^{31}P NMR OF DIISOPROPYL PHOSPHORYL α-CHYMOTRYPSIN AND CATECHOL CYCLIC PHOSPHATE α-CHYMOTRYPSIN. DIRECT OBSERVATION OF TWO CONFORMATIONAL ISOMERS.

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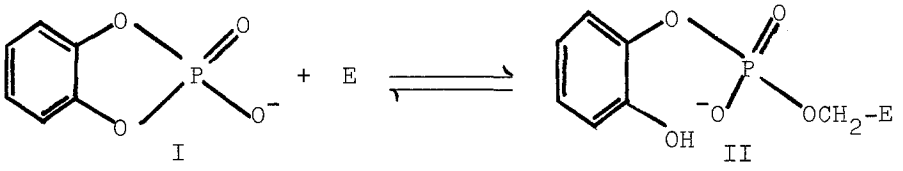
Summary. Two peaks separated by ca 2 PPM are observed in the ^{31}P NMR spectrum of diisopropyl phosphoryl α-chymotrypsin. The ratio of intensities of the two peaks is dependent upon the pH of the solution, and the two signals are assigned to two slowly interconverting conformational isomers of the enzyme complex. The ^{31}P NMR spectrum of the catechol cyclic phosphate α-chymotrypsin complex shows that the phosphate ester has reacted with the enzyme with opening of the five-membered phosphate ring.

INTRODUCTION

Phosphorylated derivatives of α-chymotrypsin have provided important structural and mechanistic information on the enzyme.[1] Thus, an acyl-chymotrypsin intermediate in which serine-195 is covalently linked to an acyl group of the substrate is uniformly proposed in any mechanism of action of this enzyme. Part of the evidence for this conclusion is provided by studies on the diisopropyl fluorophosphate irreversibly inhibited α-chymotrypsin. High resolution x-ray diffraction studies have provided three-dimensional structures of the diisopropyl phosphoryl α-chymotrypsin in which the diisopropyl phosphate moiety is esterified to the Ser_{195} $^{\gamma}O$ group.

Kaiser and coworkers[2] have studied the binding of catechol cyclic phosphate, I, to α-chymotrypsin and have proposed covalent attachment of the phosphate to the Ser_{195} $^{\gamma}O$: II.



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Interestingly, the cyclic phosphate binds in a reversible fashion, with intramolecular attack of the phenolic hydroxyl on the phosphorylated enzyme regenerating the free enzyme

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and the cyclic phosphate. It has been suggested that this reversible phosphorylation could serve as a model for the mode of activation of cyclic adenosine monophosphate protein kinase by the cAMP.

We wish to present ^{31}P NMR spectra of the diisopropyl phosphoryl and catechol cyclic phosphate complexes of chymotrypsin which confirms certain structural features of these complexes and allows, for the first time, direct observation of two interconvertible, enzyme conformational isomers.

METHODS

Three-times recrystallized α-chymotrypsin and diisopropyl phosphoryl chymotrypsin were purchased from Sigma. The enzymes were dialyzed at pH 3.00 and 4° vs. 0.01M EDTA (16 hrs.) and doubly distilled water (2x, 12hr. each), followed by lyophilization. The catechol cyclic phosphate was prepared by the method of Gross, et. al.[3] and confirmed by ^{1}H and ^{31}P NMR, uv spectrum and mp 85-94° (lit[3], 86°).

The diisopropyl phosphoryl chymotrypsin (ca, 4mM) NMR samples containing 1mM EDTA were adjusted to the desired pH with 1.0mM NaOH or HCl. The catechol cyclic phosphate chymotrypsin NMR samples contained 4mM chymotrypsin in .02M cacodylate buffer, pH 5.86, 1mM EDTA. Approximately 5 µl of catechol cyclic phosphate (0.3M in dry acetonitrile) was added to the enzyme solution prior to the NMR run.

^{31}P NMR spectra were recorded on a Bruker B-KR 322-S pulse spectrometer/HFX-90 (36.4 MHz spectrometer, operated in the block averaging Fourier transform mode.[4] All spectra were measured at 30° and reported chemical shifts are relative to 85% H_3PO_4.

RESULTS AND DISCUSSION

As shown in Figure 1, two peaks are observed in the ^{31}P NMR spectrum of the catechol cyclic phosphate chymotrypsin complex. The downfield peak (-1.1 PPM) corresponds to the hydrolysis product, o-hydroxylphenyl phosphate, as verified by the NMR of the hydrolysis product of the cyclic phosphate in the absence of enzyme. The upfield peak (+1.29 PPM) is the covalently bound, ring-opened complex, as evidenced by the upfield shift[5] and broader line-width.[6] Within the time period required for acquiring the FT NMR spectra (10 hr), there is no evidence for the cyclic diester, I, either bound or unbound, which should be easily observable since its chemical shift (-12.4 PPM) is so very different from that of acyclic esters (such as o-hydroxyphenyl phosphate). Further confirmation of the assignment is provided by the gradual increase in intensity of the downfield peak relative to the upfield peak over a period of time consistent with the kinetics of hydrolysis of the complex.

Although,we have confirmed the covalent, ring opened structure of the catechol cyclic

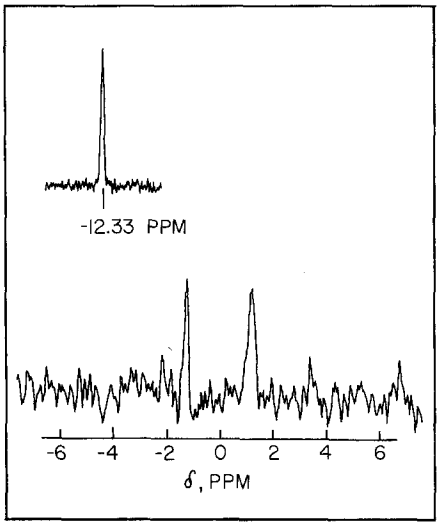

Figure 1. ^{31}P NMR spectrum of catechol cyclic phosphate α-chymotrypsin complex at pH 5.68, H_2O. Inset, ^{31}P NMR spectrum of catechol cyclic phosphate in acetonitrile.

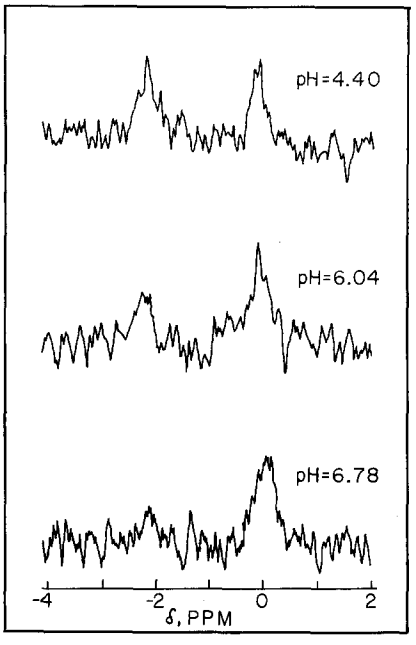

Figure 2. ^{31}P NMR spectrum of diisopropyl phosphoryl α-chymotrypsin.

phosphate chymotrypsin complex, we have no evidence supporting (or not supporting) the postulated recyclization of the phosphorylated enzyme prior to dissociation of the phosphate ester.[2] Apparently, hydrolysis or reattack by free chymotrypsin prevents the build-up of any observable catechol cyclic phosphate (consistent with Kaiser's data).

As shown in Figure 2 two peaks are also observed in the ^{31}P NMR spectra of the diisopropyl phosphoryl chymotrypsin. However, in contrast to the catechol cyclic phosphate chymotrypsin both peaks correspond to phosphorylated enzyme complexes. We believe that the two peaks represent slowly interconverting conformational isomers of the diisopropyl phosphoryl chymotrypsin. Evidence for this claim is summarized below:

1) Both peaks are broad, (as is the signal of the catechol cyclic phosphate complex) indicating the phosphate moiety is attached to a large, slowly tumbling[4] enzyme. The hydrolysis products (such as diisopropyl phosphate) would have a much sharper signal (as o-hydroxyphenyl phosphate in Fig. 1).

2) The ratio of the two peaks is dependent on pH, as shown in Figure 3. In addition, over a long period of time (days) the downfield peak appears to increase slightly. Although it is known that phosphorylated derivates of α-chymotrypsin and other enzymes "age" (that is, hydrolyze one or more of the ester bonds), this aging should increase with increasing pH.[7,8] Since the rate of increase of the downfield peak is larger at low pH, it is felt that aging is not responsible for the two different signals.

3) The pH dependent ratio of the two peaks is reversible. Thus, increasing the pH of a previously run pH 4.4 sample (ratio 1.5) results in a decrease in intensity of the downfield peak (at pH 6.0, ratio ≃ .5). If the downfield peak corresponds to the "aged" (i.e., partially hydrolyzed) phosphorylated enzyme, then the ratio of peaks should certainly not be reversible.

Both peaks in the ^{31}P NMR spectrum of diisopropyl phosphoryl chymymotrypsin thus likely correspond to chemically similar, phosphorylated species. The simplest explanation for the origin of two different forms of the same complex is that they represent two different conformations of the enzyme complex, similar to the "active" and "inactive" free enzyme conformational states.[9,10] The pH dependence of the percentage of "active" conformation bears a striking similarity to the pH dependence of the percentage of ^{31}P

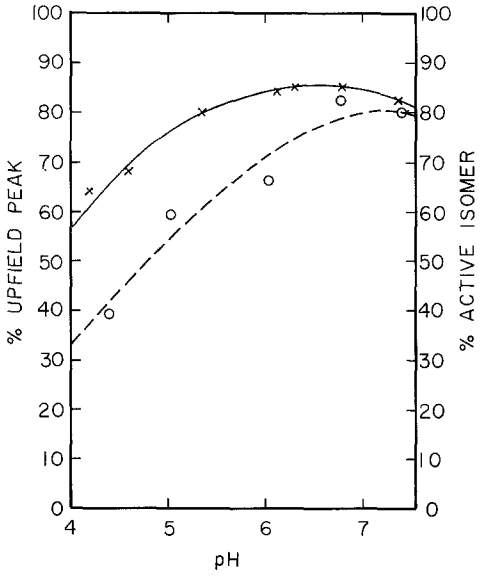

Figure 3. Plot of percent upfield peak in the ^{31}P NMR spectrum of diisopropyl phosphoryl α-chymotrypsin vs. pH (o), and plot of percent active conformation of α-chymotrypsin vs. pH (x, data from ref 9).

signal associated with the upfield peak of the complex (Figure 3).

4) The observation of separate signals for each of the isomers is consistent with the slow rate of interconversion of the chymotrypsin isomers. Thus, two NMR signals will be observed for a nucleus which chemically exchanges between two sites if the rate of exchange, $1/\tau$, is less than $2\pi\Delta\nu$, where $\Delta\nu$ is the chemical shift difference between the two sites.[6] Since $\Delta\nu$ is 70 Hz ($2\pi\Delta\nu \sim 300$ sec^{-1}) and the observed rate of exchange[9] in chymotrypsin is ~ 1 sec^{-1}, slow exchange is confirmed[11] (assuming that the phosphorylated and free enzymes isomerize at similar rates).

What is the basis for the difference in ^{31}P chemical shifts for the two isomers?

A highly speculative, but potentially significant explanation, associates the isomerization to phosphoryl transfer between the Ser_{195} $^{\gamma}O$ and the imidazole nitrogen of His_{57}. The downfield signal might represent the normal serine O-phosphorylated ester and the upfield signal the His_{57} imidazole N-phosphoramide. Some years back the concept of acyl N⇄O interconversion was fashionable;[13] however recent data appears to rule out this possiblity. Our interpretation is consistent with the expected ~ 2 PPM

upfield shift of a neutral imidazole, diester phosphoramide relative to a neutral triester.[14] The decrease in the intensity of the upfield peak (phosphoramide) at low pH is consistent with the acid lability of amides and stability of esters.[15] Finally, other explanations for the 2 PPM chemical shift difference based upon altered geometry[5,16] or environment[17] in the two isomers are possible, but not any more compelling.

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11. These results are also probably consistent with slow monomer $\rightleftharpoons$ dimer exchange (ref. 12), although the ratio of the two peaks at higher pH does not fit this hypothesis. Actually, both isomerization and dimerization processes probably contribute to the observed pH dependence of the ratio of signals.

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