Association by Hydrogen Bonding of Mononucleotides in Aqueous Solution
(base pairing/ribonucleotides/220-MHz PMR/restricted amino group rotation/proton exchange)

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ABSTRACT Evidence for hydrogen bonding between 5′-ribonucleotides in water has been obtained from a 220-MHz proton magnetic resonance study of nitrogenous protons. The amino groups of GMP, AMP, and CMP exhibit proton resonance lines which are somewhat broadened by proton exchange with the solvent at 0°; their downfield shifts in mixtures of mononucleotides provide the basis for the following order of base-pairing tendencies: GMP-CMP > AMP-UMP. Hydrogen bonding is also observed in other pairs of mononucleotides, notably GMP-UMP, AMP-CMP, and CMP-UMP, to a lesser extent in GMP-IMP, CMP-XMP, and possibly in CMP-IMP. In agreement with previous reports, hydrophobic interactions of mononucleotides have also been observed; base pairing occurs in addition to vertical stacking of these bases, their hydrogen bonding to water, or self-association. Only CMP shows clear evidence of self-association via hydrogen bonding in water; the evidence for GMP is less direct, and that for AMP is negative. This lack of observable self-association may occur as a result of competition from strong stacking interactions. Only CMP shows restricted rotation of the amino group at 0° and neutral pH. As expected, higher temperatures increase the rate of rotation of the amino group for CMP, as well as accelerate the rate of proton exchange between water and the amino protons of mononucleotides.

High-resolution proton magnetic resonance spectroscopy could prove to be a valuable tool in mapping out the specificities conferred by hydrogen bonding between biomolecules in aqueous solution.

Hydrogen bonding between the monomers of nucleic acids has been the object of numerous investigations. The initial success of infrared spectroscopy in demonstrating hydrogen bonding between A and U derivatives in deuterochloroform (1) was quickly followed by more extensive infrared studies (2, 3), as well as by two proton magnetic resonance (PMR) reports (4, 5) that showed the remarkable Watson–Crick specificity of G·C, and the much weaker A·U interaction in organic solvents. Other possible combinations of bases showed little or no association, as analyzed by infrared and PMR spectroscopy.

Although interactions occurring in aqueous solvents are more applicable to biological systems, little work has been done on hydrogen bonding of nucleotide bases in water. Several workers (6–8) have interpreted their results in terms of base pairing, but these chromatographic and solubility studies, as well as an extensive osmometric study (9), were incapable of distinguishing between hydrogen bonding and stacking interactions. Raman spectroscopy studies of complementary mononucleotides failed to detect any spectral changes (10), whereas PMR spectroscopy studies generally revealed upfield shifts of nondissociable protons; this result is consistent with the view that stacking of mononucleotides is predominant in D2O (11–13).

We believe that we have PMR evidence of hydrogen bonding between mononucleotides in aqueous solution. This evidence is based on the detection and measurement of chemical shifts for the protons directly involved in hydrogen bonding, i.e., those of the amino groups. There are many similarities between the behavior of nucleotide amino protons in water and in organic solvents. In water, however, a wider range of base-pairing possibilities can be observed than in organic solvents.

PMR spectroscopy is ideally suited for differentiation between vertical ring stacking and hydrogen bonding. In most cases, upfield spectral shifts of ring protons are observed upon stacking of aromatic bases, whereas hydrogen bonding results in downfield shifts for the participating protons. Complications arising from proton exchange of nucleotide amino protons when water is the solvent can be controlled by a judicious choice of pH and temperature (14), and any broadening of proton resonances due to the quadrupole moment of nitrogen can be estimated from heteronuclear decoupling experiments (15).

MATERIALS AND METHODS

All ribonucleoside-5′-monophosphates were purchased from P-L Biochemicals, Inc. as sodium salts, except AMP—acid form—which was neutralized in solution with 6 N NaOH and lyophilized. Mononucleotide stock solutions were prepared volumetrically in distilled water, containing 2 mM DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate, Merck), adjusted to pH 7.5, and pressure filtered through 0.45-μm Millipore filters (Millipore Corp. no. HAWP 01300). Dilutions and mononucleotide mixtures were prepared by pipetting appropriate volumes of stock solutions into NMR sample tubes. Measurements of pH after spectroscopy gave values of 7.5 ± 0.2.

Spectra were recorded on a Varian HR-220 NMR spectrometer operating in field-sweep mode. Lower temperatures were obtained with the aid of a Varian Variable Temperature Controller and monitored with a standard methanol sample. Chemical shifts were measured relative to internal DSS by means of audio frequency side bands and chart calibrations. Interaction shifts were reproducible to ±2 Hz in separate runs. Heteronuclear 1H-14N decoupling was performed on a Varian HA-100 NMR spectrometer equipped with a Nuclear

References:
RESULTS AND DISCUSSION

Restricted rotation of the amino group in CMP

A recent PMR study of 1-methylytosine in dimethylformamide (16) reported the assignment of the two cytosine amino protons. In the unprotonated molecule, the amino group exhibited restricted rotation. This restricted rotation resulted in separate proton resonances, which could be made to coalesce at higher temperatures. A coalescence temperature of about 25° provided a rate constant of 138 sec⁻¹.

An analogous situation occurs for CMP in water at neutral pH, as illustrated in Fig. 1. Extrapolation gives an approximate coalescence temperature of 30°. Above this temperature, the coalesced line does not continue to sharpen (as when the solvent is dimethylformamide), because proton exchange with water becomes accelerated*. As a result, the coalesced line becomes increasingly broad, although still somewhat detectable at 50°.

Below 30°, broadening of individual amino resonances arises from two effects: intermediate rates of amino group rotation and proton exchange with the solvent. The two effects are not easily separated from one another. However, in the present case only amino group rotation will produce a coalescence of the two amino proton resonances. Hence, a rate constant for the amino group rotation of CMP can be calculated from:

\[ k_{\text{coalescence}} = \frac{\sigma}{\sqrt{2}} \Delta \nu_{ab} \]

where \( \Delta \nu_{ab} \) is the line separation in absence of rotation (17). An estimate of \( \Delta \nu_{ab} \) can be obtained from a measurement at -5°, where the effects of amino-group rotation on line separation are small. Assuming that \( \Delta \nu_{ab} = 122 \text{ Hz} \) holds also at 30°, we calculated \( k_{ab} = \sim 270 \text{ sec}^{-1} \).

By contrast, the amino groups of AMP and GMP exhibit fast rotation at 0°, and a single PMR line is observed. In chloroform, however, a dimethylamino derivative of adenosine gave evidence of restricted rotation, with a coalescence temperature about 0° (18). Due to their significant amide character, the amino groups of nucleotides are believed to be coplanar with the rings (19). Evidence indicates that various degrees of restricted rotation of the nicotinamide amino group can be demonstrated in DPN⁺ and its derivatives (manuscript in preparation). We have observed similarities between nucleotide amino and amido protons with respect to their restricted rotation, proton exchange kinetics, and feasibility of hydrogen bonding in aqueous solution.

Self-association of mononucleotides in water

An indication of mononucleotide self-association can be obtained by monitoring PMR chemical shifts at different concentrations of nucleotide. Fig. 2 shows the results at 220 MHz and 0°, compared to the internal reference compound DSS, for three mononucleotides. In agreement with previous reports (12), pronounced upfield shifts are observed for the ring protons and H-1’ of GMP and AMP, and to a lesser extent for CMP. Significant stacking interactions at 0° for GMP and AMP can be inferred. PMR intensity loss is evident above 0.2 M GMP or 0.5 M AMP, which indicates the formation of relatively rigid, soluble aggregates and limits the available range of concentrations†.

The amino protons of these two mononucleotides show concentration-dependent upfield shifts comparable to those of ring protons. It has been demonstrated for adenosine that if the amino group is methylated, the methyl-amino protons exhibit concentration-dependent PMR shifts similar to those of the aromatic protons (20). However, for GMP, it is possible that the amino group is additionally involved in hydrogen bonding self-association, as judged by its relatively smaller upfield shift when the GMP concentration is increased.

On the other hand, both amino protons of CMP exhibit a large downfield shift with increased concentrations of CMP. By analogy with PMR findings in organic solvents, nucleotide amino protons that undergo downfield shifts suggest the occurrence of hydrogen bonding. This mononucleotide is less

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*Heteronuclear ¹H–¹⁵N decoupling failed to produce observable sharpening of the amino resonances, which might suggest that the line broadening is due to proton exchange rather than interaction with the quadrupole moment of ¹⁵N (14, 15). A detailed report on linewidths and chemical shifts of nucleotide nitrogenous proton resonances will appear in the future.

† At concentrations greater than 0.2 M, several broad PMR lines appear in the GMP spectrum, including three or four lines in the region of NH-1 absorption (∼ 11 ppm from DSS). The chemical shifts, but not the intensities, of these lines are independent of concentration, suggesting the formation of a tight, soluble complex that is slow molecular exchange with free GMP.
Fig. 2. Chemical shifts at 220 MHz and 0° of mononucleotides in water, pH 7.5 ± 0.2. Broken lines indicate loss of PMR intensity and formation of aggregated complexes. Vertical scale: Hz downfield from 2 mM DSS as internal reference.

stacked than the others, but the equal downfield shifts of the two amino protons cannot be attributed to hydrogen bonding which involves only the CMP amino-ß proton. Alternatively, since a finite rate of CMP amino-group rotation persists at 0°, it is possible that the downfield shift is shared by the two amino protons by the rotational mechanism described earlier.

**Interaction between GMP and CMP**

Fig. 3 depicts the 220-MHz PMR spectra of 0.2 M GMP and 0.2 M CMP in water, both alone and in a 1:1 mixture containing 0.4 M total nucleotide. The spectra were recorded at 0°, not only to enhance the association of mononucleotides, but also to decrease the rate of proton exchange between the amino protons and the solvent. Most important, a low temperature was needed to restrict the rotation of the CMP amino group.

The salient features of the GMP + CMP spectrum include a downfield shift of the GMP amino, as well as the CMP amino-ß, absorption, compared to the spectra of mononucleotides. The amino proton shifts in the 1:1 mixture differ significantly from self-association shifts observed when the concentration of a single mononucleotide is increased from 0.2 to 0.4 M (Fig. 2). It is the CMP amino-ß proton that is in the correct position for a Watson–Crick base pairing with GMP; moreover, the same amino proton was implicated in 1-methylcytosine self-association, via hydrogen bonding in dimethylformamide (16).

The CMP aromatic ring protons and H-1' of the ribose

Fig. 3. PMR spectra at 220 MHz and 0° of GMP, CMP, and an equimolar mixture of GMP and CMP. Each mononucleotide 0.2 M in water at pH 7.5 ± 0.2. Bottom scale: ppm from 2 mM DSS as internal reference. Field (H) increases to the right.
undergo upfield shifts when in the presence of GMP, which may be a consequence of intercalation into the GMP stacks. GMP is believed to be strongly self-associated in water—by vertical stacking and hydrogen bonding—but only its five-membered ring supports significant dihedral ring current (12). Partial overlap with this ring might induce the observed upfield shifts in the CMP ring protons.

Pyrimidine nucleosides also do not support significant ring currents, although they self-associate extensively in aqueous solution (21). Therefore, intercalation of CMP into GMP stacks would not be expected to induce an upfield shift in GMP H-8 and H-1'; our results indicate that there is no shift under these conditions.

**Interaction between other mononucleotides in pairs**

Table 1 lists the interaction shifts observed for mononucleotides in various combinations. The interaction shifts for ring protons may be interpreted as an indication of stacking (upfield) or destacking (downfield), while the downfield shifts of amino protons provide evidence of hydrogen bonding in addition to any already present, as a result of interaction with water or self-association. The amino protons, however, may also undergo stacking or destacking shifts; the interpretation in terms of hydrogen bonding must be made with caution, after the interaction shifts exhibited by ring protons are taken into consideration. The effect of hydrogen bonding is particularly unambiguous for the amino groups of GMP and AMP, since they show shifts in the opposite direction upon self-association.

Table 1 presents evidence of hydrogen bonding in the complementary pairs G-C and A-U, as well as in several non-standard pairs. When UMP is added to GMP, for example, the downfield shift of the GMP amino line indicates hydrogen bonding, whereas the upfield shifts displayed by UMP protons suggest that it additionally engages in base stacking with GMP.

A G-U interaction is permitted by Crick's "wobble hypothesis" (22), and is expected to be weaker than a G-C interaction, as observed on the basis of respective GMP amino interaction shifts. Less expected is the observed interaction of A-C and U-C base pairs, as well as C-C self-association. In contrast to the G-C interaction, both CMP amino protons show downfield shifts in the nonstandard pairs. Donohue (23) has listed two acceptable base-pairing possibilities for A-C and two for U-C, both of which involve CMP amino-b; hence, no downfield shift would be expected for the amino-a proton. As shown in Fig. 2, equal downfield shifts for both CMP amino protons were observed with increased CMP self-association. The origins of equal downfield shifts for CMP amino protons (in all hydrogen-bonding interactions except G-C), and of unequal linewidths, should be studied in greater depth.

Crick's wobble hypothesis also permits the interaction of I with either C, U, or A. Hydrogen bonding of I with C has been reported in chloroform (24), and a PMR study indicated base pairing between I and U in CHCl3 (25). In water, the observation of I-U association is not feasible by the present method, since neither of these nucleotides contains an amino group. Interactions of I are further complicated by possible tautomerization to its enol form, whose stability has been estimated to be similar to that of its keto form in aqueous solution (26). Recently, reports of tautomeration of CMP (27) and GMP (28) in deuterium oxide have appeared. Table 1 presents evidence of weak hydrogen bonding for the G-I pair (compared to G-C). The A-I pair shows primarily a stacking interaction. Some hydrogen bonding between C and I can be inferred from Table 1 by noting that CMP amino protons experience less of an upfield shift than H-6 and H-5; the interaction shifts for C-X further corroborate this view.

**Conclusions.** The principal finding of the present study is that hydrogen bonding can be demonstrated between mononucleotides in water. Hydrogen bonding apparently occurs in competition with stacking interactions, and possibly involves inter-stack bonding. Costacking, as well as hydrogen bonding, occurs in mixtures of two different mononucleotides.

An approximate order of mononucleotide hydrogen bonding tendencies can be assigned from the downfield shifts of respective amino groups as follows:

- For GMP: CMP > UMP > IMP >> AMP
- For AMP: UMP ~ CMP >> IMP, GMP
- For CMP: GMP ~ CMP > UMP > XMP, AMP, IMP

If we assume that the limiting downfield shift upon 100% complex formation is the same for the amino protons of GMP and AMP (as has been found for G and C derivatives in deuterochloroform (29)), then it is evident that in water the
G-C base-pairing tendency is favored over A-U by a factor of two or three, based on association constants‡.

Despite a large number of possible geometrical orientations, hydrogen bonding between mononucleotides in water exhibits the Watson–Crick specificities used by nucleic acids during replication and transcription. The present results, however, do not show the absolute specificities that might account for the observed low error rates in biological syntheses, where a considerable amount of enzymatic amplification of elementary specificities takes place (30).

By means of PMR spectroscopy, it is possible to monitor the elementary specificities conferred by hydrogen bonding between biomolecules in aqueous solution. This may lead to a better understanding of structure–function relationships at the molecular level. Despite its very high concentration, water is apparently not as effective a competitor for hydrogen bonding sites as has been assumed.

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‡ If the association constants are below about 3.5 (a one-point fit leads to an estimate of $K_{G-C}$ about 0.6 M$^{-1}$), then the downfield shifts of the amino protons are proportional to the binding constants at low concentrations of interactants (see ref. 29).