A ^{31}P NMR STUDY OF THE ASSOCIATION OF URIDINE-3′-MONOPHOSPHATE TO RIBONUCLEASE A

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Summary. The association between ribonuclease A and its product inhibitor uridine-3′-monophosphate has been studied by ^{31}P nmr spectroscopy. Analysis of the chemical shifts and the linewidths of the ^{31}P resonance of the inhibitor over a wide range of enzyme concentrations yielded the apparent dissociation rate constant of 3200 ± 300 sec^{-1} at 32°C for the enzyme-inhibitor complex and an activation energy of 3.5 ± 1.0 kcal/mole for this process. The chemical shift data suggest that the phosphate group of this inhibitor is simultaneously bound to two positively charged amino acid residues at the active site of the enzyme.

Nuclear magnetic resonance spectroscopy (nmr) is now extensively used to probe the active sites of enzymes in solution.[1,2] Most of these studies have been concerned with enzyme-inhibitor interactions[3-7] and information about the active site is often inferred from the effects which the enzyme has on the chemical shifts,[8,9] linewidths,[10] as well as the relaxation times[11,12] of specific resonances of the inhibitor. Of the various types of nmr experiments, high resolution proton magnetic resonance spectroscopy (pmr) has been the most widely employed. While it is often possible to infer structural information from these studies, it is in general difficult to obtain kinetic information because of small chemical shifts and the resultant long time scales of observation.

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In this communication, we wish to report a ^{31}P nmr study of the association of ribonuclease A (RNase A) with its product inhibitor uridine-3′-monophosphate (3′-UMP). Because ^{31}P chemical shifts are usually large, the nmr observation time is in general comparable to the time scales of the enzyme-inhibitor interactions, even at relatively low enzyme concentrations. Moreover, the solvent in these experiments can be H_2O instead of D_2O, so one is no longer faced with questions often raised in connection with the pmr studies regarding the isotopic effect of the solvent on the protein conformation and the enzyme-inhibitor interactions under consideration.

The RNase A used in this work was isolated from bovine pancreas and was obtained from Calbiochem, Los Angeles, California, as an A grade reagent. Its biological activity was shown to be 65 Kunitz units/mg.[13] A grade 3′-UMP was purchased from Calbiochem in the acid form, and was converted to the sodium salt on a Dowex 50W-X8 cation exchanger.

Solutions containing 0.1 M of the inhibitor and various concentrations of the enzyme (1×10^{-4} M to 2×10^{-3} M) were prepared in H_2O, and their pH was maintained at 7.5 ± 0.01 using 0.1 M tris buffer and 0.05 M NaCl. The ^{31}P nmr experiments were performed at a magnetic field of 53 kgauss and the resonance frequency of 89 MHz on a modified Varian HR-220 superconducting nmr spectrometer equipped with frequency sweep and multinuclei capabilities. Proton noise decoupling was used to remove all phosphorous and proton spin-spin couplings and a C-1024 time averaging computer was used to enhance the signal to noise ratio. The ^{31}P chemical shifts were measured relative to an external reference of trimethylphosphite sealed in a capillary. Unless specified otherwise, all the experiments were done at the normal probe temperature of 32 ± 2°C. 5 mm O.D. sample tubes were employed.

The effect of RNase A at various enzyme concentrations on the ^{31}P resonance of 0.1 M 3′-UMP is shown in Fig. 1. The ^{31}P resonance was observed to shift progressively to higher field and to become broader with increasing enzyme concentration. These chemical shifts and linewidths are

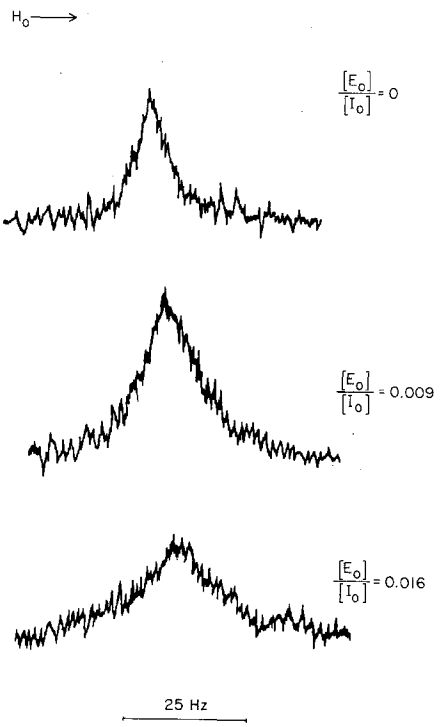

Fig. 1. ^{31}P nmr spectra of 0.1 M 3′-UMP in the presence of Ribonuclease A at various enzyme/inhibitor concentration ratios. (a) $[E_0]/[I_0] = 0$: 5 scans; (b) $[E_0]/[I_0] = 0.009$: 10 scans; (c) $[E_0]/[I_0] = 0.016$: 25 scans.

summarized in Fig. 2 as a function of the enzyme/inhibitor concentration ratio. The observed monotomic variation of the chemical shift and linewidth with enzyme concentration suggests that the kinetics of the enzyme-inhibitor binding is either intermediate or rapid compared to the reciprocal of the ^{31}P chemical shift difference of the inhibitor between the complexed and the unbound states. Although the observed broadening may reflect the shorter transverse relaxation time of phosphorous nucleus when it is bound to the enzyme, an estimation of this transverse relaxation ($\sim$0.02 sec) reveals that it is too long to account for the observed linewidth at the low concentrations of the enzyme used. We therefore surmised that the observed line broadening arises predominantly from the intermediate chemical exchange of the inhibitor between the free and the bound states. In order to substantiate this

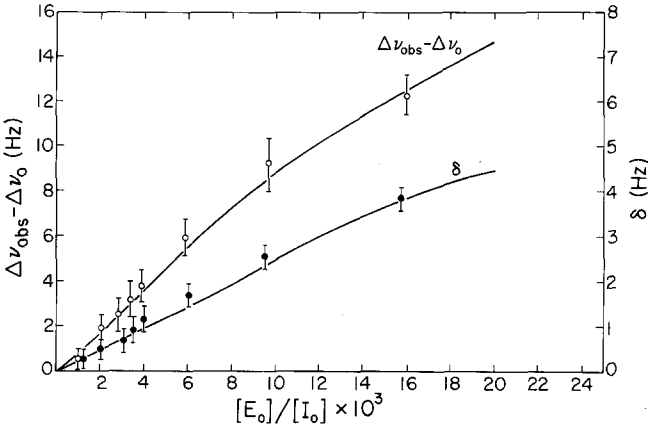

Fig. 2. The chemical shifts (⊥) and the linewidths (⊥) of the ^{31}P resonance of 3′-UMP at various enzyme/inhibitor concentration ratios. $\Delta\nu_{obs}$, $\Delta\nu_0$ denote the linewidths in the presence and the absence of the enzyme respectively; and δ is the observed chemical shift of the inhibitor in the presence of the enzyme relative to that for the free inhibitor. Solid curves were calculated for $\Delta = 800$ Hz and $k_{-1} = 3200\ sec^{-1}$ (see text).

point, we have simulated the ^{31}P spectrum on the digital computer for different enzyme/inhibitor concentration ratios over a range of chemical shifts of the inhibitor between the free and bound states (Δ) and as a function of the kinetic parameters for the overall enzyme-inhibitor reaction:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI$$

E, I and EI denote the enzyme, inhibitor and enzyme-inhibitor complex, respectively, and k_1, k_{-1} are the association and dissociation rate constants. Although it is known that the interaction between RNase A and its inhibitors involves a multistep equilibrium, our nmr measurements are unfortunately only sensitive to those steps with rates comparable to the nmr time scale.

Computer simulation of ^{31}P spectrum clearly indicated that the ^{31}P broadening of the inhibitor arises from the intermediate chemical exchange of the inhibitor between its free and complexed states. In our analysis we have assumed that $[EI]/[I] \approx [E_0]/[I_0]$, so that $k_1[E] = k_{-1}[EI]/[I] \cong k_{-1}[E_0]/[I_0]$. This should be an extremely good approximation at high

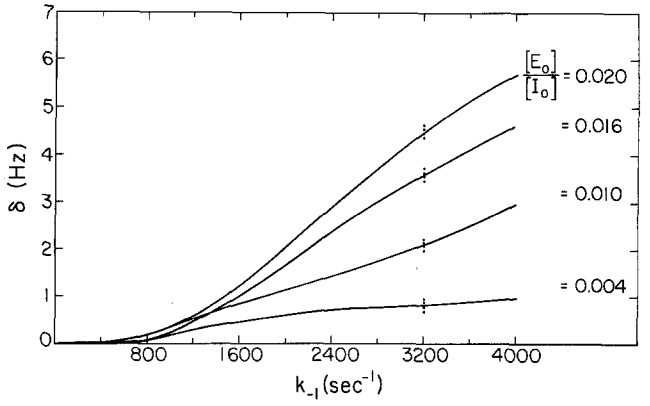

Fig. 3. Variations in chemical shift of the ^{31}P resonance of 3′-UMP with the dissociation rate constant (k_{-1}) for various enzyme/inhibitor concentration ratios and an assumed chemical shift difference (Δ) of 800 Hz between the free and the bound states.

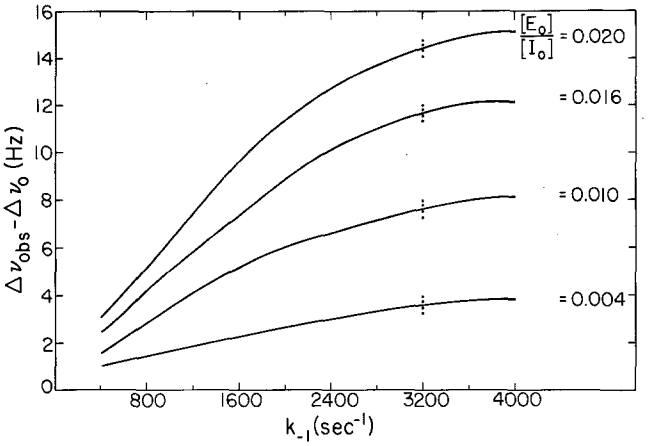

Fig. 4. Variations in the linewidth of the ^{31}P resonance of 3′-UMP with k_{-1} for various enzyme/inhibitor concentration ratios and an assumed Δ of 800 Hz.

inhibitor concentrations and at low $[E_0]/[I_0]$ ratios. The equilibrium constant for the association process is $\geq 10^3\ M^{-1}\ \ell$. Hence at an inhibitor concentration of 0.1 M, $[E]/[EI] \leq 10^{-2}$, and the enzyme should be totally complexed for $[E_0]/[I_0]$ ratios of 10^{-3} to 2×10^{-2}.

Plots of the calculated chemical shifts and linewidths of the ^{31}P resonance versus k_{-1} for $\Delta \approx 800$ Hz are presented in Figs. 3 and 4. Since the

first order dissociation rate constant is independent of the enzyme or the enzyme/inhibitor concentration ratio at a given temperature, the Δ which characterizes the system must be that which yields the observed linewidths and the observed chemical shifts for the same value of k_{-1}. The best fit to our data was obtained for $\Delta \approx +800 \pm 50$ Hz and $k_{-1} \sim 3200 \pm 300$ sec^{-1} at 32°C. The agreement between the observed and the calculated chemical shifts and the linewidths for various enzyme/inhibitor ratios is depicted in Fig. 2 and can be seen to be excellent.

The activation energy for the dissociation process can be determined from a temperature study of ^{31}P resonance widths at fixed enzyme/inhibitor ratios. Examination of the linewidth changes for three enzyme/inhibitor ratios over the temperature range of 10° to 30°C yielded an energy of activation of 3.5 ± 1.0 kcal/mole.

The kinetic parameters which we have deduced from the present ^{31}P nmr study of the association of 3′-UMP to RNase A are in general agreement with the temperature jump measurements of G. Hammes et al.,[14] who succeeded in resolving the kinetics of each step of the enzyme-inhibitor association. Jardetzky et al.[7] have suggested, on the basis of their pmr studies of RNase A-nucleotide complexes and the x-ray structure of RNase S[15, 16] completed by the Wyckoff group at Yale, that the phosphate group of cytidine-3′-monophosphate is simultaneously bound in the dianionic form to the positively charged side-chains of two amino acid residues, lysine 41 and histidine 119, at the active site of the enzyme. Although similar pmr work on 3′-UMP has not been reported, one would nevertheless expect similar bonding of the phosphate group at the active site for this inhibitor. If this were the case, we would expect the ^{31}P resonance of the inhibitor to shift upfield by ~800 Hz at 89 MHz upon binding to the enzyme at a solution pH of ~7.5, where the phosphate exists as the dianion prior to association to the enzyme. Preliminary ^{31}P studies of nucleotides undertaken in this laboratory have shown that protonation of a primary phosphate in general shifts

the ^{31}P resonance upfield by ~400 Hz (at 89 MHz) per protonation step. Thus, the ^{31}P chemical shift of the inhibitor upon association can help to elucidate the involvement of the phosphate group in the enzyme-inhibitor interaction.

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