrating between pH 3 and 8.5 or the ^{31}P signal of the neutral triesters is sensitive to the ionization state of the H-bonding active-site histidine, while the monoanionic diesters are not. It is quite possible that the pK of His_{57} is raised from ~7 in the free enzyme and neutral phosphorylated derivatives of the enzyme (Markley, 1979) to >9 in the monoanionic transition-state analogue α-MIPCT and α-MPPCT complexes. Robillard and Shulman (1974) reported that the N^{π} proton signal of His_{57} in a boronic acid transition-state analogue complex of α-CT did not titrate over the pH range 6–9.5. They suggested that the proximity of the negatively charged tetrahedral boronate transition-state analogue raised the pK of the active-site histidine to over 9.5.

Alternatively, protonation of the active-site histidine and H-bonding to the phosphoryl group is not directly responsible for the ^{31}P chemical shift titration (or absence of titration). Thus, in contrast to the effect at high pH, the DIP zymogens show a second titration at low pH, with the ^{31}P signal shifting *upfield* with decreasing pH (Porubcan et al., 1979). Finally, the ~2 ppm upfield shift of the α-DIPCTgen relative to α-DIPCT (Porubcan et al., 1979; Reeck et al., 1977; van der Drift et al., 1985) is also inconsistent with the H-bonding explanation for these shift differences. A major distinction between the zymogen and the active enzyme is the lack of a well-formed substrate specificity binding pocket and the poorly formed oxyanion hole in the zymogen (Freer et al., 1970; Stroud et al., 1974; Kossiakoff et al., 1977). The NH's in the oxyanion hole should thus more favorably H-bond to the phosphoryl oxygen in the native α-DIPCT, which again should produce shifts in the opposite direction than observed.

Conformational Effects on ^{31}P Chemical Shifts. These anomalous titration results can, however, be attributed to a pH-dependent conformational change about one or more of the P–O ester bonds. On the basis of semiempirical molecular orbital chemical shift calculations, Gorenstein and Kar (1975)

had suggested that one factor that could influence ^{31}P chemical shifts is the P–O ester torsional angles ω,ω', which we defined as a stereoelectronic ^{31}P chemical shift effect (Gorenstein, 1984). [The two torsional ω and ω' are defined by the R–O–P–O(R′) dihedral angles.] These chemical shift calculations indicated that a phosphate diester in a gauche, gauche (g,g) conformation should have a ^{31}P chemical shift several parts per million upfield from a phosphate diester in more extended conformations, such as gauche, trans (g,t) or trans, trans (t,t) conformations. Pullman and co-workers (Giessner-Pettre et al., 1984; Prado et al., 1979) have also supported a torsional-angle sensitivity to the ^{31}P chemical shifts with generally more accurate ab initio, gauge-invariant-type, molecular orbital, chemical shift calculations.

Support for the suggestion that conformational changes are at least partially responsible for the chemical shift differences between the various DIP enzymes as well as the histidine titration results comes frm additional studies on the conformationally locked phosphorylated derivative of α-CT (2-oxo-*trans*-5,6-tetramethylene-1,3,2-dioxaphosphorinane-α-chymotrypsin, **1** (Shah et al., 1983). The ^{31}P signal of the phosphorylated enzyme and the model compound 2-*N*-carbobenzoxy-L-serine methyl ester)-2-oxo-*trans*-5,6-tetramethylene-1,3,2-dioxaphosphorinane, **2** are also independent of pH. The active-site histidine in the cyclic phosphorylated



1, R = CH_2 $CH(CO_2CH_3)$ NHCBZ

2, R = Ser_{195} – αCT

chymotrypsin must be capable of being protonated in this pH range as found in the acyclic DIP derivatives, yet there is no effect on the ^{31}P chemical shift. Of course, torsion about the hydrolyzed P–O ester bond in the monoester aged enzymes is not possible and could explain the insensitivity of the ^{31}P signal to changes in pH. Similarly, the cyclic phosphorylated chymotrypsin derivative cannot undergo any conformational change about the ring ester bonds. It is possible, therefore, that while the pH dependence of the ^{31}P chemical shift of the DIP-serine proteinases and esterases is correlated to the protonation state of the histidine-57, the effect is attributable to pH-dependent conformational changes about P–O ester bonds. As revealed in the X-ray structure of MIP-trypsin (Stroud et al., 1974), one of the isopropyl groups is partially buried in the entrance to the specificity binding pocket. If the conformation about this RO–P bond is trans, it is possible to explain the 0.6–3.0 ppm upfield shift of the ^{31}P signal of the native aged phosphorylated derivatives (the alkyl ester α-MIPCT and the aryl ester α-MPPCT), the native triester α-DIPCT, and the native triester α-DIP-chymotrypsinogen upon denaturation. The lowest energy conformation for a phosphate ester is gauche (Gorenstein, 1987), which as described above in the stereoelectronic theory for ^{31}P chemical shifts is both predicted and experimentally confirmed (Gorenstein, 1984) to have a ^{31}P chemical shift several ppm upfield of a phosphate ester in a trans conformation. The conformational effect on the perturbation in ^{31}P chemical shifts is additive, so that a phosphate ester in which two of the ester bonds are in a trans conformation is expected (and observed; Gorenstein, 1984) to be 4–5 ppm downfield of an ester in which both bonds are in gauche conformations. We would thus interpret all or part of the over 7.0 ppm difference in ^{31}P chemical shifts between the various phosphorylated DIP serine enzymes and zymogens as arising from this stereoelectronic effect. It is quite likely, however, that bond angle, H-bonding effects, and other possible factors also contribute to these substantial differences.

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Multinuclear NMR Studies of the Divalent Metal Binding Site of NADP-Dependent Isocitrate Dehydrogenase from Pig Heart†

Robert S. Ehrlich and Roberta F. Colman*
Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716
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ABSTRACT: The metal activator site of NADP-dependent isocitrate dehydrogenase from pig heart has been probed by using ^{113}Cd and ^{25}Mg NMR as well as manganese paramagnetic relaxation of nuclei in the fast-exchanging ligands α-ketoglutarate and adenosine 2′-monophosphate. Cadmium NMR shows that cadmium, bound to the enzyme in the presence of isocitrate, has a resonance at 9 ppm relative to cadmium perchlorate, while the free Cd–isocitrate complex has a resonance at −23 ppm. Comparison with model compounds and previously studied proteins indicates that cadmium is coordinated with six oxygen ligands. Measurements as a function of cadmium concentration give a dissociation constant of 66 μM and a dissociation rate constant of 1.5×10^4 s^{-1} at pH 7.0. ^{25}Mg NMR demonstrates that the line width of the magnesium resonance is increased upon binding to isocitrate dehydrogenase. A further increase in line width is observed upon addition of isocitrate. Measurement of line widths as a function of temperature reveals that in the binary complex between magnesium and enzyme, exchange is the major contributor to broadening while in the ternary complex containing isocitrate, the intrinsic relaxation in the bound state is also important, suggesting an increase in the dissociation rate constant for magnesium from the ternary complex. Paramagnetic relaxation studies of nuclei of α-ketoglutarate, bicarbonate, and adenosine 2′-monophosphate locate the divalent metal within the active site. The results with adenosine 2′-monophosphate show that atoms in the adenosine moiety of the coenzyme are at least 8 Å from the metal site. In the enzyme complex with α-ketoglutarate, Mn is more than 7 Å from the 1-, 2-, and 5-carbons, but Mn is only 5.0 Å from the carbon of the inhibitor, bicarbonate. A postulated role for Mn is to activate the β-carboxyl of isocitrate that is removed in the enzymatic decarboxylation reaction.

The oxidative decarboxylation of isocitrate to form α-ketoglutarate catalyzed by NADP-dependent isocitrate dehydrogenase from pig heart [*threo*-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] requires a divalent metal (Villafranca & Colman, 1972; Colman, 1983). This requirement may be satisfied by several divalent metals including manganese, magnesium, or cadmium (Colman, 1972a). The binding of manganese has been extensively

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