Contrasting observations on buffer catalysis of guanosine amino proton exchange

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ABSTRACT

The two amino protons of 3',5'-cyclic guanosine monophosphate are shown to differ drastically in their solvent exchange properties: One is rapidly exchanging and sensitive to buffer catalysis; the other slow and insensitive. This observation accounts for the marked contrast between stopped-flow and NMR observations on buffer catalysis of amino proton exchange in guanosine monophosphates. The amino protons of guanine compounds traverse a "fast" solvent exchange position through the process of amino rotation, which together with kinetic considerations and comparative data on adenine and cytosine compounds, supports proposals of solvent exchange mediated by events at the guanine (N-3) site, rather than the (N-7) site. Exchange does not conform to rate expressions used by different workers for amino proton exchange.

INTRODUCTION

We had reported earlier that amino proton exchange in guanosine compounds was exceptional in relation to that of adenosine and cytidine, since protonation at the titratable endocyclic nitrogen of the nucleobase ring (N-7) would not represent an initial process for exchange of the amino protons in helical polynucleotides (1). This conclusion was based on the observation that guanosine amino proton exchange measured by NMR line shape was not catalyzed by buffers shown to be effective in the case of adenosine and cytosine compounds (2). However, it is shown in a recent report by Hartman, Lavery and Ramstein (3) that solvent exchange of the amino protons of 5'-GMP is indeed quite sensitive to buffer catalysis when observed by the method of deuterium stopped-flow. Here, this issue is resolved with evidence showing that the two G amino protons successively traverse a "fast" solvent exchange position in the molecule by the process of slow (C-2)-to-amino bond rotation. In the case of restricted rotation seen here in solutions of 3',5'-cGMP only one of the two protons is "fast" and sensitive to catalyst. Thus, in any compound of guanosine NMR would provide an 1H resonance reflecting only the exchange properties of the slow (buffer insensitive) amino proton, while stopped-flow would detect exchange of its faster partner. However, because of slow rotation,
the stopped-flow measurements might reflect rotation rate as a term in a useful kinetic expression.

In addition, an examination of kinetic expressions and associated buffer rate constants supports the conclusion that exchange of amino proton of guanosine is not described by kinetic expressions appropriate to the amino protons of the other nucleobases in DNA and RNA. While ribose phosphate-amino contact is a consideration, these items lend support to the proposal of Hartman, et al (3) of exchange mediated by events at the guanine N-3 site and underline the importance of using alternative methods to study exchange, which in the case of nucleobase amino protons, is critical to active development in the use of exchanging protons as valuable probes in the study of DNA and RNA polymeric structural dynamics (4,5).

MATERIALS AND METHODS

Guanosine monophosphates (Sigma) were used as supplied. Solutions of the cyclic monophosphates were tested by pH titration for possible hydrolysis and the presence of buffering contaminants. Sodium cacodylate was used as supplied and added as small volumes of concentrated (1M) stock solutions, where needed. All solutions were brought to the desired pH with HCl. NMR spectra were obtained on the Nicolet 300 MHz spectrometer with the 1280 computer on 0.05 M nucleotide solutions containing 20% D_2O. Spectra were obtained using the low power soft pulse of Redfield, which minimizes the tipping of the water moments to provide solvent suppression (6). Therefore, resonance areas of signals within 2 PPM of the water resonance are reduced and indeterminate in area.

RESULTS

A key assumption in the study of amino proton exchange by NMR line shape was that for guanine, the two amino resonances were coalesced as a single 2-proton area signal by rotation in the "rapid" rate condition. This assumption was consistent with early results at 100 and 360 MHz for guanosine monophosphates (1,7,8). In most of these studies NMR exchange data was obtained from 2',3'-cyclic nucleotides to provide adequate solubility without injecting self association artifacts (9) and catalysis due to the secondary phosphate in 5'-mononucleotides. Also, temperature studies reveal no evidence of rotationally discrete signals from the amino protons of 2',3'-cGMP (not shown, see ref. 1). However, the most likely explanation for the present disagreement between these NMR experiments (which observe the exchanging atoms directly) and those of the stopped-flow experiments of Hartman, et al (which do not) is the double premise that the two amino protons may 1) differ greatly
Figure 1. 300 MHz $^1$H NMR Spectra of 3',5'-cGMP at 5 Deg. C. Solutions of 3',5'-cyclic guanosine monophosphate (0.05M) were sequentially adjusted to indicated pH values and transferred to separate 5 mm NMR tubes. Spectra were identical in the pH range 5.0 to 6.0, i.e., except for the cacodylate (0.03 M) the pH 5.3 and pH 5.6 spectra would be equivalent. Spectral assignments are indicated in the pH 7.0 spectrum. The PPM axis is approximate, taken from stored parameters based on sodium 3-(trimethylsilyl)-1-propane sulfonic acid as reference.

In their tendency to exchange with solvent and 2) provide NMR resonances that are not rotationally coalesced in "slow" exchange or only approaching rotational coalescence at a slow rotation rate. An example of slow rotation and solvent exchange is seen in the case of cytidine compounds (10,11).

To test the possibility of "slow" or "moderately fast" rotation approaching signal coalescence, the amino $^1$H resonances of 3',5'-cyclic guanosine monophosphate were examined at 300 MHz at lower temperatures to reduce the rates of rotational and solvent exchange. As shown in Figure 1, two distinct amino resonances emerge in the pH range 5 to 6 at 5 degrees C. As suspected, the separate resonances exhibit widely different solvent exchange properties; one is quite sharp (7 Hz) and insensitive to the addition of catalyst, while the other is much broader (30 Hz) and broadens further with the addition of one of the weaker buffer catalysts (cacodylate, pK = 6.2).
Figure 2. The Effect of Temperature on 300 MHz Spectrum of 3',5'-cGMP Amino $^1$H Resonances. Spectra were obtained after equilibration to each temperature indicated on a 0.05 M solution of 3',5'-cGMP adjusted to Ph 5.6. The PPM axis was established under the same conditions as in Fig. 1.

The broadening to 60 Hz in the presence of .03 M cacodylate is in semi-quantitative agreement with the measurements of Hartman, et al, under different conditions (3). As shown in Figure 2, the broader resonance melts into the baseline as the temperature is increased to 23 degrees C. These observations are unchanged with a 10-fold dilution of 3',5'-cGMP (.05 M to <.005 M). The two resonances are clearly under the "slow" rotational exchange condition, where the rotation rate is considerably less than their frequency separation. Fitting the observed spectrum at 23 degrees C (Fig. 2) with a two-site exchange algorithm (12) requires that the rotation rate about the C-2 to amino bond must be less than 5 sec$^{-1}$. A rotation rate of 60 sec$^{-1}$ would be required for signal coalescence at 300 MHz, based on the observed line widths and the frequency separation (90 Hz). While 3',5'-cGMP may exhibit altered solvent and rotational exchange compared to other guanosine derivatives owing to the constraints of the rigid ribose configuration, it is apparent that our published data on catalysis of amino proton exchange of 2',3'-cGMP obtained by NMR are valid, but only for one (the slower) of the two amino protons (1).

The line width of the faster proton of 3',5'-cGMP in the absence of
Catalyst (Fig. 2) clearly represents the fastest solvent exchange yet seen in any nucleobase amino system. The estimated line width of 50 Hz at 23 degrees C corresponds to a solvent rate of approximately 150 sec⁻¹, which is comparable or even exceeds that of the "fast" imino proton under the same conditions. This rate is two orders of magnitude faster than the exchange rates measured by deuterium stopped-flow, which are of the same magnitude as the above estimates of the rotation rate.

In experiments involving the cyclic nucleotides the appearance of new resonances by the process of nucleotide self-association had been effectively ruled out by the narrow line width of the guanine H-8 resonance under a variety of conditions, including extensive dilution. As shown in Figure 3 a similar attempt to detect rotationally discrete amino ¹'H resonances in 2'-deoxy, 5'-guanosine monophosphate (sodium salt) is unsuccessful, as in the case of 2',3'-cGMP. The separation of the amino resonance into two at pH 5.4, (spectrum A), pH 4 (D) and pH 6 (E) is accompanied by broadening of the non-exchanging H-8 resonance (shown at an arbitrary position of 7.7 PPM) and the appearance of new H-8 signals and is accounted for by intermolecular
association producing at least two long-lived environments for the molecule (9). As in the case with other nucleotides the addition of buffer catalyst (0.1 M cacodylate) produces no significant broadening, but does increase the apparent self-association effect (B). To determine whether the lack of catalytic exchange broadening might be due to the long lifetime of the self-associated state (9), the same amount of catalyst was added to a ten-fold dilution of the 5'-monophosphate. While self-association is still apparent for .005 M nucleotide (with the appearance of the additional H-8 resonance at 8.1 PPM), broadening of the amino $^1$H resonance is entirely absent. Similar experiments at 23 degrees C produce the same observations with sharper resonances, which are completely unaffected by the addition of catalyst. In all cases only the slower exchanging proton of the two amino protons is observed.

**DISCUSSION**

**NMR Signal Behavior in Rotational and Solvent Exchange**

The appearance of two resonances in 3',5'-cGMP, one broad and sensitive to catalyst; the other sharp and insensitive, is not observed in present and previous NMR studies on 5'-deoxy GMP or 2',3'-cGMP, which exhibit a single resonance in slow solvent exchange. If rotational exchange is clearly in the "slow" NMR condition as in 3',5'-cGMP ($k_{\text{rotation}} \ll v_A - v_B$, where $v_A$ and $v_B$ are the frequency positions of the amino protons) then the second proton resonance of the other guanosine compounds must reflect even faster exchange than is seen here. Alternatively, the rotation rate may approach or exceed that required for coalescence ($v_A - v_B$ is small). If the two protons are rotationally coalesced, then the question is whether this coalescence would obscure the observation of buffer-induced line NMR broadening for a system comprised of two protons with significantly different solvent exchange rates. In this regard some earlier unexplained observations on the NMR of 2',3'-cGMP take on relevance: First, in early NMR experiments a reduction of amino $^1$H resonance intensity from the two-proton area to 1.5 protons occurred with the reduction of solution pH from 5 to pH 3. Second, NMR signal broadening induced by the addition of phosphate was not monotonic, as expected from the rate equations, but reproducibly exhibited a biphasic reduction in line width in the pH range 4 to 5.5 (see Figure 1 of ref. 1).

It can be shown that a condition of rotational coalescence, as well as the "slow" condition can provide an NMR signal reflecting the exchange properties only of the slower of the two amino protons. Figure 4(a) shows
Figure 4. Calculated NMR Lineshapes from a Two-Site Exchange Algorithm. (A), progressively increasing line widths ("solvent exchange") of the second proton (indicated in Hz) for a first proton 10 Hz wide, separated from the second by 20 Hz at a constant "rotational" exchange rate of 10 sec\(^{-1}\). (B), same as in (A), except for a frequency separation of the two resonances of 10 Hz.

plots of NMR line shape calculated from a two-site exchange algorithm (12) for the case, where only one of the two resonance contributions is variable in solvent exchange, i.e., the T\(_2\) rate of one of the protons is constant (10 Hz line width), while the other is variable to give "solvent exchange" line widths from 10 to 1000 Hz. Here, rotational coalescence is established, not by variable rotation rate, but by increasing transverse relaxation ("solvent exchange broadening") of the fast-exchanging proton at constant rotation (10 sec\(^{-1}\)). As shown in Fig. 4(A), this coalescence occurs at a line width of 50 Hz for the faster proton with an initial frequency separation of 20 Hz between the separate proton resonances. Notable in this figure is the constant line width independent of large changes in "solvent exchange" and the progressive decrease in signal intensity as the broader component disappears into the base line. This would account for the observed decrease in intensity and the constant line width in the presence of strong buffer.
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catalysts. As shown in Fig. 4(B), the line width can actually sharpen several Hz with increasing exchange of the faster proton for a small frequency separation (10 Hz) at the same rotation rate, which could account for the irregular pH profile for phosphate broadening just described (1). Therefore, the lack of a second, broad proton resonance for nucleotides other than 3',5'-cGMP could be accounted for by rotational coalescence of fast- and slow-exchanging protons at the amino site. Moreover, previous observations of marked line broadening of the guanosine amino proton resonance at pH < 4 may reflect a rotational vis-a-vis solvent exchange contribution to line shape.

Comparison and Validity of Kinetic Analyses

Based on the evidence obtained here and the kinetic parameters obtained by Hartman, et al, (3) it is useful to consider at this point whether or not the kinetic analysis generally used in both laboratories for amino proton exchange is valid for the case of guanosine. The observation of buffer catalysis in nucleobase amino proton exchange carries the implication that the nucleobase must first be protonated before proton transfer from the amino group to the buffer conjugate base can occur. This notion is based on the fact that proton transfer from the amino group to a weak buffer acceptor does not occur with the neutral nucleobase species, which gives up its amino protons only to hydroxyl ion at a slow rate suggestive of a higher basicity than the OH⁻ acceptor (2,8,13,14). However, the observation of buffer catalysis per se does not carry the general implication of formation of a steady-state (protonated) intermediate. This determination requires the collection of experimental data over a broad range of pH values that encompasses both dissociation of both the nucleobase endocyclic site and the buffer catalyst. If the protonated nucleobase represents the steady state intermediate, buffer catalysis should decrease both at lower pH, reflecting the loss of buffer conjugate base, and at higher pH, reflecting the loss of nucleobase conjugate acid, i.e., the pH profile for catalysis would go through a maximum midway between the (usually lower) pK of the nucleobase and that of the buffer (usually higher). This is shown in Figure 5, curve labelled "eq. 1". Such pH profiles have been demonstrated for A and C (2,13,14) and are accounted for completely by the relation,

\[ k_{ex} = P_N k_B [B] \] (1),

where \( k_{ex} \) is the observed first order rate (sec⁻¹), \( P_N \) is the mole fraction of the protonated nucleobase species and \( k_B \) is the second order rate constant for proton transfer to the buffer conjugate base [B]. (Note: This characteristic
Figure 5. Calculated pH Profiles for Buffer Catalysis of Solvent Exchange. The first order rate constant for exchange calculated from eq. 1 (solid line) or eq. 2 (dashed line) of text, as indicated.

The pH profile is accounted for equally well by general acid catalysis, in which exchange is initiated by an interaction between protonated buffer and the neutral nucleobase. However, this is not expected to contribute in aqueous solution, if the concentration of the protonated nucleobase is kinetically predominant, as in the case of adenosine and cytosine (17)).

The rate expression used by Hartman, et al is not the same as eq. 1: In their expression (see eq. 3, reference 3) the mole fraction of protonated nucleobase $P_N$ of eq. 1 is replaced by the product of the formation constant ($K_f$) for nucleobase protonation and the hydrogen ion concentration ($[H^+]$),

$$K_{ex} = K_f [H^+] k_B [B]$$  \hspace{1cm} (2),

As shown in Figure 5 (curve labelled "eq. 2") the pH profile described by eq. 2 is different from that of eq. 1 and that of the experimental pH profiles observed for A and C. Instead, eq. 2 produces a typical buffer titration curve with high constant rates at pH values below the pK of the buffer. The two equations converge in quantitative agreement at a pH value above the maximum produced by eq. 1, since the derivations of eqs. 1 and 2 differ only in the form used for the concentration of nucleobase; total concentration for eq. 1 and variable free concentration for eq. 2. Here, $K_f [H^+]$ (eq. 2) becomes an approximation of the mole fraction of protonated species (eq. 1) in the...
limited pH range, where 1/K_f > [H^+], i.e., at pH values above the pK of the nucleobase endocyclic protonation site. Thus, eq. 1 provides a test for a steady-state protonated intermediate, while both eq. 1 and eq. 2 are equally serviceable in obtaining estimates of k_b in a limited pH range well above the pK of the nucleobase, i.e., the range (pH = 6.5) used by Hartman, et al (3).

For guanosine, there are problems with the above expressions as a basis for a kinetic analysis. First, because of high specific acid- and base-induced NMR line broadening for the amino protons of guanosine compounds, (8) the use of eq. 1 to test for the protonated intermediate would be very difficult experimentally. Second, it would appear that the estimates of the diffusion encounter rate constants of Hartman, et al (3) are too high for a single route for buffer catalysis. Estimates of k_b for amino exchange in A and C compounds based on eq. 1 lead to estimates of diffusion encounter rates of 10^5 to 10^9 M^{-1}sec^{-1} (2) which appear reasonable for direct encounters between two solutes without solvent intervention (14,16). However, estimates for diffusion encounter rates obtained by Hartman, et al (3) are much larger (>10^{10}) and are of the same magnitude as those measured for direct donor interaction between solvent (OH^−), rather than solute species (17). Moreover, the estimates of the OH^− diffusion encounter rate by Hartman, et al, (10^{12}) exceeds the maximum limit for hydroxyl ion recombination in liquids (10^{11}) and is much larger than that reported experimentally (10^5 to 10^{10}) (19). Third, the above NMR spectra show a large difference in the solvent exchange properties of the two protons, a remarkably rapid exchange rate for one and complete absence of buffer catalysis for the other.

Although the existing data do not permit the formulation of a mechanism for guanosine amino proton exchange for the present, the simplest explanation for the high buffer increments of Hartman, et al (3) is that there is more than one route for buffer catalysis of the "fast" amino proton. The most obvious additional route for exchange catalysis would be that mediated by the ribose phosphate, which in the case of 5'-GMP can be brought into virtual contact with the guanosine amino protons by ribose rotation about the C-1'-to-N-9 bond. This same syn-anti conformational process produces interactions with the N-3 site, as well, as discussed below. Such interactions are stabilized, since guanosine compounds are known for their syn conformational preference (20) and amino deprotonation would be initiated without the necessity for diffusional processes by the secondary phosphate in response to lower pH or to added buffer, i.e., the 5'-phosphate could act as a proton shuttle between amino and buffer. Accordingly, lower buffer increments and
related diffusion constants might be expected for 2',3'-cGMP, owing to the lower proton accepting function of its primary phosphate, which is further removed from amino (or N-3) contact in any case. On the other hand, the greater restriction to syn-anti rotation expected in the case of 3',5'-cGMP would enhance exchange mediated by its primary phosphate through a sustained contact position.

The Effect of Solvent and Rotational Exchange on Stopped-Flow Results.

Although the observations made here on rotationally discrete NMR signals resolves the contrasting observations on buffer catalysis made by NMR and stopped-flow, an issue now remains in reconciling the two methods in terms of what is being observed. The "slow" amino proton can be considered to be essentially non-exchanging at pH 5.2 - 5.7, since high concentrations of catalyst have no effect on NMR line width at temperatures approaching 70 degrees C (1). On the other hand, exchange of the "fast" amino proton is the most rapid yet observed in amino containing nucleobases, approaching or exceeding that of the guanine N-1 proton at pH < 6 (14). While NMR is a measure of the slower proton's exchange properties, it is unlikely that stopped flow would be a direct measure of the faster proton, based on its rate estimated from NMR line width in solutions of 3',5'-cGMP. If this were the case, then it follows that half the deuterium exchange would be completed "instantaneously" before stopped-flow data collection would begin in the time frame shown by Hartman, et al (3). At an uncatalyzed rate of 100 sec⁻¹, based on the line width of the "fast" proton (Fig. 1 and 2), 90% of the "fast" exchanges would be completed in .02 seconds and 40% completed within the dead time of a stopped-flow instrument. Since the absolute rates measured by stopped-flow are in the same range as the rotation rate estimated here, it is conceivable that the rotation rate is rate-limiting, i.e., after "instantaneous" deuteration of the faster proton, deuteration of the second would await a 180 degree of rotation placing the remaining proton in the "fast" position. However, if this were the case, no buffer increments could be measured. To account for the existence of the "non-exchanging" proton and the stopped-flow results the rotation rate would have to be within the range of solvent exchange rates for the proton in the "fast" position. While this situation is not consistent with the observed rate of the faster proton in 3',5'-cGMP, it is possible that the solvent rate is slower in the compounds used by Hartman, et al, which would be consistent, as well, with the line shape analysis discussed above. Therefore, to reconcile the results of both methods one would postulate that 3',5'-cGMP differs from the 5'-deoxy and the
2',3'-cyclic compounds in having a much slower rate of rotation and a faster solvent exchange rate for the "fast" rotational position. The slower rotation rate for 3',5'-cGMP can be justified by examination of space-filling models showing the severely restricted syn-anti ribose rotation (the N(5)-to-C(1') bond) and its contact proximity to the 2-amino group. Moreover, this restricted syn-anti conformational change could favor increased solvent exchange of the "fast" proton by increasing the lifetime or probability phosphate contact or of hydronium ion interaction at the N-3 site, as discussed below. With this postulate, the condition is met, wherein the rotation rate is in the same range of values as the solvent exchange rate of the faster proton and becomes part of the kinetic expression, although rotation is not completely rate-limiting. It is noteworthy that limitation of exchange by amino rotation does not conform to the usual "non-exchanging" \[ \Rightarrow \text{"exchanging" formalism (15)} \], since rotation does not produce a reverse reaction leading to the "non-exchangeable" situation; rather, it rotates the deuterium to the slower site, while placing the remaining proton in the faster exchanging position, e.g.,

\[
k_{\text{obs}} = k_{\text{rot}} [B] \left( k_B[H^+] = k' \right)
\]

where \( k' \) is a term representing an additional route for buffer catalysis as discussed above.

The Nature of the "Fast" and "Slow" Sites

Finally, the remaining question is how to account for the surprising indifference of one of the amino protons to the addition of buffer in a system where this proton is quite sensitive to variations in pH and further, is attached to the same nitrogen atom as another proton that is rapidly exchanging and highly sensitive to exchange catalyst. A scheme involving a tautomeric equilibrium initiated by G(N-3) protonation can be written, which would account for all the data:
The role of hydronium interaction at the N-3 site in I, in support of the suggestion of Hartman, et al (3), is consistent with earlier studies, in which we had shown that both A and G amino proton exchange occurred by a low pH route that was conspicuously absent in C (1,2). Structurally, C differs markedly from A and G in the absence of a second endocyclic nitrogen one or two atoms away from the amino group, which could act as a second protonation site to promote exchange. This notion would lead to the expectation of greater specific acid catalysis for G than for A, which is observed. Moreover, a route for guanosine amino proton exchange involving hydronium ion interaction at G(N-3) had been proposed some years ago by Bucher, Blomberg and Ruterjans on the basis of nitrogen-15 NMR studies (21). Although the imino tautomer, II, is not commonly held to predominate in aqueous solution, the preference for the imino form in cyclic guanidines and amidines is well documented (22). The extremely low acidity expected of its single imino proton would account for the absence of buffer base catalysis by simply evoking an appropriate relationship between the rate constants establishing the I II equilibrium and proton transfer rates for the second proton. For amidinium ions analogous to I the selectivity of amino protons for transfer to base has been demonstrated (23). The formation of II and its reversal would provide a route for exchange of the "fast" proton, would be a mechanism for the marked specific acid-base catalysis of the "slow" proton and would determine the rate of amino rotation possibly as an integral part of the exchange process. Such an equilibrium, modified by ribose interactions may form the basis for a valid rate expression for this interesting and exceptional nucleobase.

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