PROTON EXCHANGE OF NUCLEIC ACIDS: THE AMINO PROTONS OF 5'-AMP AND POLY A*

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SUMMARY: Approximate values of the hydronium and hydroxyl second order rate constants for the exchange of the amino protons of 5'-AMP and Poly A were obtained from the observed variation with pH of the 100 MHz PMR resonance signal line width for the amino protons. These values are several orders of magnitude lower than corresponding rate constants for amino protons and purine hydrogens, which may resolve some important interpretative difficulties that are encountered both in the hydrogen exchange of helical polynucleotides and in recent data on the exchange of the non-hydrogen bonded amino protons of DNA.

INTRODUCTION

It has been observed recently that all the amino protons of helical polynucleotides exchange at rates that are slow enough to be measured by the gel filtration technique, whose earliest kinetic point is about 10 seconds (1,2,3). It was expected that only half of the amino protons of the macromolecules would exchange at slow rates, since it was assumed that the amino groups of adenine, guanine and cytosine are "normal bases" (fast exchange) and only one of the protons from each amino group would be hindered in exchange by its involvement in internucleotide hydrogen bonding (4,5). Thus far, no definitive studies on the proton exchange of the amino groups of DNA monomeric units are available to support the assumption that the exchange of the amino groups of the bases is as expected; diffusion controlled with rate constants for hydronium and hydroxyl catalyis of the order of 10^{10} M^{-1} sec^{-1}.

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We report estimates of proton exchange and the catalytic rate constants, which are derived from the $-NH_2$ proton resonance of 5'-AMP and poly A measured from spectra taken at 100 and 300 MHz. Rate constants for H_3^+O and OH^- are estimated from an empirical method involving the observed variation of the half-height width as a function of pH. These values are of sufficient accuracy to demonstrate that the rate constants are two to three orders of magnitude lower than those for amines (6) and purine hydrogens (7). In so far as these rates represent controls for the exchange of DNA hydrogens in the absence of secondary structure, a revision of the interpretations on hydrogen exchange of polynucleotides and calculation of helical parameters (4,10) is necessary.

MATERIALS AND METHODS

Adenosine 5'-monophosphate · Na^+ (Sigma) and polyadenylic acid · K^+ (Miles) were dissolved in double distilled water without further purification, brought to the desired pH and filtered through Millipore HA.45μ filters. The pH was checked once more immediately after the experiment. PMR spectra were obtained with the Varian HA 100 Nuclear Magnetic Resonance Spectrometer in the frequency sweep mode and locked on solvent water. In the case of 5'-AMP at 28°, the RF phase was adjusted to give complete absorption mode by maximizing the signal symmetry of the H-2 and H-8 protons. Signal intensity for the amino proton, which was about equal to the half-height width in this case (signal/noise = 10 to 20), was determined by adjusted extension of the recorded water base line through the $-NH_2$ absorption to give a two proton area (from H-2) for the $-NH_2$ resonance (8). Temperature was controlled with a Varian Variable Temperature Controller and monitored by chemical shift measurements of acidified methanol. Experiments at 300 MHz were performed with the Varian HR 300 NMR Spectrometer at Varian Associates, Inc., Palo Alto, California.

RESULTS

The amino proton signals for 5'-AMP and poly A (0.2 M with respect to

phosphorus for each) lie about 2 ppm downfield of the water signal where the water base line makes an equal contribution to the total intensity. For 5'-AMP, the assignment of the signal was verified by taking the spectra at 300 MHz, where the increased ($-NH_2$)-water proton line separation (5.5 ppm) permitted an accurate estimate of the amino signal area (two protons) in comparison to the single proton H-2 and H-8 resonances. The half-height width of the amino proton signal obtained from the 300 MHz spectrometer was the same (or smaller) than that from the equivalent 100 MHz experiments. This indicates that the exchange is at the slow exchange limit and, therefore, that line width measurements are sufficient for approximate estimates of proton lifetimes (9). As has been seen with the pyrimidines (12) and Tris (13), N-H decoupling** did not sharpen the amino proton signal at 100 MHz. Since this indicates that the nitrogen quadrupole is not a sensible factor contributing to linewidth we define the half-height width as a sum of line widths corresponding to relaxation and exchange processes:

$$(\Delta\nu_{1/2})_{obs} = (\Delta\nu_{1/2})_{T_2+ex} + (\Delta\nu_{1/2})pHex \qquad (1)$$

where $(\Delta\nu_{1/2})_{obs}$ is the observed half-height width and $(\Delta\nu_{1/2})_{T_2+ex}$ is the line broadening due to all processes that are independent of pH. These include the individual contribution of pH-<u>independent</u> exchange (ex) and the natural line width in the absence of exchange (T_2) due to relaxation processes and field inhomogeneity. The last term, $(\Delta\nu_{1/2})pHex$, represents all pH-dependent processes that contribute to line broadening and we assume further that these constitute exchange only, i.e.,

$$(\Delta\nu_{1/2})pHex = \frac{1}{\pi} \left(\frac{1}{\tau}\right)pHex \qquad (2)$$

where $\left(\frac{1}{\tau}\right)pHex$ is the specific exchange rate, or pseudo first order rate constant ($\frac{1}{[-NH_2]}$ x exchange velocity) and $\frac{1}{\pi}$ remains from conversion of

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**Nuclear Magnetic Resonance Specialties, Inc., HD60A Heteronuclear Decoupler

radians sec^{-1} to cycles sec^{-1} in the calculation of exchange (9). If the pH is varied, then

$$\frac{1}{\pi}\left(\frac{1}{\tau}\right)pHex = \frac{1}{\pi} k_{H_3^+0} [H_3^+0] + k_{OH^-} \frac{Kw}{[H_3^+0]} \quad (3)$$

can be substituted into eq. 1 for calculation of a theoretical pH dependence of the observed line width, where $k_{H_3^+0}$ and k_{OH^-} are the second order rate constants for hydronium and hydroxyl ion catalysis respectively and K_w is the ion product of water (10^{-14} at 28° and 10^{-15} at 0°). The variation of $(\Delta\nu_{1/2})_{obs}$ with pH for poly A (28°), 5'-AMP (28°) and 5-AMP (0°) is illustrated in Figure 1, and the dashed lines through each of the three sets

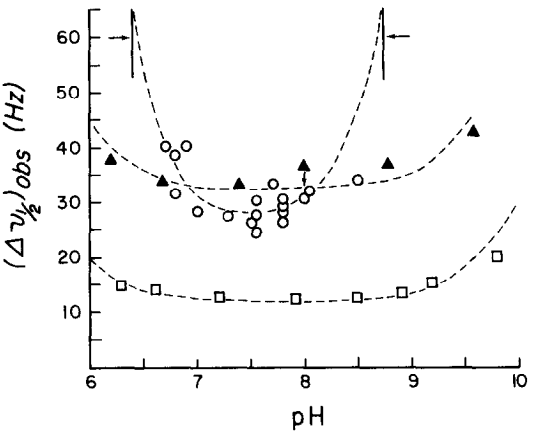

Figure 1. The effect of pH on the observed line width of the amino proton resonance signals of Poly A at 28° (Δ), 5'-AMP at 28° (0) and 5'-AMP at 0° (□). The curves (----) were obtained from eq. (1) and (3) and the assignments listed on Table I. For 5'-AMP at 28°, the amino proton resonance signal was too broad for detection outside the region indicated by the arrows.

of points are obtained from equations (1), (2), and (3). The assignments for $(\Delta\nu_{1/2})_{T_2+ex}$ and the second order rate constants used in the equations to obtain the calculated curves are listed in Table I along with rate constants for "normal" nitrogen bases for comparison. The latter, which represent diffusion controlled rate processes (6) are two to three orders of magnitude larger than those for the adenine amino protons. The values of $(\Delta\nu_{1/2})_{T_2+ex}$ represent 80% of the observed line width for 5'-AMP at 28° and 90% for poly A at 28° and 5'-AMP at 0°. Strictly speaking, this term for 5'-AMP is not

TABLE I

Assignment for calculation of $(\Delta\nu_{1/2})_{obs}$ vs pH[d].

Compound	$(\Delta\nu_{1/2})_{T_2+ex}$	$k_{H_3^+O}$ (M^{-1} sec^{-1})	k_{OH^-} (M^{-1} sec^{-1})
5'-AMP (28°)[a]	22 Hz	3×10^8	2.5×10^7
5'-AMP (0°)	11 Hz	$\leq 3 \times 10^7$	$\leq 6 \times 10^6$
Poly A (28°)	32 Hz	$\leq 4 \times 10^7$	$\leq$ 2-5 $\times 10^6$
Aliphatic and aromatic amines[b]	--	$1\text{-}5 \times 10^{10}$	1×10^{10}
Purine[c]		2.5×10^{10}	

a. All compounds at 0.2 M (P).
b. Taken from reference (6).
c. Taken from reference (7).
d. Equation (1) and (3).

entirely pH-independent, since added phosphate ion exhibits concentration-dependent line broadening (8). Some intra-species catalysis may occur from the secondary ribose phosphate ion, whose mole fraction varies in the pH range used. Also, part of the phosphate influence on the line width may be due to possible alterations in proton relaxation times associated with its interaction with the purine ring (11). These effects will be considered in a later publication (8) and for purposes of discussion on helical polynucleotides (below) we neglect these, since they are not large and not relevant to the macromolecule. We assume further that the effect of the phosphate ion on $k_{H_3^+O}$ and k_{OH^-} associated with the attraction and repulsion of the ion catalysts is not large here (6).

DISCUSSION

In spite of the marked scatter of the data for 5'-AMP at 28° the empirical extraction of rate constants from broad lines by curve fitting provides fairly unique values. This arises from the additive nature of eq. 2 and the high sensitivity of the second term to changes in pH (see eq. 3).

Thus, manipulation of the assigned magnitudes of $(\Delta\nu_{1/2})_{T_2+ex}$, $k_{H_3^+0}$ and k_{OH^-} are quite limited if the proton line changes from a visible absorbance (however broad) to one that is undetectable in the span of less than 0.4 pH units. The calculated curve for 5'-AMP was fitted to the bias of overestimation and is accurate to within a factor of two. This and the profound lack of pH dependence for 5'-AMP at 0^o and poly A at 28^o is sufficient to illustrate clearly that the rate constants are several orders of magnitude lower than previously assumed values based on rates for aliphatic and aromatic amines (4,5).

The kinetic description for the hydrogen exchange of native DNA is:

$$k_{obs} = K\, k_3 \qquad (4)$$

where k_{obs} is the first order rate constant for the disappearance of labeled hydrogen from the macromolecule and K is the equilibrium constant for the opening process required for the exchange of hydrogens from the macromolecule to solvent, ie., $K = \frac{[open]}{[closed]}$ (4).

In this equation k_3 is the rate of exchange in the absence of secondary structure and is equivalent to $(\frac{1}{\tau})$pHex, since estimates of k_3 were based on the relation

$$k_3 = k_{H_3^+0}\ [H_3^+0] + k_{OH^-}\ \frac{Kw}{[H_3^+0]}$$

and the assumption that $k_{H_3^+0} \simeq k_{OH^-} \simeq 10^{10}\ M^{-1}\ sec^{-1}$. Thus, at neutrality K was estimated from eq. 4 to be 10^{-6}, since k_{obs} for the DNA macromolecule was $\simeq 10^{-3}\ sec^{-1}$ (4). However, $k_{H_3^+0}$ and k_{OH^-} obtained for the monomer amino protons yield a value of $K = 5 \times 10^{-4}$ to 10^{-3} (at pH 7.5), which is more consistent with the low activation energy character of helical proton exchange (10). This also indicates that the slow exchange observed recently for non-H-bonded amino protons of the double helix is only $\simeq$ 100-fold slower than that of the monomeric amino protons. This is a more reasonable comparison than that encountered from the assumption of fast amino proton exchange and is easily rationalized by ionic effect and steric

hindrance of the helical grooves (8). Similar values for $k_{H_3^+O}$ and k_{OH^-} have been obtained for the amino protons of the 3'-, 5'- and cyclic phosphates of adenosine and guanine (8) and evaluation of the separate catalytic and relaxation processes that broaden these resonance lines is underway to confirm the use of mononucleotide exchange as a model for hydrogen exchange in the macromolecule.

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