Amino Proton Exchange Processes in Mononucleosides

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Abstract: Amino proton exchange with water could be a useful probe of nucleic acid structure and kinetics. This requires an understanding of exchange processes in the mononucleosides, which has been only partial up till now. We investigate these processes by nuclear magnetization transfer in cytidine, cAMP, cGMP, and endocyclic-methylated derivatives. From the effect of pH on the exchange rate, we disentangle the exchange processes which involve the neutral nucleoside or the nucleoside protonated on an endocyclic nitrogen, and those which operate by basic catalysis (proton transfer to OH⁻ or to water) or by acid catalysis (proton transfer from hydronium). (*Cyclic AMP*) At basic pH, the amino proton transfers from the neutral nucleoside to OH⁻. Between pH 7 and pH 5, it transfers from the endocyclic-protonated nucleoside to water. Endocyclic protonation lowers the deprotonation pK of the amino group by about 9 units. This is confirmed by the effect of proton acceptors such as ammonia on amino proton exchange. Below pH 5, another process sets in. By reference to proton exchange in primary amides, we identify it tentatively as acid catalysis by transfer from hydronium to the amino group of the neutral nucleoside. A protonation pK of −6 is derived. (*Cytidine*) At basic pH, amino proton exchange proceeds by transfer from the neutral nucleoside to OH⁻. Below pH 6, the amino proton transfers from endocyclic-protonated cytidine to OH⁻ (pH >4) or to water (pH <4). As in cyclic AMP, endocyclic protonation lowers the deprotonation pK of the amino group by about 9 units. Acid catalysis is not observed. (*Cyclic GMP*) At basic pH, the amino proton transfers from the neutral nucleoside to OH⁻. Endocyclic protonation (at N7) does not accelerate exchange. This indicates that it has only a weak effect on the deprotonation pK of the amino group, an explanation supported by the lack of an effect of proton acceptors such as ammonia on amino proton exchange. Below pH 6, exchange is acid-catalyzed as it is in cAMP. A protonation pK of −6.3 is derived. Similar processes occur in the methylated derivatives. In 7-methylguanosine, there are two base-catalyzed and two acid-catalyzed exchange processes. In all cases, a quantitative description of the exchange rates is obtained. Application to base-paired oligonucleotides is discussed briefly.

Introduction

In the last decade, the study of imino proton exchange kinetics has led to a description of nucleic acid base-pair opening,[1−3] providing information on internal motions and/or the structure of t-RNA, Z-DNA, B′-DNA curving sequences, drug−DNA complexes, and the intercalated C·C⁺ duplexes of the i-motif.

Amino proton exchange could be similarly useful. It is much slower in B-DNA,[4,5] in tRNA,[6] in chromomycin complexes,[7] and in Z-DNA[8] than in mononucleosides (minutes rather than seconds). This is particularly surprising for the external amino proton which would be expected to exchange quickly by transfer to hydroxyl. In the intercalated duplexes of the i-motif[9] and in triple helices,[10] the amino protons provide markers of paired protonated cytidine. The spectral resolution of A and G amino protons is poor in B-DNA, but it is better in four-stranded G-quartets,[11,12] in structures containing a protonated adenine,[13] and in the A·A and G·G base pairs of π-DNA duplexes.[14]

A prerequisite for the interpretation of amino proton exchange in base-paired oligomers is the understanding of the exchange processes in mononucleosides. However, this is complicated by rates which may be inconvenient for NMR measurements, by rotation of the amino group, and by chemical shifts in a spectral range crowded by aromatic and H1′ protons. Furthermore, the high deprotonation pK of the amino group, in the range of those of peptidic protons, ca. 20, slows proton donation. A consequence is that, at neutral or acidic pH, protonation of the amino group by proton transfer from H_3O^+ may be a more efficient path for exchange than deprotonation by proton transfer to water.

Amino proton exchange of nucleic acid monomers has been studied previously by stopped-flow UV spectroscopy,[15−18] by tritium labeling,[19] by proton NMR,[20−27] and by ¹⁵N NMR.[28] At

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basic pH, abstraction of an amino proton by OH^- produces exchange with water. At neutral and acidic pH, exchange in A and C is catalyzed by general bases, as shown by McConnell and Politowski,[26] who thus identified the nucleoside protonated on an endocyclic nitrogen as the amino proton donor. Using methylated analogs, they determined that the amino proton pK in this species is shifted down to 9. In the case of guanosine, the catalytic effect of general bases was first negated[25] and later affirmed,[18,29] which leaves standing the question of a pK shift.

Büchner et al.[28] studied the pH dependence of the exchange rate in nucleoside monophosphates by ^{15}N NMR. For GMP, they observed a sharp increase at low pH, in contrast to CMP and AMP. They tentatively ascribed this to protonation of the amino group.

These early studies were hampered by technical limitations: stopped-flow UV spectroscopy and tritium labeling do not directly assign the exchanging protons; exchange studies in nucleoside monophosphates are complicated by the effects of the phosphate group; NMR line broadening measurements are limited to exchange times shorter than 100 ms.

This prompted us to undertake a systematic investigation of amino proton exchange by proton NMR. Our measurements span the pH range of $0-10$. Magnetization transfer is measured in 90% D_2O, where it is essentially unaffected by the dipolar interaction between amino protons, so that one can determine exchange and rotation times as long as 3 s. Measurements at very low pH expose the role of protonation of the amino group of the neutral molecule. The set of all measured rates is interpreted quantitatively by definite exchange mechanisms. We have been guided by the illuminating investigation of proton exchange in primary amides by Redfield and Waelder.[30]

Materials and Methods

(1) Nomenclature. The nucleic acid monomers used here, either nucleosides or cyclic monophosphates, are designated "nucleosides" for simplicity. For each nucleoside, the states of interest in the pH range considered (pH $0-10$) are the neutral state, designated N, and a charged state, protonated or methylated on an endocyclic nitrogen, designated E (Chart 1). Exchange processes are labeled by the nucleoside state, with the catalytic group as a superscript (water (W) or hydroxyl (OH), proton acceptors; OH_3^+ (P), proton donor) e.g., E^W. A pK may be designated by the donor group, always in its protonated state, and by the state of the nucleoside; e.g., $pK(N6H_3^+;A,N)$ (also $pK(N6H_3^+;N)$) is the pK for protonation of the amino group of neutral adenosine. Similarly, $pK(H_2O)$ refers to the H_2O/OH^- equilibrium.

(a) Proton Exchange Mechanisms. (a) Nucleoside Donor. Proton transfer from a nucleoside, nuH, to an acceptor, acc, proceeds via a transition complex, which may form by a diffusion-controlled collision:[1,31]

$$nuH + acc \Leftrightarrow \{nu-H\cdots acc\} \Leftrightarrow \{nu^-\cdots H^+-acc\} \Leftrightarrow$$
$$nu^- + accH^+ \quad (1)$$
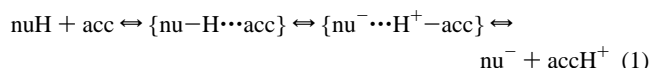
The complex is assumed to dissociate to the left or right side of eq 1 according to the free energies of the products. Exchange occurs by dissociation to the right, followed by reprotonation.

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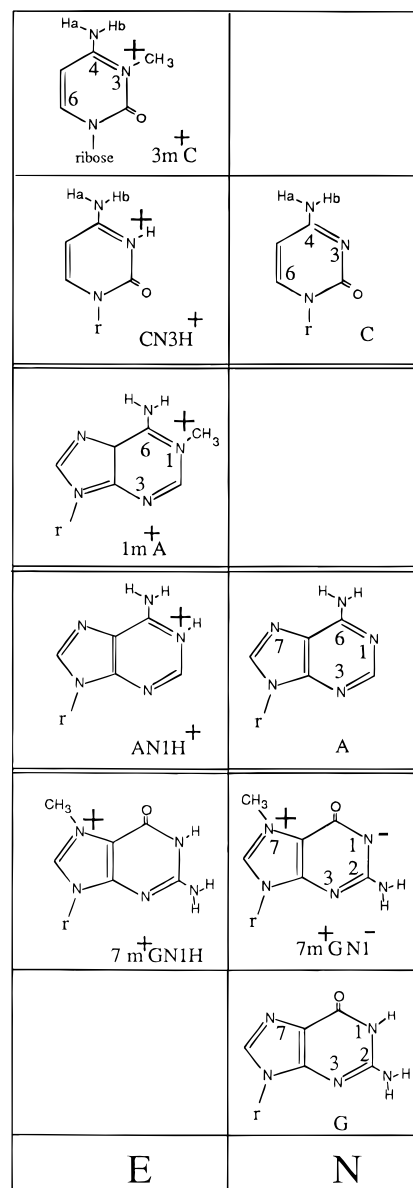(27) Englander, S. W.; Kallenbach, N. R. *Q. Rev. Biophys.* **1984**, *16*, 521−655.
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Chart 1. Species Involved in Amino Proton Exchange

The transfer rate is given by

$$k_{ex} = q_{acc}[acc][1 + 10^{pK(nuH)-pK(accH^+)}]^{-1} \quad (2)$$

where q_{acc} is the rate constant for the formation of the complex and $[1 + 10^{pK(nuH)-pK(accH^+)}]^{-1}$ is the proton transfer yield, a function of the nucleoside and acceptor pK values, respectively, $pK(nuH)$ and $pK(accH^+)$. At equilibrium, the flux of the inverse reaction (from right to left; protonation of nu^-) is equal to the flux of the direct reaction. This entails that the rate constant of the inverse reaction is the same as that for the direct reaction, q_{acc}. The acceptor concentration, [acc], is given by

$$[acc] = [acc]_t[1 + 10^{pK(accH^+)-pH}]^{-1} \quad (3)$$

where $[acc]_t$ is the sum of concentrations of the acceptor base and of its conjugate acid. In the case of the hydroxyl acceptor, one has

$$[OH^-] = 10^{(pH-pK_i)} \quad (4)$$

and

$$pK(H_2O) = pK_i + \log [H_2O] \approx pK_i + 1.7 \quad (5)$$

where pK_i is the ionization product of water, $pK_i \approx 14$ at 20 °C, $pK_i \approx 15$ at 0 °C.

If pK(nuH) < pK(H$_2$O), the exchange rate is therefore

$$k_{OH} = q_{OH}10^{(pH-pK_i)} \qquad (6)$$

If pK(nuH) > pK(H$_2$O)

$$k_{OH} = q_{OH}10^{(pH+1.7-pK(nuH))} \qquad (7)$$

When water is the proton acceptor, the complex is permanently formed. However, the rate which must appear in the expression for the exchange rate, for dimensional reasons, may be conveniently, if arbitrarily, decomposed into a product, $q_{H_2O}[H_2O]$, so that the form of eq 2 is conserved, with [acc] = 55 mol/L and pK(accH$^+$) = $-$log [H$_2$O] = -1.7.[32]

(b) Nucleoside Acceptor. A proton transfers from a donor to the nucleoside acceptor. Equation 1 may be used once more. For convenience, we invert the right and left sides and change the symbols: the initial reactants are the nucleoside acceptor NU and the proton donor DONH$^+$:

NU + DONH$^+$ ⇌ {NU···DONH$^+$} ⇌

{NU$-$H$^+$···DON} ⇌ NUH$^+$ + DON (8)
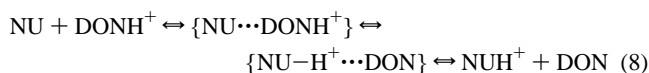
The exchange rate is

$$k_{ex} = q_{DON}[DONH^+][1 + 10^{pK(DONH^+)-pK(NUH^+)}]^{-1} \qquad (9)$$

Amino protons may exchange in this way at acidic pH. The amino group is protonated by proton transfer from a hydronium ion, OH$_3^+$. The collision rate constant for this reaction is evaluated as follows: (a) It is equal to that of the inverse reaction, namely, proton donation from the protonated amino group to water. (b) The exchange rate by proton donation to water is measured in a series of reference compounds whose pK values are known, for example, ammonium (see the Results). (c) The collision rate constant for proton donation to water is then derived by eq 2.

(c) Particulars of the Amino Group. The fact that there are two protons complicates the exchange situation. Each proton has its own exchange time and its own pK. There is also rotation, which may merge the proton NMR peaks, and which introduces a third time constant. There are a variety of cases which will be discussed as required. Here we consider two extreme cases. If the two peaks are resolved and if rotation is slower than exchange, exchange of each amino proton may be considered separately. If the peaks are merged and rotation is faster than exchange, and the exchange process is dependent on the amino proton pK, exchange by deprotonation (respectively, protonation) of the amino group proceeds mainly from the position with the lower (respectively, higher) pK.

In the case of exchange by protonation, the catalytic efficiency depends on the rate of rotation as compared to the rate of deprotonation.[30] Our analysis makes the assumption that rotation is not limiting.

(3) Amino Proton Exchange Processes. They were identified using principally the variation of the amino proton exchange rates vs pH. Exchange in the endocyclic-protonated state of the nucleosides was studied using as models the nucleosides methylated at the same position:[26] ribo-*N*3-methylcytidine (3m$^+$C), ribo-1-methyladenosine (1m$^+$A), and ribo-7-methylguanosine (7m$^+$G).

(4) Standard Collision Rate Constants of Hydroxyl and Water. The exchange time of ammonium is plotted as a function of pH in Figure 1, left panel. The pH-independent process at low pH is assigned to proton donation to water. A similar process is observed with triethanolamine, Tris, and piperidine (not shown), and with 1m$^+$A and 3m$^+$C (the E^W process; see below, Figure 5). In the right panel of Figure 1, the pH-independent exchange time is plotted as a function of the pK, whose value is either taken from tables or, in the case of 3m$^+$C

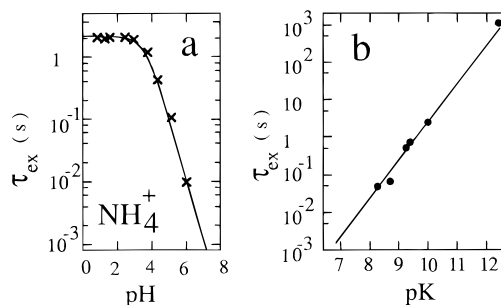(32) Nonin, S.; Leroy, J. L.; Guéron, M. *Biochemistry* **1995**, *34*, 10652−10659.

Figure 1. Proton transfer to water in reference compounds. (Left panel) Proton exchange time vs pH for the ammonium ion at 0 °C. Above pH 4, proton exchange is catalyzed by OH$^-$. The pH-independent process is direct proton transfer to water. (Right panel) Time for pH-independent proton exchange vs pK at 0 °C. In order of increasing pK, the donors are triethanolamine, Tris, 1m$^+$A, 3m$^+$C(Hb), ammonia, and piperidine. The pK values are given in Table 2. The fit of eq 2 corresponds to $q_{H_2O} = 10^{9.6}$ s^{-1}.

Table 1. Chemical Shifts Which Report on Nucleoside Titration[a]

| | titrating group | | reporter proton | |
nucleoside	site	pK^b	site	chemical shift[c] (ppm)
C	N3 imino	4.48[d]	H4a, H4b	8.2/7.03; 9.08/6.52
A	N1 imino	3.91[d]	H6a, H6b	9.04/6.76; 8.71/6.76
G	N7	2.21[d]	H8	9.06/7.88
7m$^+$G	N1 imino	7.4[d]	H8	9.10/8.86
3m$^+$C	N4 amino	9.6[e]	H6	8.16/7.26
1m$^+$A	N6 amino	9.3[e]	H2	8.56/8.03

[a] $T = 0$ °C. [b] The pK value is determined by NMR titration of the chemical shift of the reporter proton. [c] Chemical shifts of the reporter protons in the protonated/deprotonated state of the titrating group. [d] See also ref 36. [e] Lowest of pK(Ha) and pK(Hb). See also ref 26.

Table 2. Parameters of Proton Exchange Kinetics in Reference Compounds at 0 °C

donor	pK	τ_{H_2O} (s)	log(q_{H_2O})[b]	log(q_{OH})[b]
piperidine	12.5	1100	9.46	
ammonia	10.1	2.0	9.8	10
3mC$^+$ (Hb amino)	9.6	0.63	9.8	10.8
1mA$^+$ (amino)	9.3	0.5	9.6	10.4
Tris	8.78	0.19	9.5	10
triethanolamine	8.26	0.05	9.6	9.8

[a] The exchange time for direct transfer to a water molecule (low-pH plateau, Figure 1a). [b] Computed from eq 2.

and 1m$^+$A, measured by NMR titration (Table 1). As predicted by eq 2, the logarithm of the exchange time is proportional to the donor pK, except for the triethanolamine anomaly. The fit corresponds to q_{H_2O} = 10$^{9.6}$.

Above pH 4, proton exchange is catalyzed by OH$^-$. The rate constant q_{OH} is close to 10^{10} for neutral compounds, and to 10$^{10.6}$ for cationic compounds (Table 2).

These rate constants, valid for $T = 0$ °C, will be used in eq 2 for the derivation of amino proton pK values from the observed exchange rates (Table 3).

(5) NMR Methods. Unless stated otherwise, the experiments were carried out in water solutions with a H/D ratio of 0.1, in order to avoid cross-relaxation between amino protons. This procedure is particularly useful for the measurement of amino group rotation, as described in The Supporting Information. Proton exchange times were measured as described previously.[1] Magnetization was selectively inverted with DANTE sequences.[33] It was selectively saturated by a 90° DANTE sequence followed by a homospoil gradient of 5 ms, and this was repeated continuously during a specified delay.

(6) Sample Preparation. The ribonucleosides cytidine, 2′,3′-cyclic adenosine monophosphate (cAMP), 3′,5′-cyclic guanosine monophos-

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Table 3. Amino Proton Exchange Processes[a]

	process[b]	nucleoside species	nucleoside amino proton pK[d]	catalyst	transfer yield (log)	collision rate constant[c] log(q_{OH})	log(q_{H_2O})	log($q_{H_3O^+}$)
3m$^+$C	E^{OH}	3m$^+$C	Ha, 10.5;[e] Hb, **9.6**	OH$^-$	0	10.8		
	E^W	ibid.	Ha, 10.5; Hb, **9.6**	H$_2$O	pK(H$_3$O$^+$) − pK(N4H$_2$;E)		Ha, (9.6); Hb, 9.8	
C	N^{OH}	C	Ha, 18.8; Hb, 18.35	OH$^-$	pK(H$_2$O) − pK(N4H$_2$;N)	(10)		
	E^{OH}	CN3H$^+$	Ha, 10.5;[f] Hb, 9.6[f]	ibid.	0	Ha, 11.1; Hb, 11.3		
	E^W	ibid.	ibid.	H$_2$O	pK(H$_3$O$^+$) − pK(N4H$_2$;E)		Ha, 10; Hb, 9.7	
1m$^+$A	E^{OH}	1m$^+$A	**9.3**	OH$^-$	0	10.2		
	E^W	ibid.	ibid.	H$_2$O	pK(H$_3$O$^+$) − pK(N6H$_2$;E)		9.6	
	E^P	ibid.	−9.9	H$_3$O$^+$	pK(N6H$_3^+$;E) − pK(H$_3$O$^+$)			(9.6)
cAMP	N^{OH}	A	19.4	OH$^-$	pK(H$_2$O) − pK(N6H$_2$;N)	(10)		
	E^{OH}	AN1H$^+$	9.3[f]	ibid.	0	11.3		
	N^P	A	−6	H$_3$O$^+$	pK(N6H$_3^+$;N) − pK(H$_3$O$^+$)			(9.6)
7m$^+$G	N^{OH}	7m$^+$GN1$^-$	~20.3	OH$^-$	pK(H$_2$O) − pK(N2H$_2$;N)	(10)		
	E^{OH}	7m$^+$G	17.4	ibid.	pK(H$_2$O) − pK(N2H$_2$;E)	(10.6)		
	N^P	7m$^+$GN1$^-$	−3.75	H$_3$O$^+$	pK(N2H$_3^+$;N) − pK(H$_3$O$^+$)			(9.6)
	E^P	7m$^+$G	−8.9	ibid.	pK(N2H$_3^+$;E) − pK(H$_3$O$^+$)			ibid.
cGMP	N^{OH}	G	18.6	OH$^-$	pK(H$_2$O) − pK(N2H$_2$;N)	(10)		
	N^P	GN2H$_3^+$	−6.3	H$_2$O	pK(N2H$_3^+$;N) − pK(H$_3$O$^+$)			(9.6)

[a] According to eq 2 or eq 9, as appropriate, one has log(exchange rate) = log(transfer yield) + log(collision rate constant) + log(active nucleoside state fraction) + log(catalyst concentration). The last term is 1.7, pH − 15 (at 0 °C) and −pH for the water and hydroxyl acceptors and for the hydronium donor, respectively (eqs 4 and 5). The active nucleoside state is either N or E. All data are for $T = 0$ °C. [b] The nomenclature is defined in the Materials and Methods. [c] The standard values of the collision rate constants, in parentheses, are obtained as described in the Materials and Methods. Other values are determined from the measured exchange rates, using the stated amino proton pK, which in such cases is obtained independently of the exchange measurement. [d] Unless stated otherwise, the pK values are derived from the measured exchange rate by eq 2 or eq 9, using the standard value of the collision rate constant. The pK values obtained by chemical shift titration are indicated in bold type. [e] This pK is taken from the line below. [f] This pK is set equal to that of the methylated derivative.

phate (cGMP), 3-methylcytidine (3mC), 1-methyladenosine (1mA), and 7-methylguanosine (7mG) were used as provided by the manufacturer (Sigma). Unless stated otherwise, the nucleoside concentration was 20 mM and that of NaCl 50 mM, and the H/D ratio of the solution was 0.1. Samples at very low or high pH were processed rapidly to avoid degradation. As judged from spectral modifications, degradation of 7m$^+$G was particularly fast, occurring in about 1 h at 0 °C at pH 9.

(7) NMR Titrations and pH Measurements. Some pK values were determined with titrations monitored by the chemical shift of reporter protons: these were the amino protons for cytidine N3 and adenosine N1, and the aromatic proton for titration of quanosine and of the methylated nucleosides (Table 1).

The solution pH was measured in the NMR tube. It was buffered by the protonation equilibrium of the nucleoside or of the added proton exchange catalyst. In the study of exchange catalysis by ammonia, the pH was measured at the temperature of the NMR experiment. Otherwise, it was measured at room temperature, and therefore differs from the pH at the temperature of the NMR experiment, usually 0 °C, due to the pK shift of the effective buffer with temperature. This shift is estimated at 0.1−0.4 pH unit. The pH values stated in this work do not take it into account.

Results

(1) Spectral Identification and Amino Group Rotation. Hydroxyl proton resonances were characterized by comparing cyclic nucleosides, ribonucleosides, and deoxyribonucleosides (Figure 2). Due to broadening, they could be observed only between pH 4.5 and pH 6.5, and at temperatures below 25 °C. The imino protons of 3′,5′-cyclic GMP and of 7m$^+$G were identified by their chemical shift, around 11 ppm. Those of CN3H$^+$ and AN1H$^+$ were not detected, due to their low pK (Table 1) which induces fast exchange with water. The remaining peaks from exchangeable protons were assigned to the amino protons. They are resolved in cytidine and 3m$^+$C and, at 0 °C, in 1m$^+$A and acidic cAMP. In other cases, they are merged into one peak of double intensity, due to rotation of the amino group.

(a) Cytidine. The cytidine spectrum (Figure 2) shows two amino proton lines whose positions shift with pH, reflecting N3-protonation (ref 26; Table 1). The hydroxyl protons are detected at 6.25, 6.05, and 5.85 ppm.
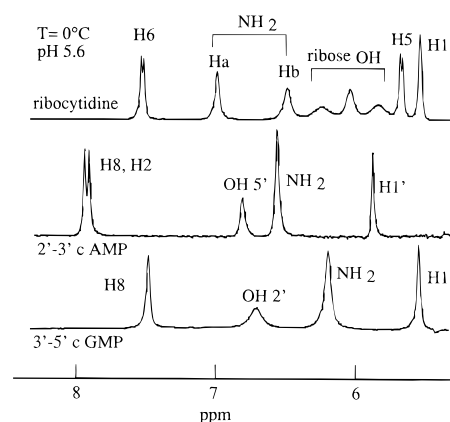

Figure 2. Proton spectra of cytidine, 2′,3′-cyclic adenosine, and 3′,5′-cyclic GMP. The ribose hydroxyl protons are broadened out except between pH 4.5 and pH 6.5. Experimental conditions: [NaCl] = 50 mM, proton resonance frequency 360 MHz.

Magnetization transfer experiments designed for amino proton identification and for determination of the rotation rate of the amino group are displayed in Figure 3. The low-field amino proton peak is assigned to Ha by its NOE coupling to H5 (5.9 ppm), an assignment which extends by continuity to neutral cytidine (not shown).

(b) 3-Methylcytidine. Amino proton assignments are the same as in cytidine, according to the NOE connectivities between the downfield amino proton and H5, and between the upfield amino proton and the 3-methyl protons (data not shown). The chemical shifts of H5 and H6 titrate at pH 9.6, reflecting the loss of one amino proton.

(c) 2′,3′-Cyclic AMP. Two exchangeable peaks are detected in the NMR spectrum (Figure 2). The 6.7 ppm peak, whose intensity corresponds to two protons, is assigned to the amino protons. The peak at 6.82 ppm is assigned to 5′-OH. Upon N1-protonation, the amino proton peak shifts downfield (Table 1) and splits into two components.

(d) 1-Methyladenosine. The chemical shifts of H8 and H2 titrate at pH 9.3, corresponding to the loss of an amino proton.
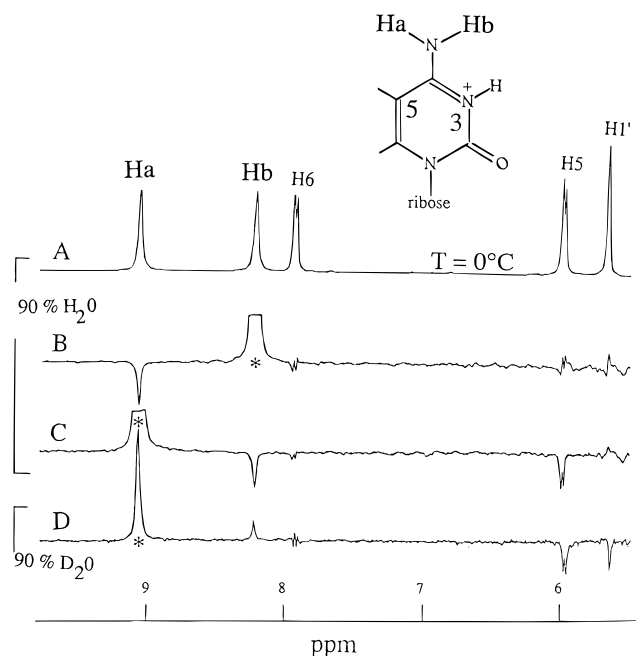
Figure 3. Magnetization transfer between amino protons of protonated cytidine: (A) reference spectrum in 90% H_2O/10% D_2O solution (0.1 D/H solution), (B–D) difference spectra obtained by subtracting the spectrum collected after irradiation of the amino proton line labeled by an asterisk from the reference spectrum (a negative peak corresponds to enhanced magnetization). In 90% H_2O (spectra B and C), the magnetization *enhancement* of the other amino proton shows that cross relaxation dominates. In 10% H_2O, the magnetization is *reduced*: the effect of amino group rotation dominates. The NOE from the down-field peak to H5 assigns this peak to Ha, the amino proton close to H5 (spectra C and D). The spectra were recorded at pH 1 and $T = 0$ °C in order to slow rotation of the amino group and amino proton exchange. [cytidine] = 40 mM; [NaCl] = 50 mM.

(e) 3′,5′-Cyclic GMP. The exchangeable peak at 6.7 ppm is missing in the dG spectrum and is thus assigned to the 2′-OH proton; this corrects the earlier assignment to an amino proton.[29] The 6.19 ppm peak, whose intensity corresponds to two protons, is assigned to the amino protons. Around pH 2.2, its chemical shift titrates in response to N7-protonation (Table 1).

(f) 7-Methylguanosine. At 0 °C, the chemical shift of H8 titrates around pH 7 in response to deprotonation at N1 (Table 1).

(2) Amino Proton Exchange. The exchange times of cytidine, cAMP, and cGMP are displayed in the lower panels of Figure 4, and those of the methylated derivatives similarly in Figure 5. In all cases, exchange is accelerated at high pH. In adenosine, guanosine, and the methylated derivatives, it is also accelerated at low pH. Endocyclic protonation, whose pK is indicated in the figures, is reflected in a change of slope in the pH dependence of the exchange time. The upper panels show the fractions of the different nucleoside states, and their involvement in exchange.

(3) Proton Exchange Catalysis by Bases. (a) Cytidine and cAMP. The effect of ammonia on the amino proton exchange times is pH-independent between the pK of the endocyclic nitrogen and that of ammonia, as expected from eq 2 if NH_3-catalyzed exchange occurs from the species protonated on the endocyclic nitrogen (Figure S2 in the Supporting Information).

(b) cGMP and 7m⁺G. The exchange rates of the amino protons are not affected by the proton acceptors ammonia (pH 7, 0.1 M), Tris (pH 6.2, 0.5 M), cacodylate (pH 6.5, 0.25 M), glycine, and proline (pH 6.7, 0.25 M). But they are affected by phosphate. The effect is less than proportional to the phosphate concentration, and there is also a change in chemical shift, downfield by 0.26 ppm at pH 7.1 and by 0.09 ppm at pH 4.2 for 1.5 M phosphate. These effects suggest complexation of phosphate by the amino group. The variation of the chemical shifts is consistent with binding constants of 0.47 and 0.8 M^{-1} for $H_2PO_4^-$ and HPO_4^{2-}, respectively (data not shown).

Discussion

In the present study, we elucidate the processes of amino proton exchange, the principal result being the explanation of the exchange rates of Figure 4. This has required the examination of exchange in methyl derivatives (Figure 5), of exchange
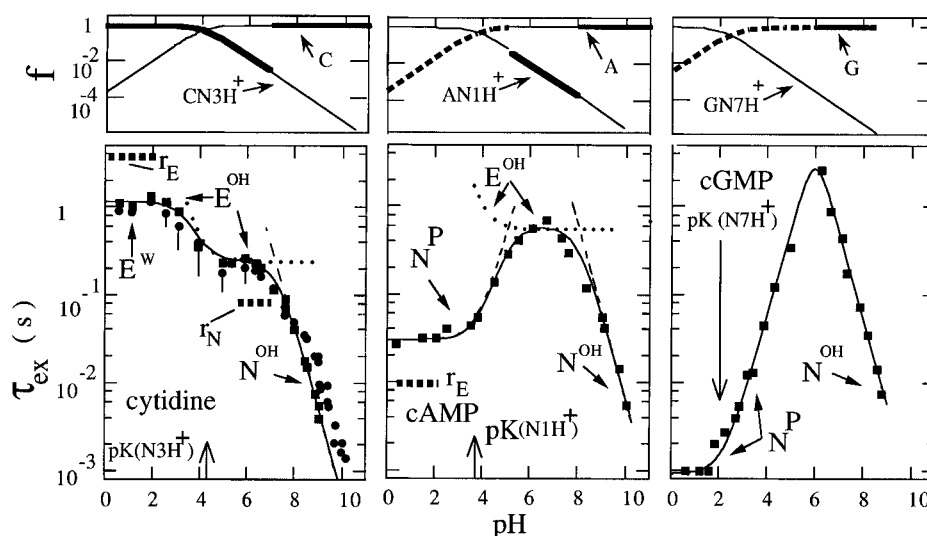

Figure 4. Amino proton exchange in cytidine, cAMP, and cGMP vs pH at 0 °C. (Lower panels) Left, cytidine: exchange times of Ha (squares) and Hb (circles), corrected for self-catalyzed exchange as explained in the Supporting Information. The exchange time before correction is indicated for selected data points by the lower tip of the vertical line; center, cAMP; right, cGMP. The curves in full lines are computed according to eq 2 (deprotonation) or 9 (protonation) with the parameters displayed in Table 3. The exchange processes are indicated. The heavy dashed lines labeled r_N and r_E indicate the rotation time of the amino group of protonated (300 ms) and unprotonated (80 ms) cytidine and of protonated cAMP (10 ms). (Upper panels) Fractions of the nucleoside states vs pH. The state whose contribution to exchange is predominant is drawn in thick font: full line for exchange by deprotonation, dashed line for exchange by protonation.
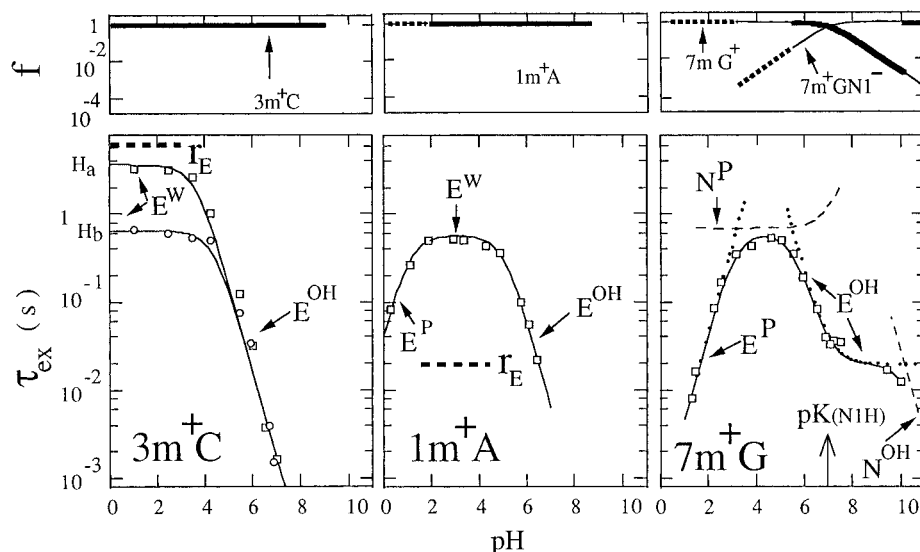
Figure 5. Amino proton exchange in methylated nucleosides vs pH at 0 °C. (Lower panels) Left, $3m^+C$: exchange times of Ha (squares) and Hb (circles); center, 1-methyladenosine; right, 7-methylguanosine. (Upper panels) Fractions of the nucleoside states vs pH. Same conventions as in Figure 4.

catalysis by bases, and of the rotation of the amino group. The discussion centers on the identification of the exchange processes. We consider briefly its extension to amino proton exchange in base-paired nucleic acids.

(1) Processes of Amino Proton Exchange. An exchange process is characterized by the species subject to exchange, the exchange agent, and the nature (donation or acceptance) of the exchange reaction. The nucleoside may be neutral or protonated/methylated on an endocyclic nitrogen. When exchange proceeds by deprotonation of the amino group, the relevant pK, that of the NH_2/NH^- couple, is in the range of 20 for the neutral nucleoside, and it is much reduced, to ca. 10, for the protonated/methylated forms of A and C derivatives (Table 3). The proton acceptors are H_2O, OH^-, and the basic form of buffers such as ammonia or phosphate. At low pH, exchange by protonation of the amino group may become the faster process. It involves the NH_3^+/NH_2 couple, whose pK is in the range of -3 to -10.

Acceleration of exchange with increasing pH indicates hydroxyl catalysis. A pH-independent exchange time reflects proton transfer to hydroxyl from a nucleoside state whose concentration increases as pH decreases, to H_2O from a nucleoside state whose concentration is pH-independent, or from hydronium to a nucleoside state whose concentration decreases with decreasing pH.

Acceleration of exchange with decreasing pH indicates transfer from hydronium to a species whose concentration is constant; it could also indicate transfer to H_2O from a protonated species whose concentration increases as the pH is lowered, but the latter case is not observed here.

The six nucleosides differ in their exchange behavior. The lower panels of Figures 4 and 5 show the measured exchange times together with the fit (full line) to the individual processes indicated in dotted or dashed lines. The upper panels show the concentrations of the two states of the nucleoside. The state which controls exchange is indicated by a thick line, full when it acts as proton donor, dashed when it is the proton acceptor.

Table 3 includes all parameters of the 17 different cases. When unknown otherwise, the pK is fitted to the data by eq 2 or 9, using the standard collision rates with OH^- or H_2O, which are shown in brackets (see the Materials and Methods). When the pK is known independently, it is the collision rate constant which is fitted. It is always within 0.6 unit of the corresponding standard one. The small difference between the fitted collision

rates and the corresponding standard ones supports our assignments of exchange mechanisms.

(a) 3-Methylcytidine. This is the simplest case because only one state of the nucleoside requires consideration, and the amino proton peaks are resolved. The NH_2/NH^- pK is close to that of ammonia (Table 1), and the exchange behavior is therefore similar (compare Figure 5 to Figure 1a). Exchange is catalyzed by OH^- above pH 4.4, the E^{OH} process. At lower pH, transfer to water (the E^W process) is faster. Acid-catalyzed exchange by acceptance of a proton is absent.

Since the amino proton pK values are less than the H_2O/OH^- pK and more than the H_3O^+/H_2O pK, the rate of the E^{OH} process is independent of the amino proton donor pK, and that of the E^W process is proportional to $10^{-pK(amino)}$, according to eq 2. Accordingly, the exchange rates of Ha and Hb are equal in the first case and unequal in the second, as shown in Figure 5 where the rate of rotation is also indicated. This rate is less than the slowest exchange rate (Ha), and we believe the difference to be significant. In this situation, the pK values of the two amino protons can be derived. The difference between them is less than 1 pK unit. The rate of rotation is presumed to be independent of pH, but it could not be measured above pH 4, due to line broadening.

(b) Cytidine. Above pH 7, exchange occurs from the neutral nucleoside. The rate of hydroxyl catalysis (the N^{OH} process, Figure 4) gives pK values of 18.85 (Ha) and 18.35 (Hb). Below pH 7, exchange from the N3-protonated nucleoside (the E state) dominates. Hydroxyl catalysis (the E^{OH} process) is pH-independent as long as the product $[CN3H^+][OH^-]$ remains constant, i.e., for pH > pK(N3H$^+$). At lower pH, this process slows until the pH-independent process E^W takes over, with an exchange time quite close to that of $3m^+C$.

(c) 1-Methyladenosine: Acid-Catalyzed Exchange. Above pH 2, the exchange rates are similar to those in $3m^+C$ and they are ascribed to the same processes, E^{OH} and E^W (Figure 5). Below pH 2, an acid-catalyzed process appears, reminiscent of acid-catalyzed exchange of amide protons in primary amines, which has been ascribed to N-protonation of the amide group, $O=C-NH_3^+$.[30] A protonation pK of -10 was derived from those observations, assuming protonation and deprotonation rates of 5×10^{11}. We observe that deprotonation of the N-protonated group is a reaction of transfer to water, for which the rate in our formalism is $55q_{H_2O}$. With our standard value of q_{H_2O}, this

is $55 \times 10^{9.6}$, or 2.2×10^{11}, close enough to the value of ref 30. As proposed earlier by McConnell,[24] we assign the acid-catalyzed exchange in 1-methyladenosine to the same mechanism. The observed exchange rate corresponds to a pK of -9.9 for the NH_3^+/NH_2 couple (Table 3), as computed from eq 9.

A cautionary note is in order. In the study of primary amides,[30] protonation on the carboxyl rather than the amine could not be ruled out, and the situation is the same here. Acid catalysis might proceed from a protonated species (at N3 or N7 in the case of $1m^+A$) if the pK of the NH_2/NH couple is lower in this species. For an example using plausible but unknown values, suppose that the pK for protonation on N3 or N7 is -1, and that this protonation lowers the amino proton pK by 2 units, from 9.3 to 7.3 (a change comparable to that of the imino proton pK of cGMP upon N7-protonation[32]), the amino proton exchange rate would be enhanced by a factor of 10 at pH 0, as observed (Figure 5). It is worth noting that acid-catalyzed exchange is missing in cytidine or in $3m^+C$, the only two among the six molecules examined which have no available endocyclic nitrogen in the E form.

Lacking independent information on the real pK values, such a process cannot be excluded, or argued. We ignore it in the discussion of the other nucleosides.

(d) cAMP. Above pH 6, the amino proton exchange rates are similar to those in cytidine and they are ascribed to the same processes, N^{OH} and E^{OH} (Figure 4).

Below pH 4, N1-protonation saturates, so that the E^{OH} process becomes inefficient, as indicated by the dotted line. But an acid-catalyzed process appears, which is much more efficient than that occurring in $1m^+A$ since it appears at higher pK, and which levels off at the pK of the N1 proton. This points to a new exchange process, one not available in $1m^+A$. The obvious candidate is exchange through protonation of the amino group, from the species which is *unprotonated* at N1 (the N^P process). We derive a pK of -6 for amino group protonation of neutral cAMP, close to that of -5 similarly derived for NADH.[30]

(e) 7-Methylguanosine. This case involves four different exchange processes. Above pH 10, the donor is $7m^+GN1^-$, the acceptor is OH^- (the N^{OH} process, Figure 5), and the fitted pK is 20.3. The shoulder between pH 10 and pH 7 is assigned to transfer from $7m^+G$ (protonated on N1, p$K = 7.3$) to OH^-, the E^{OH} process; protonation at N1 has shifted the amino proton pK by 3 units, to 17.4. This process slows at lower pH, and a plateau is observed around pH 5, due to amino group protonation of the minority species $7m^+GN1^-$ (the N^P process). The fit yields a pK of -3.75 for the NH_3^+/NH_2 couple. This process is faster than the E^W process because of the high amino proton pK of $7m^+G$ (17.4, see above). This is in contrast to $3m^+C$ or $1m^+A$, where the low amino proton pK, around 10 (Tables 2 and 3), makes E^W efficient, leading to plateaus around pH 2–3. Lastly, below pH 3, amino group protonation of $7m^+G$ takesover (the E^P process). The pK derived from the exchange rate is -8.9.

(f) cGMP. Above pH 6, amino proton exchange is catalyzed by OH^-, corresponding to a pK of 18.6 (Table 3). At lower pH, an acid-catalyzed process sets in without any intermediate plateau. This is explained by assuming that the amino proton pK of N7-protonated G remains high, like that of $7m^+G$, so that the E^W process is inefficient. As in the case of adenosine derivatives, acid-catalyzed exchange is faster in G than in $7m^+G$, and is therefore assigned to amino group protonation from unprotonated G (the N^P process). The fit is to a pK of -6.3 for the NH_3^+/NH_2 couple.

(2) Proton Exchange Catalysis by Bases. Proton acceptors such as ammonia (p$K = 10.1$, Table 2) are poor catalysts of the exchange of amino protons of neutral nucleosides, due to the high amino proton pK (ca. 19, Table 3). Catalysis is nevertheless observable in cytidine and in adenosine, the donor being the minority species protonated on the endocyclic nitrogen, whose amino proton pK is lower by close to 10 units. For a constant total concentration of ammonia, the exchange rate of this process should therefore be pH-independent between the pK of ammonia and that of the endocyclic proton, and it should diminish outside this range, as is indeed observed (Figure S2 in the Supporting Information).

In contrast, ammonia does not catalyze the exchange of G amino protons, as expected if protonation of guanosine (known to occur at N7 from ^{15}N titration[28]) has little effect on the amino proton pK (see above). The difference between A and C on one hand and G on the other may be understood in terms of charge delocalization. In the former, ring protonation should give double-bond character to the C$-$N bond of the amino group.[34] This is in agreement with the observations of a slower rotation of the amino group (Figures 4 and 5) and of changes in the chemical shifts of the proton (ref 26; Table 1) and ^{15}N[28] resonances. By contrast, all the mesomeric structures of N7-protonated guanosine have the positive charge localized in the five-membered ring. Indeed, protonation has little effect on the chemical shifts of the G amino group (refs 22 and 27; Table 1) and presumably on its rotation which remains fast on the NMR scale.

Catalysis of guanosine amino proton exchange by phosphate[25] is a special case. The nonlinear effect on the exchange rate and the change in chemical shifts point to the binding of phosphate to amino protons. In agreement with this suggestion, we note that a H-bonded complex between guanosine and dimethyl phosphate has been proposed previously to account for changes in chemical shifts similar to those reported here.[34]

Because catalysis of exchange by phosphate is related to specific interactions with the amino group of guanosine, it is not evidence for a low amino proton pK of N7-protonated guanosine. In combination with the reassignment of the proton NMR spectrum, the present analysis of phosphate catalysis and lack of catalysis by other bases solves the contradictions of the literature on this point,[18,25,29] so that the same degree of understanding of amino proton exchange is now achieved in guanosine as in cytidine and adenosine.

(3) Amino Proton Exchange in Base-Paired Polynucleotides. Our results provide a basis for the analysis of amino proton exchange in oligonucleotides, where it is subject to intra-base-pair H-bonding, reduced accessibility, and a hydrophobic environment. The H-bonded and exposed amino protons of closed Watson–Crick base pairs within B-DNA and A-RNA duplexes are sterically accessible to solute and solvent molecules. One or perhaps both amino protons might therefore exchange from the closed base pair, in contrast to imino protons whose exchange requires base-pair opening.[32] The internally bonded proton should exchange more slowly than the exposed one, but the difference may be masked if amino group rotation is faster than exchange of the exposed amino proton. This may explain the similar exchange times of the two amino protons in RNA,[4,19] in B-DNA (ref 5, and unpublished results), and in Z-DNA.[8]

The exposed amino proton is presumably hydrogen-bonded to water. But the situation is quite different for the amino nitrogen, whose axial access to water is hindered by base stacking. Were amino proton exchange to involve concerted donation of an amino proton and acceptance by the amino

(34) Lancelot, G.; Hélène, C. *J. Biol. Chem.* **1984**, *259*, 15046–15050.

nitrogen of an axially H-bonded proton from a water molecule, the absence of the latter could hinder transfer.

It could also increase the pK of the NH_2/NH^- couple, in analogy to the effect of a solvent change from water to an organic solvent. For example, the pK of acetic acid increases from 4.8 in water to 12.4 in *N,N*-dimethylformamide.[35] In the C·CN3H$^+$ pairs of the i-motif quadruplex, the exchange behavior of the external amino proton is indicative of a pK shift from 9.6, the amino proton pK for CN3H$^+$, to about 18.[9] The

phenomenon is easy to detect and could become a useful structural and motional probe. A pK shift may also explain the slow exchange, noted in the Introduction, of external amino protons in B-DNA and in other DNA structures.[9] The amide and side-chain protons of proteins could be similarly affected.

Supporting Information Available: Sections on the rotation of the amino group, including a figure, and on autocatalysis, plus one figure showing exchange catalysis of amino protons by ammonia vs pH (7 pages). See any current masthead page for ordering and Internet access instructions.

JA962022K

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