Stereochemistry of the Phosphorylation Reaction of α-Chymotrypsin by a Cyclic Phosphate Triester

Dinesh O. Shah, Deborah Kallick, Robert Rowell, Rouhlwai Chen, and David G. Gorenstein*

Contribution from the Department of Chemistry, University of Illinois at Chicago, Chicago, Illinois 60680. *Received January 24, 1983*

Abstract: The reaction of α-chymotrypsin with the axial triester 2-(2,4-dinitrophenoxy)-2-oxo-*trans*-5,6-tetramethylene-1,3,2-dioxaphosphorinane (**1a**) yields a stable serine ester enzyme complex. The ^{31}P NMR spectrum of the native and denatured triester complex shows a single peak at ca. −4.7 ppm. ^{31}P NMR of synthetic model compounds 2-(*N*-carbobenzoxy-L-serine methyl ester)-2-oxo-*trans*-5,6-tetramethylene-1,3,2-dioxaphosphorinanes (**3a,b**) shows two peaks at −5.8 and −4.7 ppm under the same conditions corresponding to the axial epimer (**3a**) and equatorial epimer (**3b**), respectively. This suggests that the enzymatic reaction at phosphorus in compound **1a** proceeds with 100% inversion of configuration.

The enzymatic stereochemistry of reaction at phosphorus has been of considerable current interest, and so far in all enzymatic phosphoryl transfer reactions inversion of configuration at phosphorus in single-displacement steps has been observed.[1-9] Observation of overall retention of configuration in an enzymatic reaction has also been observed. However, this has been interpreted as evidence for formation of a transient covalent phosphorylated enzyme intermediate, with each phosphoryl transfer step (phosphorylation of the enzyme followed by dephosphorylation) proceeding with inversion of configuration at phosphorus. The double-displacement enzymatic reaction thus gives a product of net retention of configuration. Overall retention stereochemistry could equally well result if each of the two steps proceeded with retention of configuration. In none of the double-displacement enzymatic reactions has it been proven, however, that the individual phosphoryl transfer steps proceed with retention or inversion of configuration. In this paper we provide the first direct proof that at least the first step involving phosphorylation of an enzyme (although one not involving a normal substrate) does indeed proceed with inversion of configuration.

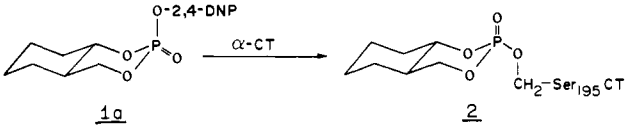
Phosphorylated derivatives of α-chymotrypsin have provided important structural and mechanistic information on this enzyme.[10] Thus, the diisopropylphosphoryl-α-chymotrypsin species, in which serine-195 is covalently phosphorylated, has contributed to our understanding of the important nucleophilic role played by the γ-oxygen of serine-195 in the enzymatic mechanism of hydrolysis of acyl substrates.[11] Indeed, the tetrahedral phosphorylated enzyme may be considered a "transition-state analogue" of the tetrahedral carbonyl addition intermediate/transition state.[12,13]

Experimental Section

General Procedures. ^{31}P NMR spectra were recorded on a Bruker WP-80 spectrometer at 32.4 MHz (^{31}P) and ^{1}H NMR on a 60-MHz Varian T-60 spectrometer. Chemical shifts in parts per million for ^{1}H NMR spectra are referenced to external Me_4Si and for ^{31}P NMR spectra are referenced to 85% H_3PO_4. Mass spectra were taken on an AEI MS-30 spectrometer. Infrared spectra were obtained on a Perkin-Elmer 727 B 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 °C overnight. Pyridine was refluxed over and then distilled from calcium hydride. Diethyl ether was refluxed over and distilled from sodium metal. α-Chymotrypsin (Bovine pancreatic) was purchased from Sigma Chemical Co. as a 3 times crystallized and lyophilized salt-free type II powder. Active site titration of the enzyme followed the method of Schonbaum et al.[18] and routinely yielded 80–85% active sites. Protease type XIV (Pronase E) was purchased from Sigma Chemical Co.

The axial and equatorial epimers of 2-(2,4-dinitrophenoxy)-2-oxo-*trans*-5,6-tetramethylene-1,3,2-dioxaphosphorinane [2-(2,4-dinitrophenoxy)-1,3-dioxa-2-phospha-*trans*-decalin-2-one] (**1a,b**) were prepared as described by Gorenstein et al.[14]



1a 2

1a,3a 1b,3b

1: R = 2,4-DNP

3: R = $CH_2CH(CO_2CH_3)NHCBZ$

2-Oxo-*trans*-5,6-tetramethylene-1,3,2-dioxaphosphorinane-α-chymotrypsin Complex, 2. The axial phosphorinane epimer, **1a** (7.5 mg), was added slowly to 100 mg of 3 times recrystallized α-chymotrypsin in 20 mL of 11% acetonitrile, aqueous Tris (0.5 M) buffer, pH 7.8, with stirring at room temperature (some preparations used 10% dioxane as cosolvent instead of the acetonitrile). After overnight stirring, an additional 7.5 mg of **1a** was added and left for an additional 6 h. The sample was adjusted to pH 3 and centrifuged, and the supernatant was

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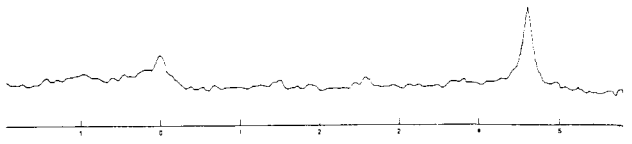

Figure 1. $^{31}P\{^1H\}$ NMR of denatured enzyme complex **2** in 8 M urea, 20% D_2O, pH 7.0, 32.4 MHz.

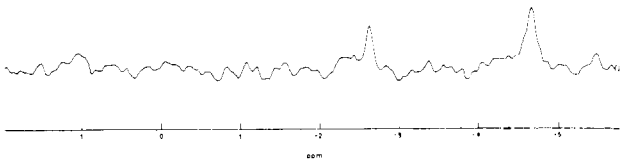

Figure 2. $^{31}P\{^1H\}$ NMR of proteolytically cleaved enzyme complex **2** in 20% D_2O, 1 mM EDTA, pH 7.0, 32.4 MHz.

dialyzed overnight at 4 °C against 1 L of pH 3 (1 mM HCl) buffer. The sample was lyophilized. Essentially no enzymatic activity (<0.3%) remained and the extent of phosphorylation was complete (1 mol of phosphorus per mol of enzyme).

2-(*N*-Carbobenzoxy-L-serine methyl ester)-2-oxo-*trans*-5,6-tetramethylene-1,3,2-dioxaphosphorinane (3a,b) 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 (**4**). 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), 1460 (m), 1440 (m), 1340 (m), 1220 (m), 1060 (s) cm^{-1}; NMR ($CDCl_3$) δ 2.5 (br s, 1 H, OH), 3.75 (s, 3 H, $COOCH_3$), 3.85–3.9 (d, 2H, -CH_2CH-), 4.25–4.55 (m, 1 H, -CH_2CH-), 5.05 (s, 2 H, -CH_2Ph), 5.6–6.15 (br, 1 H, -NH-), 7.3 (s, 5 H, aromatic).

2-Chloro-2-oxo-5,6-tetramethylene-1,3,2-dioxaphosphorinane (5) was prepared from *trans*-2-(hydroxymethyl)-1-cyclohexanol and phosphorus oxychloride in dry ether in the presence of dry pyridine according to the procedure of Gorenstein et al.[14]

A solution of 1.053 g (5 mmol) of phosphorochloridate **5** in 8 mL of dry pyridine was added dropwise to a solution of *N*-carbobenzoxy-L-serine methyl ester (1.3 g, 5.1 mmol) in 5 mL of dry pyridine at 0 °C over 10 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 50 mL of chloroform and washed with dilute HCl and finally with water. The separated organic solvent was dried over sodium sulfate, and evaporation of the solvent left a gummy product. ^{31}P NMR of crude product in $CDCl_3$ shows peaks at −4.7 and −7.4 ppm in the ratio of 1:7.

The crude gummy product was separated on a silica gel column using ethyl acetate/chloroform (1:1) as eluent with a *N,O*-dicarbobenzoxy-L-serine methyl ester eluting first, followed by the axial isomer (**3a**) and then the equatorial isomer (**3b**). The product **3a** was obtained in about 55% yield: mp 122–124 °C; IR (KBr) 3540 (m), 3260 (m), 3040 (w), 2940 (s), 2870 (m), 1755 (s), 1715 (s), 1535 (s), 1450 (m), 1350 (s), 1273 (s), 1255 (s), 1210 (s), 1090 (s), 1060 (s), 1020 (s), 980 (s) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.8–2.2 (m, 9 H, ring), 3.85 (s, 3 H, $COOCH_3$), 3.82–4.8 [m, 6 H, (3 H for H-1,2,4; 2 H for -CH_2CH-; 1 H for -CH_2CH-)], 5.25 (s, 2 H, -CH_2Ph), 5.7–6.0 (m, 1 H, -NH-), 7.3 (s, 5 H, aromatic); MS, molecular ion at *m/e* 427; ^{31}P NMR ($CDCl_3$) −7.4 ppm.

The product **3b** was obtained in the form of a colorless semisolid product in about 8% yield: IR (KBr) 3440 (m), 3020 (m), 2950 (s), 2430 (w), 1750 (s), 1720 (s), 1520 (s), 1440 (m), 1340 (m), 1250 (s), 1200 (s), 1160 (m), 1010 (s), 950 (s) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.8–2.2 (m, 9 H, ring), 3.80 (s, 3 H, $COOCH_3$), 3.85–4.7 (m, 6 H), 3 H for H-1,2,4; 2 H for -CH_2CH-; 1 H for -CH_2CH-)], 5.2 (s, 2 H, -CH_2Ph), 5.6–6.0 (m, 1 H, -NH-), 7.2–7.4 (d, 5 H, aromatic); MS, molecular ion at *m/e* 427; ^{31}P NMR ($CDCl_3$) −4.7 ppm.

Anal. Calcd for $C_{19}H_{26}NO_8P$ (**5a**): C, 53.39; H, 6.09; N, 3.27; P, 7.26. Found: C, 53.16; H, 6.03; N, 3.18; P, 7.42.

Hydrolysis of Phosphorylated Enzyme Complex. A 7-mL solution of 100 mg of **2** was added dropwise with stirring to a 7-mL solution of 70 mg of pronase (nonspecific protease, Sigma) pH 7.5, at 30 ± 2 °C. The pH was kept at 7.5 ± 0.2 by slow manual addition of ~0.2 M NaOH. All the solution of **2** was added over a period of 45 min. A total of 28 equiv of NaOH per equiv of **2** was consumed during the enzymatic hydrolysis. The reaction mixture was further stirred at room temperature for an additional 2 h and treated with ~0.5 g of Chelex-100. The pH was adjusted to 7.5, and stirring was continued for an additional 1 h. The hydrolysate was ultrafiltered through an Amicon filter (DM-5, molecular weight cut-off 5000). The filtrate was lyophilized to yield 85.0 mg of a pale yellow colored fine powder.

Results and Discussion

The reaction of α-chymotrypsin with the axial phosphorinane triester **1a** [2-(2,4-dinitrophenoxy)-2-oxo-*trans*-5,6-tetramethylene-1,3,2-dioxaphosphorinane] yields a stable phosphorylated enzyme derivative, **2**. The enzymatic transesterification reaction proceeds over 10^6 times faster than the nonenzymatic hydrolysis reaction (D. Kallick, unpublished). Interestingly, the equatorial ester epimer, **1b**, does not appear to be a substrate for the enzyme. Epimer **1a** is a racemic mixture and it appears as through both enantiomers react similarly.

Phosphorus-31 NMR spectroscopy has been used to characterize the phosphorylated enzyme intermediate **2**, and, in particular, to determine the stereochemistry of the enzymatic reaction. A freshly prepared sample of **2** shows only a single phosphate triester signal at −4.7 ppm (upfield from 85% H_3PO_4 reference). The starting material, **1a**, shows a single ^{31}P signal at −12 ppm. The −4.7 ppm signal is associated with a covalent phosphorylated species that cannot be removed even upon prolonged dialysis. Older samples show additional nondialyzable signals at 0.33 and 1.08 ppm, probably associated with "aging" of the triester to ring-opened diester phosphorylated species.

In order to assign the peak at −4.7 ppm, we have synthesized the model compounds **3a,b**. As in the case of other phosphorinane methyl triesters we expect the ^{31}P chemical shift of the axial epimer **3a** to be ~2.0 ppm upfield of **3b**.[14,15] This chemical shift difference has allowed us to quite simply determine the stereochemistry of the enzymatic reaction at phosphorus. The ^{31}P chemical shift of pure **3a** in $CDCl_3$ is, indeed, at −7.4 ppm while that of **3b** is at −4.7 ppm. To compare the phosphorylated enzyme **2** with model compounds **3a,b** under similar conditions with minimal environmental perturbation of the ^{31}P chemical shift,[16] we compared the spectra in 8 M urea, 20% D_2O, pH 7.0 (denaturing conditions for the enzyme complex). The ^{31}P signal for the phosphorylated enzyme species shifts slightly to −4.65 ppm under these conditions (Figure 1), and the ^{31}P chemical shifts for **3a** and **3b** are −5.8 and −4.7 ppm, respectively. In addition, the phosphorylated enzyme was proteolytically cleaved with Pronase, a nonspecific protease. Peptides that passed through a 5000 molecular weight cut-off ultrafilter were pooled, and the ^{31}P NMR spectrum showed a major peak (56%) at −4.66 ppm and two minor components at −2.6 (24%) and 1.08 ppm (18%) (Figure 2). The signal at −2.6 ppm is identified as the cyclic diester **1** (R = anion) by comparison with the ^{31}P NMR chemical shift of an authentic sample. Apparently the enzyme triester complex **2** is partially hydrolyzed during the pronase treatment to either aged diester product **1** (R = anion) with hydrolysis of the serine ester bond or to ring-opened diesters (1.0 ppm signal).

Clearly, however, the ^{31}P signal of the phosphorylated enzyme or peptide fragment thus corresponds most closely with that of the equatorial ester model serine ester **3b**. We can reasonably conclude, therefore, that the single-displacement, phosphorylation reaction has proceeded with 100% *inversion* of configuration. This contrasts with the stereochemistry of the methoxide reaction and the hydroxide-catalyzed hydrolysis of **1a**, which we have previously shown proceeds with only 82% inversion of configuration.[17] This enzymatic reaction, as in all previous examples,[1-9] thus enforces rigid stereospecificity to the phosphoryl transfer reaction.

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Registry No. 1a, 74431-09-7; **3**, 87450-96-2; **4**, 1676-81-9; **5**, 34384-98-0; α-chymotrypsin, 9004-07-3.