^{1}H Nmr Study on the Binding of CMP Inhibitors to RNase A. III. Chemical Exchange and Relaxation Effects.

by David G. Gorenstein* and
Alice M. Wyrwicz

Department of Chemistry, University of
Illinois, Chicago Circle, Chicago, Illinois 60680

Received May 10,1974

SUMMARY: A new dynamic ^{1}H nmr method has been developed and applied to the binding of 3'- and 5'-cytidine monophosphate (CMP) to Ribonuclease A. The study has provided information on the rates of chemical exchange and the degree of inhibitor immobilization at the active site. Evidence is presented which indicates that exchange-broadening effects on the line widths result from a slow pH-dependent conformational change of the enzyme•inhibitor complex. At pH 4.5-5.5 and 24° $1/\tau \sim 150\text{-}250$ sec^{-1} for the CMP inhibitors with the exchange rate increasing with increasing pH. The rotational correlation time for the pyrimidine ring of the complex is 4.4×10^{-9} sec and for the ribose ring of the complex is 1.2×10^{-8} sec. These results indicate that the ribose ring is rigidly bound to the enzyme while there remains some residual degree of rotational freedom about the glycosidic bond.

We have previously shown in ^{31}P and ^{1}H nmr chemical exchange studies[1,2] how information on the structure of the cytidine monophosphate•ribonuclease A complex may be obtained from chemical shift titration data. In the present communication we wish to describe the perturbations of the line widths at half height that were observed in the ^{1}H nmr experiment. In contrast to spectral line positions, line-width data provides information not only on the structures of molecules but also on the molecular motions and the rates of chemical exchange[3-6].

Jardetzky has already noted that the ^{1}H nmr signals of CMP inhibitors are broadened upon binding to RNase A.[7] Other workers have observed similar effects as well[8]; however, no attempt has been made in these studies to quantitatively analyze these line broadening effects which can provide much significant information on the nature of the enzyme•inhibitor complex.

In addition, with this study we provide the first demonstration that a direct separation of exchange broadening from other line broadening effects is possible.

All solutions of the Bovine pancreatic ribonuclease A (RNase A) and the CMP inhibitors were prepared as previously described.[2] ^{1}H nmr spectra were recorded either on a Bruker HFX-90 high resolution spectrometer or on a Bruker B-KR 322S pulsed spectrometer as described in that paper.[2] Care was taken to insure that the radio frequency power was below the saturation level, so that no distortion of the signals was observed when the same spectra were taken either in the cw or pulsed

mode operation of the nmr spectrometers. The temperature of the nmr sample in the probe was read directly from the thermocouple meter on a Bruker B-ST 100/700 temperature regulator which was standardized just prior to a run against methanol chemical shifts. Unless otherwise noted, spectra were taken at 24 ± 2^{o}.

The line width in Hertz at half height, $\Delta\nu_{\frac{1}{2}}$, of an nmr signal is related to the transverse relaxation time, T_2, by eq 1:

$$\Delta\nu_{\frac{1}{2}} = \frac{1}{\pi T_2} \cdot \qquad (1)$$

In the binding of an inhibitor to the active site of an enzyme, it may be shown that the observed line width represents a weighted average of the line width of the inhibitor in solution, I, and of the enzyme·inhibitor complex, E·I, assuming that chemical exchange of the inhibitor between these two sites is sufficiently rapid:[9]

$$(\Delta\nu_{\frac{1}{2}})_{obs} = \frac{1}{\pi(T_2)_{obs}} = (\Delta\nu_{\frac{1}{2}})_{EI} \cdot \frac{(E\cdot I)}{I_o} + (\Delta\nu_{\frac{1}{2}})_{I} \cdot \frac{(I)}{I_o} + (\Delta\nu_{\frac{1}{2}})_{ex} \qquad (2)$$

In addition to this weighted average relaxation time contribution to the observed line width (the first two terms of eq 2), a third term, $(\Delta\nu_{\frac{1}{2}})_{ex}$, the chemical exchange broadening term, appears. The line broadening observed upon addition of enzyme to the inhibitor can thus result from a decrease in the transverse relaxation time of the E·I complex relative to the T_2 of the inhibitor in solution as well as from the dynamics of the exchange process.

Under the experimental conditions in this study,

$$(E\cdot I) \sim E_o, \quad (I) \sim I_o - E_o$$

and substituting into eq 2 and rearranging

$$(\Delta\nu_{\frac{1}{2}})_{obs} = (\Delta\nu_{\frac{1}{2}})_{I} + \Delta(\Delta\nu_{\frac{1}{2}}) \cdot E_o/I_o + (\Delta\nu_{\frac{1}{2}})_{ex} \qquad (3)$$

where $\Delta(\Delta\nu_{\frac{1}{2}}) = (\Delta\nu_{\frac{1}{2}})_{EI} - (\Delta\nu_{\frac{1}{2}})_{I}$.

The chemical exchange term follows from the solution of the Bloch equations[9] for exchange between two sites:

$$(\Delta\nu_{\frac{1}{2}})_{ex} = \frac{(1-E_o/I_o)^2 \cdot E_o/I_o \cdot 4\pi\Delta_\delta^{\,2}}{k_{-1}} \qquad (4)$$

where

$\Delta_\delta = \delta_{EI} - \delta_I$ (the chemical shift difference between the tw sites in Hz) and k_{-1} is the rate of dissociation of the E·I complex. This assumes that the exchange rate is in the fast exchange region

$$k_{-1} \gtrsim (2\pi\Delta_\delta).$$

The form of eq 3 provides an unambiguous method for separating the weighted average term from the chemical exchange term. Thus, a plot of $(\Delta\nu_{\frac{1}{2}})_{obs}$ vs. E_o/I_o will be non-linear if the exchange term makes a significant contribution to the line broadening and high enough enzyme-inhibitor ratios are achieved (the cubic function $E_o/I_o(1-E_o/I_o)^2$ curves significantly only at $E_o/I_o \gtrsim .2$. See the curved dashed line in Fig. 1). Both non-linear and linear plots of the line widths are observed in the binding of 3'- and 5'-CMP to RNase A and representative plots are shown in Figs. 1,2 and 3. We believe this represents the first time such non-linear behavior

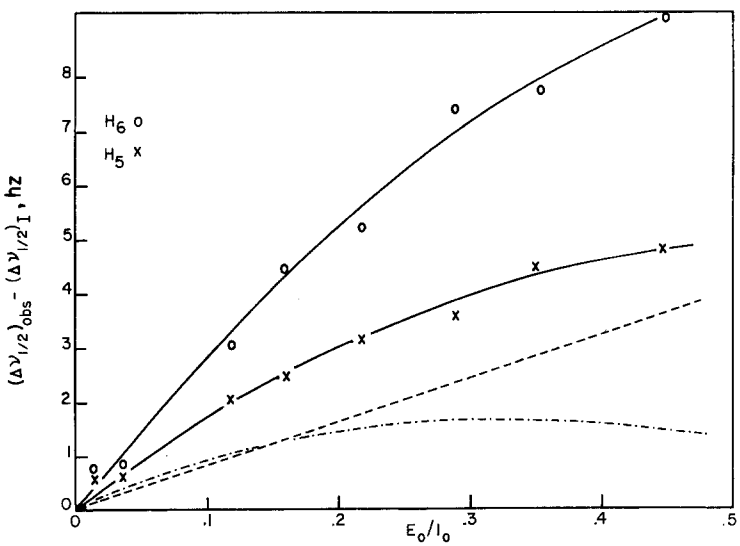

Figure 1. Plot of the line broadening in Hz as a function of E_o/I_o at pH 5.5, .2 M NaCl in D_2O (24°) for the H_6(o) and H_5(x) resonances of 3'-CMP. Dashed/dotted line (.-.-.) represents the exchange broadening contribution to the line width of the H_5 signal calculated from eq 4 with the assumption of two-site exchange, k_{-1} = 200 sec^{-1}. Linear dashed line (---) represents the non-exchange broadening contribution to the line width of the H_5 signal calculated from eq 3. (RNase A) = .009 - .018 M; (3'-CMP) = .03 - .10 M.

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has been observed in studies of this type. While the method serves as a useful alternative to the traditional relaxation times approach,[5] it may only be applicable in the case of low molecular weight, soluble proteins such as RNase where it is possible to attain sufficiently high E_o/I_o ratios at reasonable inhibitor concentrations. With the increased application of pulsed, Fourier transform (FT) nmr, however, this limitation may in the future be overcome.

Since Δ_δ is independently obtained from a study of the chemical shift effects produced by the enzyme[2], k_{-1} may be determined. A best fit estimate of k_{-1} was

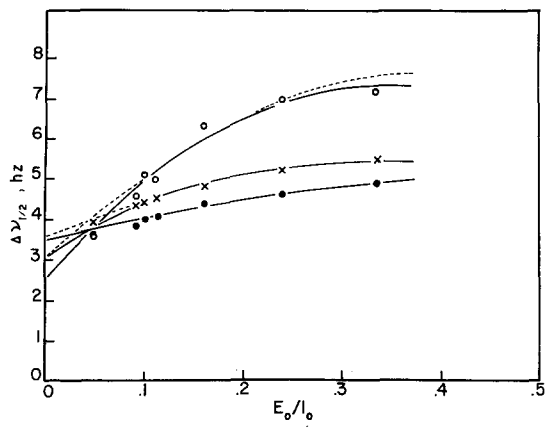

Figure 2. Plot of line width as a function of E_o/I_o at pH 5.0, .2M NaCl in D_2O (24°) for the H_6(○), H_5(●), and H_1'(x) resonances of 5'-CMP. Solid lines represent a best fit of the data to eq 3 assuming the two-site exchange model and k_{-1} = 200 sec^{-1}. The dashed lines (including overlap with the solid curves) represent a fit to the line widths calculated from a three-site exchange model. (RNase A) = .0087 M; (5'-CMP) = .026 -.18 M.

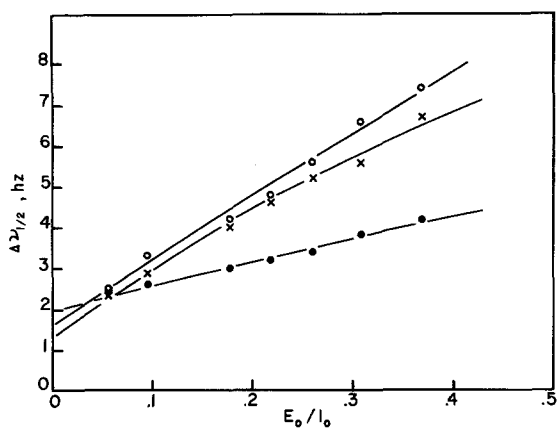

Figure 3. Plot of line width as a function of E_o/I_o at pH 6.5, .2M NaCl in D_2O (24°) for the H_6(○), H_5(x), and H_1'(●) protons of 5'-CMP. Both two-site and three-site models give a similar fit to the data with $1/\tau > 300$ sec^{-1}. (Rnase A) = .0044 - .010M; (5'-CMP) = .03 -.08M.

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selected which minimized the sum of the squares of the residuals in the two parameter equation 3, using the <u>combined</u> line-width data for the H_5, H_6, and H_1' protons of the CMP inhibitors. In the pH region 4.5-5.5 where sufficient curvature to the plots allows separation of the exchange term, analysis of the 3'- and 5'-CMP

line-width data gives a "best fit" k_{-1} = 150-250 sec^{-1}. At other pH's k_{-1} > 250 sec^{-1}.

Up to now we have only been assuming that the exchange rate falls within the fast exchange region. The temperature dependence of $1/T_2$ provides a test of this hypothesis since k_{-1} increases with temperature according to the Arrhenius activation energy expression $k_{-1} = Ae^{-E_a/RT}$, where A is the preexponential factor, R is the gas constant, T is temperature, and E_a is the activation energy. If chemical exchange contributes significantly to the line broadening then an increase in temperature should decrease the transverse relaxation rate and hence decrease the line width of the signal (see Figure 4). One must be careful to not mistake normal

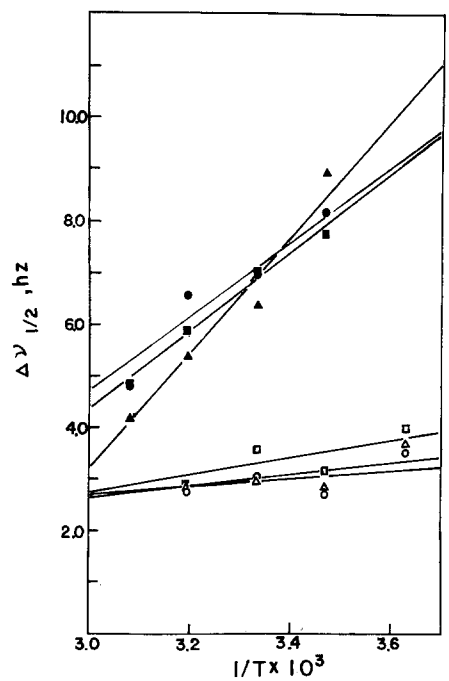

Figure 4. Temperature dependence of the line widths for the H_6(○), H_5(△), and H_1' (□) resonances of free 3'-CMP and the H_6(●), H_5(▲), and H_1' (■) resonances of 3'-CMP in a .19 E_o/I_o solution of Rnase A, pH 5.5 (D_2O). (Rnase A) = .0165M.

sharpening of non-exchanging proton signals with increasing temperature which is associated with a decrease in the correlation time,[9] τ_c, of the molecule. (Note that in Figure 4 the line widths for the inhibitor do in fact decrease with increasing temperature.) Most importantly, however, the line widths of the enzyme•inhibitor complex decrease with increasing temperature even more rapidly than the line widths for the free inhibitor. This would support our interpretation

that curvature in the line width plots results from a significant contribution of exchange broadening to the line widths.

The exchange rates calculated assuming a simple two-site exchange model are smaller by a factor of ca 10 than the rate constants measured for dissociation of the 3'- CMP•RNase A complex as determined by Cathou and Hammes[10] in a temperature-jump fast kinetics experiment. Thus, at pH 5.5, $k_{-1} \sim 250$ sec^{-1} for the 3'-CMP complex. Hammes reports $k_{-1} = 6500$ sec^{-1} for dissociation of the 3'-CMP•RNase complex. On the other hand, the nmr exchange rate is quite comparable to a second slower relaxation process which Hammes has ascribed to an isomerization of the E•I complex with $1/\tau \sim 350$ sec^{-1}, at pH 5.6 in D_2O at 25°. This indicates that the exchange broadening we do observe must originate from the much slower conformational isomerization of the enzyme•inhibitor complex. We therefore prefer to analyze the line-broadening effects in terms of a three-site exchange model shown in Scheme I.

Scheme I.
$$HE + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HE{\cdot}I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} HE'{\cdot}I \overset{K_a}{\rightleftharpoons} E'{\cdot}I + H^+$$

The inhibitor first binds to the protonated enzyme (HE) to give an initial complex HE•I. This first complex isomerizes to HE'•I which can lose a proton to give E'•I. On the nmr time scale protonation of E'•I must be fast so that a three-site chemical exchange model may be used to describe the line-width and chemical shift changes observed in this system. A three-site analysis gives comparable best fits to t data and exchange rates at lower pH similar to those obtained by a two-site analysis. Most importantly though, the line-broadening effects may now be associated with a slow pH-dependent conformational change of the enzyme•inhibitor complex, consistent with the fast kinetics analysis of Hammes.[10]

Dynamic nmr studies can provide information not only on the chemical environment and rates of chemical exchange of a small molecular probe, but also on the overall motion of the molecule in solution. A correlation time, τ_c, associated with the rotational motion of the molecule can be obtained from the relaxation times of nuclei in the molecule. An advantage to the non-linear line width approach of the present study is that the correlation time is a single valued function of the line width in the absence of exchange broadening. On the other hand in the alternative relaxation time method, the spin-lattice relaxation time defines two different possible values for $1/\tau_c$, one greater and one smaller than the Larmor frequency. Thus in the absence of other data, there can be some ambiguity in the correlation times derived from direct relaxation times studies. Intramolecular dipole-dipole interactions as modulated by rotational diffusion are often assumed[3] to be largely responsible for proton relaxation. The line width of a proton signal in the absence of exchange broadening will then be approximately given by

$$(\Delta\nu_{\frac{1}{2}}) = \frac{1}{\pi T_2} = \frac{C\tau_c}{\pi} \sum_i \left(\frac{1}{r_i}\right)^6 \qquad (7)$$

where r_i is the internuclear distance between the observed nucleus and all other proximate nuclei, i, measured in Angstroms and C is a constant equal to 8.85×10^{11} for the assumed dipole-dipole relaxation mechanism between protons only.[3]

The line widths in the absence of exchange broadening do not in general follow any apparent pH dependency. Therefore, the line widths were averaged over all pH's, giving $(\Delta\nu_{\frac{1}{2}})_{H_5} = 9.3 \pm 2.9$ Hz, $(\Delta\nu_{\frac{1}{2}})_{H_6} = 10.6 \pm 2.1$ Hz and $(\Delta\nu_{\frac{1}{2}})_{H'_1} = 11.6 \pm 2.9$ Hz for the 3'-CMP complex and $(\Delta\nu_{\frac{1}{2}})_{H_6} = 10.5 \pm 3.8$ Hz and $(\Delta\nu_{\frac{1}{2}})_{H_5} = 10.1 \pm 3.2$ Hz for the 5'-CMP complex. Using the intramolecular distances in CMP molecules having an anti conformation,[11] analysis of these line widths yields a rotational correlation time for the pyrimidine ring of the complexes of 4.4×10^{-9} sec and for the ribose ring of the complex of 1.2×10^{-8} sec (comparable to the correlation time for the backbone carbons of the enzyme [12]). These results indicate that the ribose ring is rigidly bound to the enzyme while there remains some residual degree of rotational freedom about the glycosidic bond.

Acknowledgment. Suport of this research by the National Institutes of Health (Grant GM-17575), the Research Corporation and the Research Board of the University of Illinois is gratefully acknowledged. Purchase of the Nicolet 1080 Fourier transform data system was assisted by an NSF Departmental Equipment Grant. We also thank Dr. Ben Bangerter of this department for help in the setting-up of the nmr spectrometers.

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