Interaction of Uridine and Cytidine Monophosphates with Ribonuclease A. IV. Phosphorus-31 Nuclear Magnetic Resonance Studies

David G. Gorenstein,* [1a,b] Alice M. Wyrwicz,[1b] and Jurgen Bode[1c]

Contribution from the Department of Chemistry, University of Illinois at Chicago Circle, Box 4348, Chicago, Illinois 60680, and from the Gesellschaft für Molekularbiologische Forschung mbH, D-3301 Stöckheim über Braunschweig, West Germany. *Received June 4, 1975*

Abstract: The pH dependence of the ^{31}P chemical shifts of 3′-, 5′-, and 2′-cytidine monophosphate and 3′-uridine monophosphate, both free in solution and when bound to bovine, pancreatic ribonuclease A has been determined by both chemical exchange and direct observation methods. The ^{31}P NMR titration data demonstrate that each nucleotide binds to the enzyme in the dianionic ionization state around neutral pH. Two ionizations are observed for the complex, with $pK_1 = 4.0$–5.5 and $pK_2 = 5.9$–6.7. The first pK is associated with ionization of the monoanionic inhibitor and the second with ionization of the protonated histidine$_{12}$ residue which hydrogen bonds to the phosphate. Apparently, the ^{31}P chemical shifts of the phosphate esters are only affected by the protonation state and not by the highly positive local environment of the enzyme. Thus, the chemical shift of 3′-CMP is shifted upfield by only 10 Hz at neutral pH despite the proximity of protonated histidine$_{119}$ and lysine$_{41}$ and partially protonated histidine$_{12}$. In addition, as long as the imidazole N remains the hydrogen-bonding donor, the phosphate ^{31}P chemical shift is unaffected by a hydrogen-bonding interaction. This property provides a unique opportunity to calculate microscopic ionization constants and allows a detailed description of the ionic states involved in the binding process.

The application of ^{31}P NMR spectroscopy to the study of the solution structure of molecules has been relatively limited, in spite of the useful NMR properties of the ^{31}P nucleus (spin ½, 100% natural abundance, long relaxation times, wide range of chemical shifts, key role in many molecular, particular biomolecular, structures). One reason for this neglect is the small sensitivity of the phosphorus nucleus; until recently concentrations about 0.1 M were regarded as the lower limit.[2] However, with the introduction of FT NMR spectroscopy, much lower concentrations are now routinely considered.

A much more serious limitation to the use of ^{31}P NMR in biochemical systems is the relative insensitivity of the chemical shift of the biologically important phosphate esters to changes in local magnetic environments. In contrast, ^{1}H, ^{13}C, and ^{19}F NMR have proven extremely useful probes of molecular structure, particularly enzyme small-molecule complexes, because the chemical shift of these nuclei is sensitive to changes in chemical and magnetic environments. While ^{31}P chemical shifts are relatively insensitive to the local environment, they apparently are quite dependent upon the geometry of the ester.[2a,3] Thus, ^{31}P shifts in phosphates are *not* very sensitive to the chemical identity (R or H) of the group bonded to the phosphate oxygen but are very sensitive to changes in O-P-O bond angles[3a] and phosphate-ester torsional angles.[3b,c] Perhaps most surprisingly, association of divalent metal ions[4] and (as demonstrated in this paper) even hydrogen-bonding donors has little effect on the ^{31}P chemical shift other than that explained by a shift in the pK. Secondary ionization of a phosphate monoester does produce a 4-ppm downfield shift of the ^{31}P signal,[2a] but as earlier noted,[3] this is most likely attributable to an O-P-O bond angle effect. In this paper we demonstrate how this limited sensitivity of ^{31}P shifts of phosphates may provide a unique opportunity to define the microscopic ionization states in an enzyme-phosphate ester complex.

In particular we wish to report the pH dependence of the ^{31}P signal of pyrimidine nucleotides, both free in solution and when bound to bovine pancreatic ribonuclease A (RNase A). An earlier preliminary communication of a portion of this work has already appeared.[5] By considering both the ^{1}H and ^{31}P NMR data on RNase A in conjunction with the x-ray crystallographic structures,[6] we are able to describe in detail the ionization states and chemical environment about the phosphate in the RNase nucleotide complexes.

Experimental Section

Materials. The RNase A and the nucleotide inhibitors used in this work were obtained from Sigma and Calbiochem. The phosphate-free, recrystallized 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. In other experiments, crystalline bovine RNase A/B was purchased from Sigma and freed from the B component on Bio Rex 70 cation exchange resin according to Hirs et al.[7] The protein pooled was desalted on Sephadex G25 and stored as a lyophilized powder below 4 °C. The nucleotides were routinely purified by the resin treatment. The H_2O solutions were prepared with 10^{-3} M or greater EDTA and 0.2 M NaCl. The D_2O solutions were prepared with 0.1 M ammonium formate and 1 mg/ml EDTA. The pH in H_2O 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. Smaller aliquots were measured on a Radiometer G2221C/K1301 micro electrode assembly. All solution pH's were measured before and after the NMR run and both agreed within ±0.02 pH units.

NMR Methods. The ^{31}P NMR spectra were recorded on either a combination Bruker B-Kr 322S pulsed spectrometer-HFX-90 spectrometer at 36.4 MHz with 5-mm diameter tubes or on a Varian Associates XL-100-15 spectrometer at 40.5 MHz with 12-mm diameter tubes. A Nicolet 1080 Fourier transform data system was used for signal averaging in the pulsed mode operation of the Bruker spectrometer. In the Bruker-Nicolet Fourier transform experiment 2K data points were acquired with a sweep width of 200 Hz and resolution therefore of 0.2 Hz. Proton, broad-band decoupling and a fluorine (C_6F_6) external lock were used with frequencies measured on either Hewlett-Packard 5612A 12.5-MHz or 5248M 100-MHz counters. For the pulsed FT-Varian spectrometer, an internal D_2O lock was used with spectral width of 200–1000 Hz, and acquisition time of 1.5 s. Typically 200–1000 transients were collected and a signal enhancement of 0.5 was applied to the free induction decay. All spectra were taken at 24 ± 1 °C.

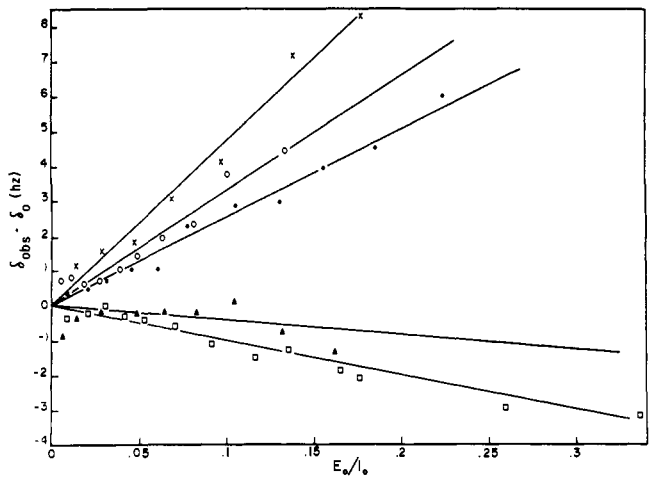


Figure 1. Plot of the ^{31}P chemical shift difference, $\delta_{obsd} - \delta_0$, vs. the E_0/I_0 ratio for 3′-CMP and RNase A at pH 4.47 (○), 5.19 (x), 5.61 (●), 6.50 (▲), and 7.50 (□). At 30 °C, positive chemical shift differences represent increasing frequencies.

Analysis of Data. A weighted, nonlinear least-squares program[8] which was modified for use on a chemistry department PDP 11/45 computer (32K words) was used to obtain "best fits" for the chemical shift, δ_{obsd}, titration curves. For simple titration curves the chemical shift data was fit to the Henderson–Hasselbalch function. In those cases where a significant deviation from a simple titration curve was observed, the data was least-squares fit to the equation for a dibasic acid (eq 1).

$$\delta_{obsd} = \frac{\alpha\beta\delta_{H_2A} + \beta\delta_{HA} + \delta_A}{1 + \beta + \alpha\beta} \quad (1)$$

Subscripts refer to the diprotonated, H_2A, the monoprotonated, HA, or the unprotonated species, A, of the dibasic acid and $\alpha = (H^+)/K_1$, $\beta = (H^+)/K_2$. K_1 and K_2 are the first and second macroscopic ionization constants of the dibasic acid.

Results

Early measurements with 0.1 M solutions of commercial grade 3′-CMP yielded a ^{31}P resonance with 50-Hz line width. By addition of 1 mg/ml of EDTA and repetition under the same conditions sharpening of the signal to 3 Hz was obtained. All spectra were noise decoupled in order to avoid splitting by the ribose protons ($J_{P-H_3'}$ = 8.6 Hz for 3′-CMP).

Either a chemical exchange or direct observation method was chosen to measure the ^{31}P chemical shift of the nucleotide RNase complex. In the chemical exchange method[9] it is assumed that the small-molecule inhibitor exchanges between two sites, the free solution and the enzyme active site. If the chemical shift of the E·I complex, δ_{EI}, is different from that of the inhibitor in solution, δ_I, and if the inhibitor exchanges between the two sites sufficiently rapidly

$$E + I \rightleftharpoons E \cdot I$$

then the observed chemical shift, δ_{obsd}, will represent a weighted average of chemical shifts for the two different environments.

$$\delta_{obsd} = \frac{(E \cdot I)}{I_0}\delta_{EI} + \frac{(I)}{I_0}\delta_I \quad (2)$$

Rearranging, and since under most of our conditions the enzyme is saturated with the inhibitor,[10] $(E \cdot I) \sim E_0$, and

$$\delta_{obsd} - \delta_I = \Delta E_0/I_0 \quad (3)$$

where $\Delta = \delta_{EI} - \delta_I$. Thus the chemical shift of the E·I complex may be obtained from the slopes of plots of δ_{obsd} vs. E_0/I_0. The results of these experiments for 3′-CMP at different pH's are presented in Figure 1. In some of the runs both enzyme and inhibitor concentrations are varied (between 0.15 and 0.007 M for 3′-CMP and 10^{-3} and 7.5×10^{-3} M for RNase A) in order to obtain the maximum possible range of E_0/I_0 ratios. Although it is better practice to keep the enzyme concentration constant in order to minimize bulk-susceptibility changes in the solvent, only small solvent shifts and line width effects were introduced by proceeding in this way. In later experiments, only the inhibitor concentrations were varied to minimize the possibility of introducing any complications in interpretation of the shifts. The chemical shifts were fitted to eq 3 with a weighted, least-squares computer program and the δ_I and δ_{EI} obtained from these plots at different pH's for the various nucleotides are presented in Figure 2 (error brackets represent standard deviations). It should be noted that δ_I obtained from eq 3 may be different from δ_I obtained in the absence of protein. The difference arises from the change in the bulk susceptibility of the solution. However, as indicated earlier, these effects are quite small and the pH-dependent shifts of δ_I in the two solutions are quite comparable.

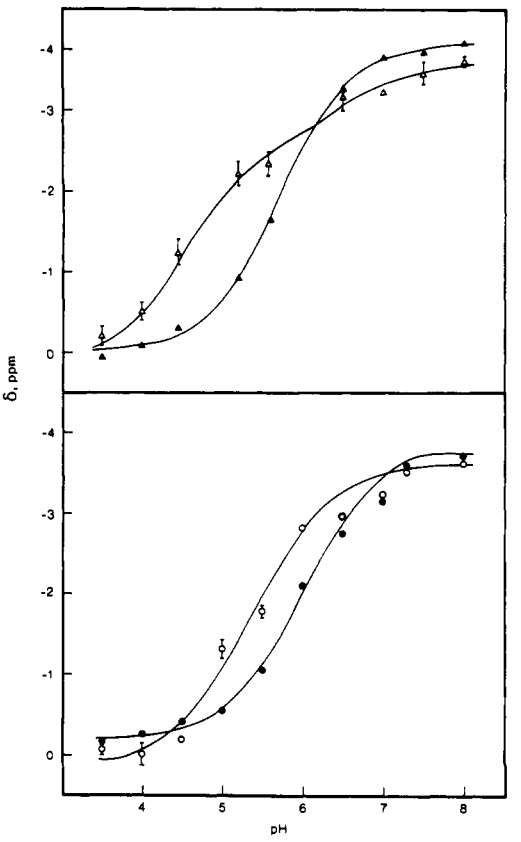


Figure 2. ^{31}P chemical shift, δ, vs. "pH" for 3′-CMP (△) and 5′-CMP (○) in H_2O as determined by the chemical exchange method. Filled symbols represent δ_I, the chemical shift of the inhibitor free in solution. Unfilled symbols represent δ_{EI}, the chemical shift of the enzyme inhibitor complex. Curves were generated from eq 1 and the "best-fit" parameters given in Tables I and II. Points at pH 6.5 and 7.0 for the 5′-CMP·RNase A complex were ignored in the computer fit because at the higher pH's the enzyme is only partially saturated with 5′-CMP.

The second method involves the direct NMR observation of an equimolar solution of the inhibitor and enzyme. Under conditions $(I) \gg K_1$, this method yields the same chemical shifts as the chemical exchange method. However, pyrimidine nucleotides have bell-shaped pH-binding constant profiles, with maximum binding occurring around pH 5.6. At pH's much lower or higher than this, the enzyme will not be saturated and the chemical shifts will therefore best be obtained only by the chemical exchange method.

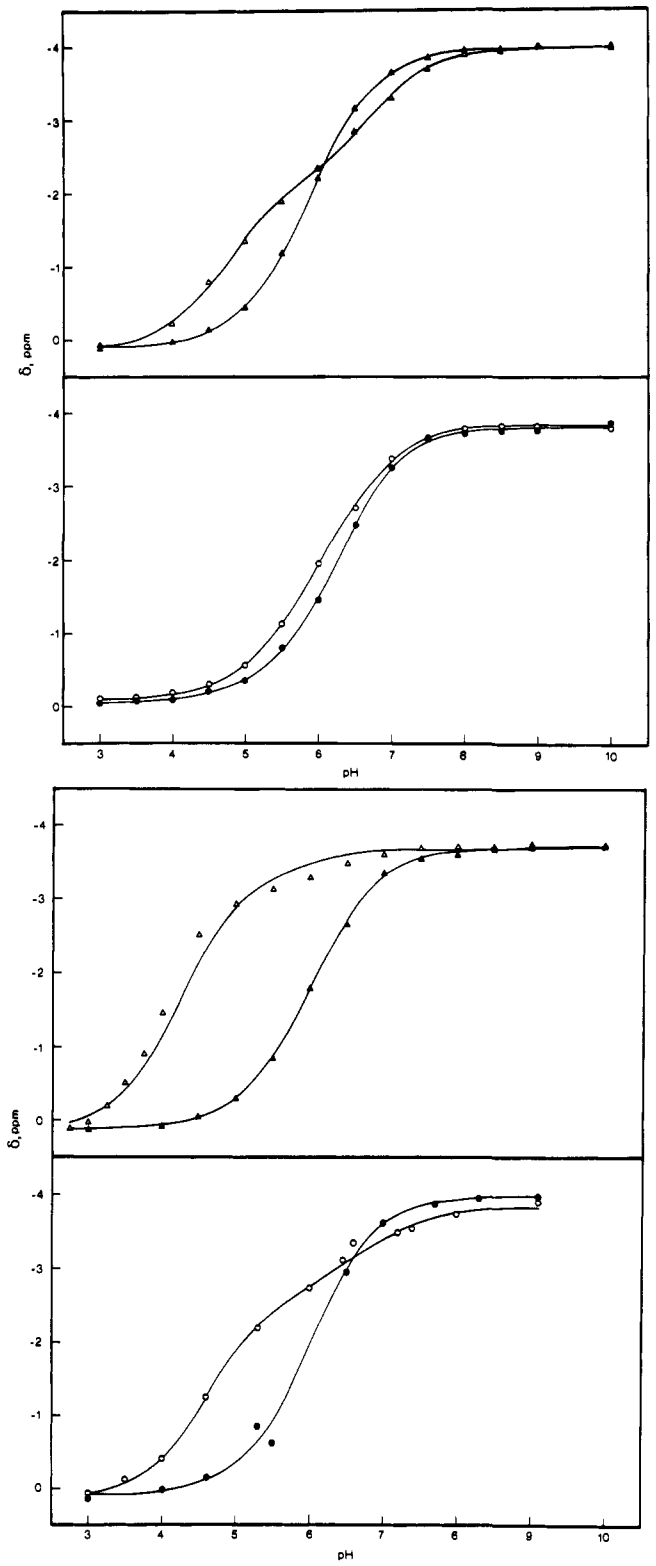


Figure 3. (A, top). ^{31}P chemical shift, δ, vs. "pH" for 3′-CMP (△) and 5′-CMP (○) in D_2O as determined by the direct observation method. (B, bottom). ^{31}P chemical shift, δ, vs. "pH" for 2′-CMP (△) and 3′-UMP (○) in D_2O as determined by the direct observation method. Filled symbols represent the chemical shift of the inhibitor in the absence of enzyme, unfilled symbols in the presence of an equimolar concentration of RNase A.

The direct observation studies were carried out by comparing 6×10^{-3} M samples of inhibitor and RNase inhibitor at various "pH" values in 0.1 M ammonium formate, D_2O solutions with 1 mg/ml of EDTA added. Plots of the observed chemical shifts vs. pH for 3′-, 5′-, 2′-CMP, and 3′-UMP are found in Figure 3. The bound state of 3′-CMP is characterized by only little line broadening (from 1.3-3 Hz in the free state to 4-6 Hz); at pH 5.5 no temperature dependence of the signal shape was found between 15 and 45 °C.

To assure that our results are not influenced by an interaction with EDTA and the enzyme (as found for a number of polyvalent ions) the following tests were undertaken.

(1) The activity of RNase toward (cyclic) 2′3′-CMP[11] was raised to 110% by addition of 1 mg/ml of EDTA; this result may indicate the presence of inhibitory metal ions[5,12] in commercial enzyme preparations.

(2) After purification by chromatography on an anion exchange column (Dowex 1X-8) 3′-CMP (without EDTA) gives rise to a signal similar to that obtained in the presence of EDTA; this signal is shifted 7 Hz in the upfield direction with regard to the untreated sample. Addition of 1 equiv of commercial RNase at pH 5.5, however, led to deshielding by 30 Hz *and* broadening to 40 Hz. Only after purification according to Crestfield[13] could sharp signals be reproducibly obtained.

These results indicate the strong association of paramagnetic ions to nucleotides in the free *and* bound state causing shortened relaxation times and additional shifts of the ^{31}P signals. In fact, these same effects can be reproduced by the addition of the paramagnetic ion, Cu^{2+}, to a purified solution of the inhibitor.[5]

Discussion

The free solution, isomeric cytidine phosphates exhibit almost identical titration curves. A slight deviation from the Henderson-Hasselbalch function in the lower pH range for 2′-, 3′-, and 5′-CMP is likely attributable to a slight change in the diamagnetic contribution to the ^{31}P shift resulting from protonation of the base.[3] Except for the poorly defined base pK's, the ionization constants derived from these plots (Table I) agree well with potentiometric titration values and confirm the utility of ^{31}P chemical shifts as effective probes of the degree of ionization of phosphate esters.

In contrast, none of the nucleotide RNase curves can be analyzed in terms of a simple titration curve. Assuming that only one ionizable group of the complex is responsible for each of the inflections of the profiles, a least-squares fit of the data of these plots to eq 1 yields the apparent pK's and chemical shifts given in Table II. It should be mentioned that the values for pK_1 and pK_2 which are based upon the uncorrected values from Figure 3 are not as reliable as those obtained from the chemical exchange method; correcting Figure 3 for incomplete association of inhibitors, however, produces profiles and pK's more like those obtained from the chemical exchange method.

These results suggest that all of the nucleotides bind around neutral pH in the dianionic ionization state. Thus, the 3′-CMP·RNase A complex is shifted upfield only 10 Hz from the free 3′-CMP between pH 6.5 and 7.5 while monoprotonation of the free dianion results in a 133-Hz upfield shift. Furthermore, the addition of the first proton to the nucleotide complex (pK_2 = 6.0-6.7) must occur largely on some site other than the dianionic phosphate since the ^{31}P signal is shifted upfield by only 1-2 ppm. The addition of a second proton (pK_1 = 4.0-5.7) to the complex shifts the ^{31}P signal further upfield so that at the lowest pH's the phosphate finally appears to be in the monoanionic ionization state.

Jardetzky has argued on the basis of the pH-binding constant curve[14] and ^1H NMR titration data that the mononucleotides are bound in the dianionic form to a diprotonated active site. On the other hand, Hammes[15] has favored on the basis of the same binding curve and his relaxation kinetic studies that the monoester inhibitors are bound in the

Table I. Apparent pK's and ^{31}P Chemical Shifts of Nucleotides

	Ionization constants[a]		Chemical shifts,[a,b] ppm		
Inhibitor	pK_1	pK_2	H_2A	HA	A
3′-CMP[f]	5.71 ± 0.22		−0.02 ± 0.04		−3.82 ± 0.04
3′-CMP[e]	5.28 ± 0.20[c]	6.11 ± 0.50[d]	+0.09 ± 0.02	−1.27 ± 0.70	−3.99 ± 0.01
	5.88 ± 1.44[g]		+0.045 ± 0.024		−3.97 ± 0.02
3′-CMP[h]	4.15[c]	5.65[d]			
2′-CMP[e]	5.10 ± 0.03[c]	6.15 ± 0.65[d]	+0.13 ± 0.03	−0.64 ± 0.53	−3.70 ± 0.01
2′-CMP[i]	4.4[c]	6.2[d]			
5′-CMP[f]	5.97 ± 0.100[g]				
	5.77 ± 0.18	6.78 ± 0.28	−0.20 ± 0.06	−2.76 ± 1.4	−3.83 ± 0.12
5′-CMP[e]	4.80 ± 0.003[c]	6.31 ± 1.00[d]	−0.049 ± 0.03	−0.43 ± 0.21	−3.81 ± 0.02
5′-CMP[h]	4.32[c]	6.06[d]			
3′-UMP[e]	5.99 ± 0.83[d]		+0.067 ± 0.09		−3.99 ± 0.08
3′-UMP[i]	5.9[d]				

[a] Errors represent linear estimates of the standard deviation (see Experimental Section). [b] Chemical shift vs. external 85% H_3PO_4. [c] pK of base. [d] pK of phosphate. [e] Direct observation method in D_2O, 0.1 M ammonium formate, 1 mg/ml of EDTA. [f] Chemical exchange method in H_2O, 0.2 M NaCl, 1 mM EDTA. Determined in the presence of enzyme. [g] Data fitted to single titration curve. [h] Potentiometric titration value (ref 9b). [i] Potentiometric titration value: "Data for Biochemical Research", R. M. C. Dawson, D. C. Elliot, W. H. Elliot, and K. M. Jones, Ed., Oxford University Press, London, 1969, Chapter 5.

Table II. Apparent pK's and Chemical Shifts for the Inhibitor RNase A Complexes

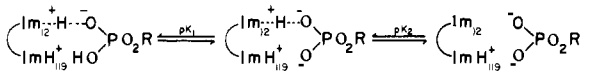
	Ionization constants[a]		Chemical shifts, ppm[a,b]		
Inhibitor complex	pK_1	pK_2	H_2A	HA	A
3′-CMP·RNase A[d]	4.52 ± 0.17	6.52 ± 0.37	+0.14 ± 0.25	−2.56 ± 0.33	−3.57 ± 0.16
3′-CMP·RNase A[c]	4.71 ± 0.97	6.72 ± 0.96	+0.11 ± 0.04	−2.14 ± 0.07	−4.01 ± 0.02
5′-CMP·RNase A[d]	4.99 ± 0.51	5.89 ± 0.59	−0.20 ± 0.06	−2.76 ± 1.40	−3.83 ± 0.12
5′-CMP·RNase A[c]	5.45 ± 0.61	6.46 ± 0.72	−0.11 ± 0.02	−1.76 ± 0.27	−3.83 ± 0.01
2′-CMP·RNase A[c]	3.99 ± 0.02	6.60 ± 0.14	−0.14 ± 0.13	−3.31 ± 0.63	−3.67 ± 0.07
3′-UMP·RNase[c]	4.57 ± 0.66	6.48 ± 0.40	+0.14 ± 0.07	−2.54 ± 0.18	−3.78 ± 0.06

[a,b] See footnotes to Table I. [c] Direct observation method in D_2O, 0.1 M ammonium formate, 1 mg/ml of EDTA. [d] Chemical exchange method in H_2O, 0.2 M NaCl, 1 mM EDTA.

monoanionic ionization state to a monoprotonated active site. Our own results now prove that both the dianionic and monoanionic species are bound to the RNase. On the basis of the ^{31}P and ^{1}H NMR, as well as the x-ray diffraction,[6] studies it is now possible to describe in 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 Scheme I His_{119} and the dianionic nucleotide monophosphates are presumed to interact only slightly.[14b] This would be consistent with our own small upfield ^{31}P 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_{119} resulting from complex formation. The substantial downfield chemical shift of the C_2-H proton of the protonated His_{12} in the 3′- and 2′-CMP complexes 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.

Scheme I[a]



The assignment of pK_2 to titration of His_{12} is largely based upon the similarity of this pK to that obtained from ^{1}H NMR titrations. Yet, as shown in Table III, the pK's for His_{12} in various complexes obtained from ^{1}H NMR studies do not quantitatively agree with the pK_2's obtained from the ^{31}P titrations. Although some of the studies were conducted in H_2O and others in D_2O and under different ionic strengths and temperatures, this cannot explain the differences. Roberts et al.[16] have previously noted that the isotope effect on the ionization constants is nearly balanced by the effect of D_2O on the glass electrode "pH" reading. Instead we propose that the difference between the pK's obtained from the ^{1}H or ^{31}P titrations is real and that, furthermore, may be shown to yield important information about the nature of the interaction between His_{12} and the phosphate group.

Table III. Comparison of Apparent Macroscopic pK's for His_{12} and His_{119} in the RNase Inhibitor Complexes

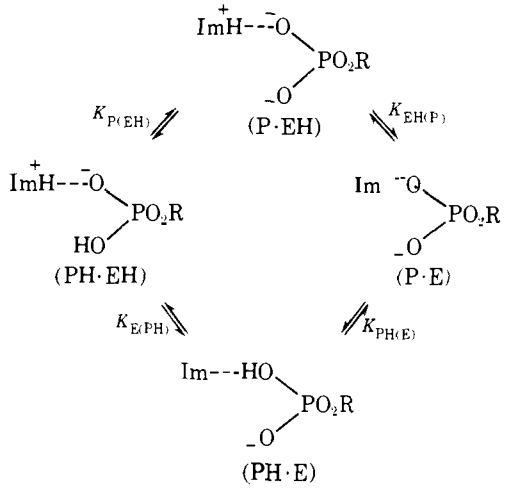
	pK	
Nucleotide	His-119	His-12
None[a]	6.2	5.8
2′-CMP	8.0[b]	>8.0[b,d]
3′-CMP	8.0[b]	7.4,[b] 7.2[d]
5′-CMP	8.0[b]	<7.0,[b] 6.2[d]
2′-UMP[c]	8.0	6.3

[a] In 0.2 M deuterioacetate-D_2O buffer (D. H. Meadows, O. Jardetzky, R. M. Epand, H. H Ruterjans, and H. A. Scheraga, *Proc. Natl. Acad. Sci. U.S.A.*, **60**, 766 (1968)) and in 0.2 M acetate-H_2O buffer (ref 16). [b] In D_2O, 0.2 M NaCl at 32 °C (Meadows et al., 1968). [c] In D_2O, 0.2 M NaCl (W. Haar, W. Maurer, and H. Ruterjans, *Eur. J. Biochem.*, **44**, 201 (1974)). [d] In H_2O, 0.1 M NaCl at 22 °C: J. H. Griffin, J. S. Cohen, and A. N. Schecter, *Biochemistry*, **12**, 2096 (1973).

The pK's obtained by fitting the titration data to eq 1 represent macroscopic ionization constants. Actually two possible monoanionic species may exist and a scheme which relates all four species and the respective microscopic pK's is given (Scheme II).

In the monoanionic state either the phosphate or histidine may be protonated. It is likely that because of the spatial

Scheme II



orientation of the two groups a strong hydrogen bond exists between them (represented by the dashed lines). With few exceptions a hydrogen bond is characterized by a double minima potential energy function with the proton located more on the average near the more basic electron donor. The energy barrier separating these two states is small and on the NMR time scale the proton will jump from one site to the other rapidly and the NMR spectrum will consist of a weighted average of the two states. NMR is potentially capable of determining the populations of the two hydrogen-bonding states if some independent measure of the NMR properties in the two states can be obtained. We propose that the ^{31}P chemical shifts can provide this information.

In general, one does not have enough data from NMR titration studies alone to uniquely define the populations of the two H-bonding states and the four microscopic pK's. In the present case, however, we have important additional information. It has been noted that the ^{31}P chemical shift in acyclic phosphate monoesters is largely determined by the protonation state of the ester and not by its local environment.[3] Cohn[4] pointed out this rather surprising phenomenon after observing little shift of the ATP γ-phosphate signal upon complexation with diamagnetic divalent metal ions other than that explained by a shift in pK. Our own data also support this observation since we find little difference between the chemical shifts of the monoanionic inhibitor bound to the *di*protonated (His_{119} and His_{12}) enzyme and the monoanion free in solution. Similarly the dianionic 2′-CMP bound to the diprotonated enzyme (both His_{119} and His_{12} protonated) also differs little from the free dianionic 2′-CMP chemical shift. Furthermore, it is believed that the ϵ-amino group of Lys_{41} which is protonated throughout the pH region of this study might also H bond to the phosphate, especially to 2′-CMP.

This highly positive active site, which is capable of perturbing the pK of the phosphate from 5.8-6.11 to 4.0-5.0, must have one or more H bonds to the phosphate over the entire pH region. Yet at the pH extrema, little, if any, perturbation of the ^{31}P chemical shift is found. It must be concluded that as with metal ion complexation, H-bond formation has little effect on the ^{31}P chemical shifts of phosphate esters! Then why is the chemical shift of the bound nucleotides influenced by a second titrable group? One possible explanation is that these two macroscopic pK's may be qualitatively viewed as arising from ionization of the same monoanionic phosphate (PH) in the presence of protonated (EH) or unprotonated His_{12} enzyme (E). This interpretation may be used to determine the microscopic pK's of Scheme II.

If we assume that the chemical shift of the phosphate species is *only* dependent on the phosphate ionization state and not on the protonation level of neighboring acids (even if H bonded to the phosphate), then

$$\delta_{P(E)} = \delta_{P(EH)} = \delta_A$$

and

$$\delta_{PH(E)} = \delta_{PH(EH)} = \delta_{H_2A}$$

where δ_P and δ_{PH} refer to the ^{31}P chemical shift of the dianionic and monoanionic phosphate, respectively, and the microscopic ionization states are defined in Scheme II.

These two constraints plus the overall thermodynamic constraint

$$K_{PH(E)}K_{EH(P)} = K_{PH(EH)}K_{EH(P)}$$

and the five resolvable parameters of eq 1 provide enough information to uniquely define the four microscopic pK's. In fact the relationship between the macroscopic and microscopic pK's is made especially simple assuming our chemical shift approximation. Defining a factor ϵ

$$\epsilon = \frac{\delta_A - \delta_{HA}}{\delta_A - \delta_{H_2A}}$$

the microscopic pK's may be related to the five macroscopic parameters (δ_A, δ_{HA}, δ_{H_2A}, K_1, K_2) of eq 1, yielding

$$K_{PH(EH)} = (1 - \epsilon)K_1$$

$$K_{EH(PH)} = \epsilon K_1$$

$$K_{PH(E)} = K_2/\epsilon$$

$$K_{EH(P)} = K_2/(1 - \epsilon)$$

The microscopic pK's calculated according to this model are presented in Table IV.

It is interesting to note that $pK_{PH(E)}$, the ionization constant of a monoanionic phosphate inhibitor bound to an *un*protonated His_{12} enzyme, is quite nearly the same as the second ionization constant of the inhibitor free in solution. There would thus appear to be little significant interaction[17] of the phosphate with the other protonated groups at the active site (His_{119} and Lys_{41}) as indicated earlier and as shown in Scheme I. On the other hand, the pK of the monoanionic phosphate bound to the protonated His_{12} enzyme ($pK_{PH(EH)}$) is perturbed by 1.5-2 pK units except for the 5′-CMP complex. This is consistent with the stabilization afforded by the H bonding between the 3′ and 2′ phosphates and His_{12}.

Other ^{31}P NMR Studies. During the course of this work several related ^{31}P NMR studies on nucleotide RNase A complexes appeared. In a preliminary communication Lee and Chan[18] reported that the ^{31}P signal for the 3′-UMP·RNase A complex is shifted far upfield (ca. 10 ppm) from the free 3′-UMP signal. In addition they observed substantial line broadening of the signal with increasing E_0/I_0 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, as discussed earlier, these large shifts and line broadening effects disappeared.

Ruterjans[19] and co-workers reported ^{31}P NMR spectra and titration studies similar to those described here. However, they have titrated a 1:1 mixture of nucleotide and RNase A and directly observed the ^{31}P NMR signal. As we have shown in our own comparison of the chemical exchange approach to that of the direct observation method, the latter method cannot be used to obtain accurate ^{31}P chemical shifts of the complex since the enzyme will not be

Table IV. Microscopic Ionization Constants of Inhibitor Enzyme Complexes

Complex	Macroscopic constants			Microscopic constants			
	pK_1	pK_2	ϵ	$pK_{PH(EH)}$	$pK_{PH(E)}$	$pK_{EH(PH)}$	$pK_{EH(P)}$
2′-CMP·RNase[a]	3.99 ± 0.02	6.60 ± 0.14	0.121	4.06	5.68	4.92	6.54
3′-CMP·RNase[b]	4.52 ± 0.17	6.52 ± 0.37	0.272	4.66	5.96	5.08	6.38
5′-CMP·RNase[b]	4.99 ± 0.51	5.89 ± 0.59	0.295	5.14	5.52	5.36	5.75
3′-UMP·RNase[a]	4.57 ± 0.66	6.48 ± 0.40	0.316	4.73	5.98	5.07	6.32

[a] Direct observation method in D_2O, 0.1 M ammonium formate, 1 mg/ml of EDTA. [b] Chemical exchange method in H_2O, 0.2 M NaCl, 1 mM EDTA. Direct observation method data provide qualitatively similar microscopic constants. However, data are not believed to be as reliable as the chemical exchange method data because enzyme was not saturated throughout the titration.

completely saturated throughout the pH range of the titration. This is especially evident in the 5′-CMP·RNase titrations where the enzyme is only modestly saturated under Ruterjans conditions. Thus, the single $pK \sim 5.6$ that he reports for the 5′-CMP complex probably is an artifact attributable to this technique.

Other pK's reported by Ruterjans are generally consistent with those obtained here by the direct observation method. The pK difference ($pK_1 - pK_2$) is larger for the direct observation method, presumably reflecting the less accurate chemical shifts of the complex at the extreme pH's where the saturation problem becomes more important. Ruterjans fails to observe a second inflection point in the 2′-CMP complex titration. The origin of this difference is not known.

Ruterjans strongly favors the interpretation that the second pK arises from titration of the proximate His_{12} (or what he calls His_{119}). His own data appear more convincing than ours since he is able to demonstrate an apparent direct correspondence between the His_{12} pK as determined by ^1H NMR titrations and the second pK from the ^{31}P titrations. Thus a complex with 2′-AMP shows pK's of 6.3 and 6.21, and 2′-deoxycytidine 3′-phosphate shows pK's of 6.5 and 6.38 from the ^1H and ^{31}P NMR titrations, respectively.

Mechanism of Action of RNase A and the Role of Hydrogen-Bonding Interactions. Although numerous mechanisms have been proposed for the enzymatic activity of RNase A,[6] several essential features of the pathway have gained wide acceptance. In the first transphosphorylation step, the enzyme binds the 3′-nucleotide phosphate group in such a manner that $histidine_{12}$ lies very near the 2′-OH. Catalysis in the removal of the 2′-OH proton facilitates apical attack on the phosphate and formation of a trigonal bipyramidal pentacovalent intermediate or transition state.[6,20] While the two equatorial oxygens likely possess full negative charges, the proximity of the protonated His_{119} and Lys_{41} allows for considerable stabilization. Apical leaving of the 5′-nucleotide (His_{119} catalyzed) and similar enzymatic catalyzed hydrolysis of the resulting 2′,3′-cyclic nucleotide complete the mechanistic picture.

In most descriptions of the mechanism the two active site histidines are considered to act as general catalysts or hydrogen-bonding, structure stabilizing groups. Support for this view is provided by the x-ray structures of RNase A–inhibitor complexes which show His_{12} and His_{119} next to the phosphate group.[6] In all isomeric cytidine monophosphate complexes the pK of His_{119} is ~8.0. Since this ionization constant is perturbed substantially from the free enzyme His_{119} pK of 6.2, this provides strong support that in solution as well, the histidine and phosphate are spatially proximate and a strong coulombic interaction must exist between them. However, for the cytidine mononucleotides, not only is the fully protonated C_2H peak shifted upfield by only ~0.1 ppm from the free enzyme protonated C_2H signal, but also the ^{31}P shifts of the bound dianionic nucleotide are quite small as well. Possibly these small shifts reflect the condition that the population of the His_{119} protonated H-bonding state is much greater than the phosphate protonated H-bonding state. (Note His_{119} is much more basic than the phosphate.)

While different nucleotides apparently affect His_{119} in the same way, this is not the case with His_{12}. As pointed out by Meadows et al.,[14] the series order 2′-CMP > 3′-CMP > 5′-CMP reflects the order of binding constants, pK's for the His_{12} residue in the complex (8.0, 7.4, ~6.2, respectively), and the additional shift of the fully protonated C_2H proton of His_{12} in the complex (−25, −20, 0 Hz, respectively). In addition, we now find that this same ordering applies to the magnitude of ϵ and the pK's of the phosphate group as derived from the ^{31}P titrations. If ϵ is a measure of the relative fraction of phosphate protonated vs. histidine protonated states in the hydrogen-bonding pair, we expect to find the nucleotide complex with the most basic His_{12} to have the smallest ϵ (i.e., the His-protonated state predominates). Thus the 2′-CMP complex with the most basic His_{12} shows the smallest ϵ, and the 5′-CMP complex, the largest. The phosphate macroscopic pK's in the complex are 3.99 (2′-CMP), 4.52 (3′-CMP), and 4.99 (5′-CMP). A similar ordering is found for the microscopic phosphate ionization constants $pK_{PH(EH)}$. Both the histidine and phosphate ionization constants in the 5′-CMP complex are closest to their "normal" values while perturbed the most in the 2′-CMP complex. This would indicate that the protonated histidine group which hydrogen bonds to the phosphate is closest to the 2′-phosphate and furthest from the 5′ position.

The ^{31}P data and our interpretation of the interaction between the isomeric phosphates and the two histidines is consistent with the x-ray data of Richards and Wyckoff.[6] (Note that the previous reversed assignments of the two histidines would be incompatible with the following argument and thus our data provides additional support for this revision.[14c]) Apparently His_{119} is able to assume a number of different positions in the crystal state which depends upon the structure of the phosphate group bound to the enzyme.[6] In contrast His_{12} is restricted to only one site in all of the complexes. This flexibility of His_{119} and the rigidity of His_{12} explains why only His_{12} pK's and the isomeric phosphate pK's are so dependent upon the geometry of the phosphate esters. As shown by the x-ray work, His_{12} should be closest to a phosphate in the 2′ position but furthest from a phosphate in the 5′ position.

Acknowledgments. Support of this research by the National Institutes of Health (Grant GM-17575 to D.G.G.), the Research Corporation, and the Research Board of the University of Illinois (to D.G.G.) is gratefully acknowledged. Purchase of the Nicolet 1080 FT data system was assisted by an NSF Departmental Equipment Grant to UICC. J.B. was recipient of a grant by Deutsche For-

schungsgemeinschaft; the support by Professor M. A. Raftery is gratefully acknowledged.

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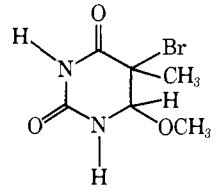
Dehalogenation of 5-Bromo-6-methoxy-5,6-dihydrothymine by Cysteine

Frank A. Sedor and Eugene G. Sander*

Contribution from the Department of Biochemistry, University of Florida, Gainesville, Florida 32610. *Received July 25, 1975*

Abstract: The pseudo-first-order rate constants for the cysteine-promoted dehalogenation of 5-bromo-6-methoxy-5,6-dihydrothymine (BrMDHT) have a strict dependence on the concentration of cysteine thiol anion with a calculated second-order rate constant ($k_2^{cys\text{-}S^-}$) equal to $1652 \pm 77\ M^{-1}\ min^{-1}$. The reaction which is not subject to catalysis by external buffers has thymine, Br^-, cystine, and some cysteic acid as products. Under conditions of excess cysteine, 2 mol of cysteine per mole of BrMDHT is consumed and about 88% of the BrMDHT is converted to thymine, a result which indicates the formation of 6-methoxy-5,6-dihydrothymine. Under conditions of excess BrMDHT relative to cysteine, the ratios of thymine produced to cysteine initially present are consistently above 0.50. These results argue for the participation of the E2 Hal mechanism in this reaction and are discussed in terms of the cysteine and bisulfite buffer promoted dehalogenation of the halouracils.

Halogenated pyrimidine derivatives are important antiviral agents which upon enzymatic dehalogenation lose their pharmacological activity. The pathways for enzymatic dehalogenation are not clearly elucidated; however, several hypothetical schemes have been advanced based on the abilities of bisulfite buffers[1-7] and thiols, such as cysteine and 2-mercaptoethanol, [8-10] to cause the dehalogenation, especially of 5-bromo- and 5-iodouracil. In the case of the dehalogenation of 5-iodouracil by cysteine, the intramolecular general acid catalyzed addition of cysteine to the 5,6 double bond of the pyrimidine ring system to presumably form 5-iodo-6-cysteinyl-5,6-dihydrouracil appears to control the overall rate of dehalogenation.[10] Two possible pathways, both of which have been identified in halopyrimidine dehalogenation by bisulfite buffers,[4-6] are possible for the subsequent dehalogenation of the iodocysteinyldihydrouracil intermediate. The first of these is the E2 Hal mechanism in which the thiol anion of cysteine attacks the halogen atom to initially yield a sulfenyl halide and an enolate anion of 6-cysteinyldihydrouracil. Further reaction then yields the final products: halide anion, cystine, and uracil. The second potential mechanism, proposed for the dehalogenation of 5-bromouracil by cysteine,[9] involves the SN2 attack of cysteine thiol anion on C-5 of the pyrimidine ring to yield halide anion and 5,6-dicysteinyl-5,6-dihydrouracil, which presumably further reacts with cysteine to yield uracil and cysteine. Very recent work in Pitman's laboratory, using both 5-bromo-6-methoxy-5,6-dihydrouracil and -dihydrothymine as a model for the dehalogenation of 5-bromo-5,6-dihydrouracil-6-sulfonate by SO_3^{2-},[5] has demonstrated the feasibility of the E2 Hal mechanism for similar reactions with simple thiols. The object of this report is to demonstrate the existence of the E2 Hal mechanism for the dehalogenation of 5-bromo-6-methoxy-5,6-dihydrothymine (I), a compound which can be considered a model for 5-bromo-6-cys-



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