^{31}P NMR STUDY ON THE BINDING OF 3'-CYTIDINE MONOPHOSPHATE TO RIBONUCLEASE A. PART I.

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SUMMARY: An nmr chemical exchange method has been used to determine the pH dependence of the ^{31}P chemical shifts of the 3'-cytidine monophosphate•RNase A complex. The results are in conflict with a related ^{31}P nmr study by Lee and Chan and we suggest that these differences may be attributed to extraneous heavy metal ion impurities in their samples. The ^{31}P nmr titration data confirm that the dianionic phosphate inhibitor binds to a diprotonated active site and two ionizations have been observed with pK_1 = 4.4 and pK_2= 6.2. A detailed description of the ionic states involved in the binding process is presented.

We wish to report in this communication a ^{31}P nuclear magnetic resonance (nmr) study on the binding of 3'-cytidine monophosphate (3'-CMP) to Ribonuclease A. This work serves as a complement to the extensive and pioneering ^{1}H nmr studies by Jardetzky[1] on the binding of the cytidine inhibitors to RNase A. In addition we wish to point out an apparent conflict between our results and those recently reported by Lee and Chan[2] on an analogous ^{31}P nmr study of the 3'-uridine monophosphate (3'-UMP) inhibitor.

The bovine pancreatic RNase A and the 3'-CMP used in this work were obtained from Sigma. The type XII-A enzyme was generally used without further purification although in several runs heavy-metal ion impurities were removed by passing the enzyme solution through a Chelex 100 ion exchange resin. The 3'-CMP was routinely purified by the resin treatment. All solutions except where specifically noted contained 10^{-3}M or greater EDTA and .2M NaCl. The pH was adjusted with 1 M NaOH or HCl and measured on a Radiometer PHM 26

pH meter fitted with a type G2222C glass semimicro electrode and type K4112 calomel electrode. All solution pH's were measured before and after the nmr run and both agreed within .02 pH units. The ^{31}P nmr spectra were recorded either on a Bruker HFX-90 High Resolution nmr spectrometer operated in the continuous wave (cw) mode at 36.4 MHz or on a Bruker B-KR 322S Pulsed spectrometer/HFX-90 spectrometer. Either a specially constructed, Pivan Engineering, data acquisition system (4K data points) or a Nicolet 1080 Fourier transform data system was used for signal averaging in the cw or pulsed mode operation of the respective spectrometers. In the pulsed, Fourier transform experiment 2K data points were acquired with a sweep width of 200 Hz and resolution therefore of .2 Hz. Proton, broad band decoupling and either proton (TMS) or fluorine (C_6F_6) external locks were used. Frequencies were measured with either Hewlett-Packard 5612A 12.5 MHz or 5248M 100 MHz counters. All spectra were taken at 30°.

Our studies have utilized the nmr chemical exchange method[3] for determining the chemical environments and structure of the inhibitor bound to the enzyme active site. By increasing the enzyme to inhibitor (E_o/I_o) ratio one may progressively "titrate" the averaged, ^{31}P nmr signal of the inhibitor as it rapidly exchanges between the solution and the active site. The results of these experiments at different pH's are presented in Fig. 1. Both enzyme and inhibitor concentrations were varied (between .15M and 0.01M for 3'-CMP and 10^{-3}M and 7.5×10^{-3}M for RNase A) in order to obtain the maximum possible range of E_o/I_o ratios. Although it is better practice to keep the enzyme concentration constant in order to minimize bulk-susceptibility[4] changes in the solvent, only small solvent shifts and line-width effects were introduced by proceeding in this way.

If we assume* that the 3'-CMP exchanges between the two sites in the fast exchange limit,[3,4] the observed chemical shift, δ_{obs}, would represent a weighted average of the chemical shifts for the enzyme·inhibitor (E·I) complex, δ_{EI}, and the inhibitor in solution, δ_I;

$$\delta_{obs} = \frac{(E\cdot I)}{I_o} \cdot \delta_{EI} + \frac{(I)}{I_o} \cdot \delta_I \qquad (1).$$

Rearranging, and since under our conditions, $(E\cdot I) \sim E_o$, $\delta_{obs} - \delta_I = \Delta \cdot E_o/I_o$ where $\Delta = \delta_{EI} - \delta_I$. Thus the chemical shift of the E·I complex may be obtained from the slopes of the

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* This assumption is reinforced by our observation of only small (<3 Hz) line broadening effects.

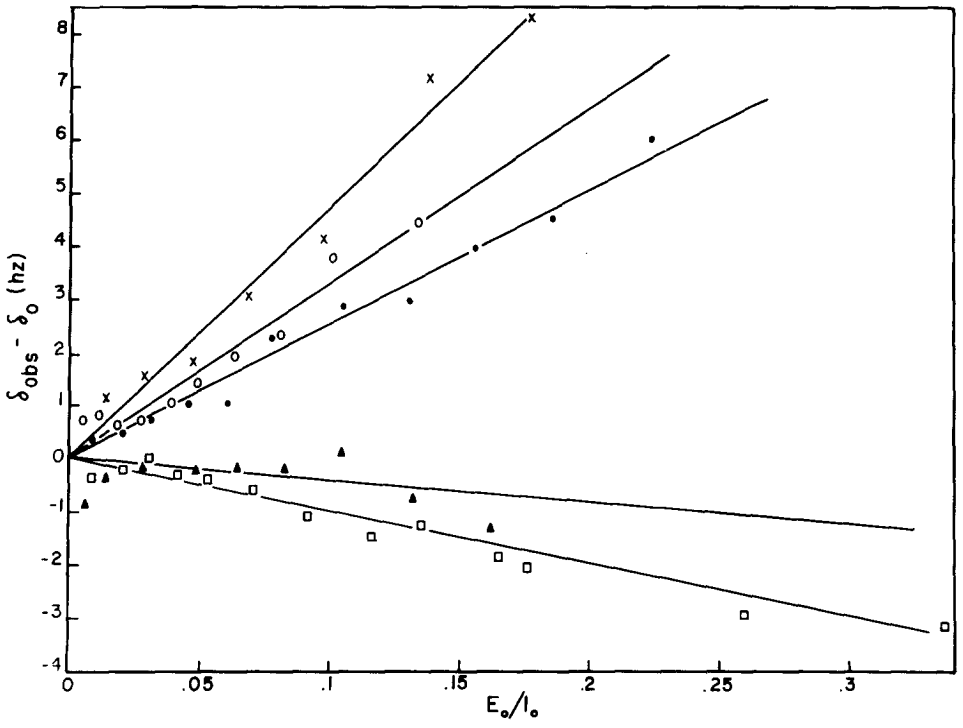

Figure 1. Plot of the ^{31}P chemical shift difference, $\delta_{obs} - \delta_o$, vs the E_o/I_o ratio for 3'-CMP and RNase A at pH 4.47 (o), 5.19 (X), 5.61 (•), 6.50 (▲), and 7.50 (□) at 30°. Positive chemical shift differences here represent increasing frequencies and decreasing field.

plots of Fig. 1. The pH dependence of the chemical shifts of the 3'-CMP in solution and when bound to RNase A are shown in Fig. 2.

Before discussing the full mechanistic implications of these figures, we must first consider the conflicting ^{31}P nmr results reproted by Lee and Chan.[2] Their work at 89 MHz indicated that the ^{31}P signal for the 3'-UMP•RNase A complex is shifted upfield 800 Hz from the free 3'-UMP signal. In addition they observed substantial line broadening of the signal with increasing E_o/I_o ratios. Before the publication of Chan's work, we had observed such effects as well, but the results were quite inconsistent and when care was taken to purify the enzyme and inhibitor solutions, these large shifts and line broadening effects disappeared.

We suspected that paramagnetic ion impurities were responsible for the very different results observed by Chan and ourselves. The

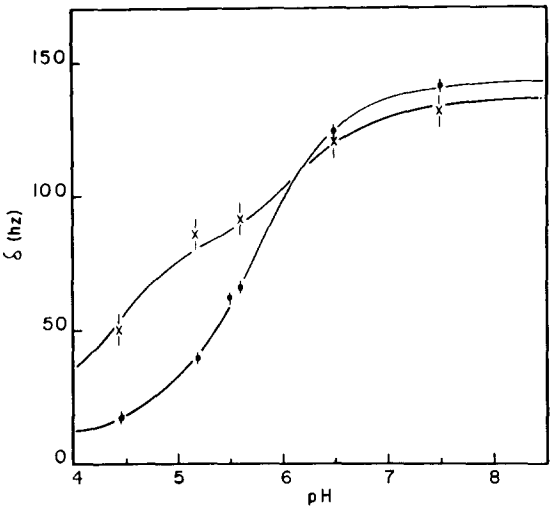

Figure 2. pH dependence of the ^{31}P chemical shifts of 3'-CMP (•) or the 3'-CMP•RNase complex (X) at 30°. Increasing frequencies represent decreasing field at 36.436190 MHz (85% H_3PO_4 ^{31}P resonance frequency is 36.436196).

pernicious problems created by these trace ion impurities have been demonstrated repeatedly in the literature[5] and, in particular, in the recent work by Lee, et. al[6] on their now refuted[7,8] claims for tautomerism in cytosine nucleic acid bases.

We therefore believe that the large effects these workers observed in the ^{31}P nmr study of 3'-UMP may actually be attributed to the presence of metal ion contamination in their samples. Thus, the large shifts and line broadening effects may be eliminated by the addition of 10^{-4} to 10^{-3}M EDTA to the solutions, and it is also possible to duplicate these large effects by the addition of a paramagnetic ion such as Cu^{++} to a purified solution of the inhibitor.

The pH dependence of the ^{31}P chemical shifts of Fig. 2 follow simple titration curves. ^{31}P nmr has previously been shown to be an effective probe of the degree of ionization of phosphate esters[9] and the pK = 5.70 we obtained from the free 3'-CMP plot agrees well with the potentiometric titration value of 5.90[10]. Only a sum of two pK's allows a fit of the 3'-CMP•RNase A data, with pK_1 = 4.4 and pK_2 = 6.2.

These results offer strong proof that the 3'-CMP binds in the dianionic ionization state to a site on the enzyme which only modestly neutralizes the negative charge. Thus, only a 10 Hz upfield shift

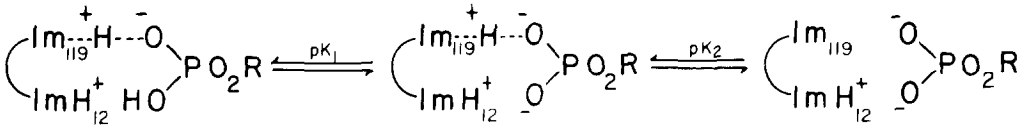

Figure 3. Ionization states for the 3'-CMP•RNase A complex. Only the imidazoles (Im) of His 12 and 119 and the phosphate monoester inhibitor are shown.

for the 3'-CMP•RNase A complex is seen between pH 6.5 and 7.5 while complete monoprotonation of the dianion results in a 133 Hz upfield shift of the free 3'-CMP. Furthermore, the addition of the first proton to the complex (pK_2 = 6.2) must occur on some site other than the dianionic phosphate since the ^{31}P signal is shifted upfield by only 50 Hz. The addition of a second proton (pK_1 = 4.4) to the complex shifts the ^{31}P signal upfield by a further 80 Hz so that at the lowest pH's this phosphate finally appears to be in the monoanionic ionization state. The upfield chemical shift of the complexed dianionic phosphate relative to the free dianionic phosphate in the entire pH region arises then from the positively charged environment of the enzyme, with this environment becoming even more positive as the pH is lowered. These shifts definitely rule out the possibility that the monoanion binds to the active site since at these pH's the enzyme active site is positively charged[1] and upfield shifts from the monoanionic ^{31}P signal would therefore be expected.

Jardetzky[1] has argued on the basis of the pH/binding constant curve[10] and ^{1}H nmr titration data that the 3'-CMP is bound in the dianionic form to a diprotonated active site. On the other hand, Hammes[10] has proposed on the basis of the same binding curve and his relaxation kinetic studies that the monoester inhibitors are bound in the monoanionic ionization state to a monoprotonated active site. Our own results now confirm Jardetzky's original proposal and on the basis of the ^{31}P and ^{1}H nmr, as well as the x-ray[11] diffraction, studie it is now possible to describe in great detail the role of the two histidines (12 and 119) which are known to be involved in the binding of the phosphate inhibitor.

As shown in Fig. 3 His 12 and the dianionic 3'-CMP are presumed to interact only slightly. This would be consistent with our own

small ^{31}P upfield chemical shift at pH's > 6.5 and the small ^{1}H upfield chemical shift observed by Jardetzky for the C(2)-H proton of protonated His 12 resulting from complex formation. The substantial downfield chemical shift of the C(2)-H proton of the protonated His 119 is similarly consistent with the upfield chemical shift of the phosphate at pH < 6.2. This is attributed to strong H-bond interaction* between the two groups.

Our results would indicate that a group with a pK ~ 6.2 is responsible for the tight binding of the dianionic 3'-CMP while Jardetzky reports pK's for the complex of 7.4 for His 12 and 8.0 for His 119. The discrepency may possibly be explained by an extraordinarily large solvent isotope effect for Jardetzky's measurements in D_2O. Finally the pK ~ 4.4 in the ^{31}P nmr titration of the complex may be assigned to protonation of the phosphate. The pK would be expected to be perturbed because of the highly positive local environment of the enzyme.

* Thus actually it may be a moot point to argue whether, at least betwee pH 4.5 and 6.5, the monoanion phosphate binds to a monoprotonated active site or the dianion phosphate binds to a diprotonated active site. Apparently both are inexact descriptions of the real hybrid situation.

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