Tautomerism of Nucleic Acid Bases. Guanine and Cytosine

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Abstract: Previous reports in the literature indicate that the H8 proton of guanine and the H5 proton of cytosine have unusually broad resonances in the ^{1}H nmr spectra under certain conditions of temperatures and pD. The line-width broadening phenomena have been interpreted as indicating that the minor tautomers of guanine and cytosine were present to the extent of 15 ± 3%. We find, however, that these line-broadening effects are not reproducible with purified samples of 2′-GMP and 5′-CMP, but with addition of paramagnetic impurities the pD and temperature dependence of GH8 and CH5 proton line-width broadening previously reported can be reproduced. This study, therefore, raises serious questions about the existence of 15% minor tautomers in guanine and cytosine at room temperature in neutral aqueous solution. Furthermore, the existence of abnormal G–U base pairing must be taken with caution in view of conflicting ^{1}H nmr and CD evidence.

In the Watson–Crick G–C and A–U or A–T base pairing scheme of nucleic acids,[1,2] the nucleic acid bases are assumed to have the amino or the lactam structure (see Figure 1). Although it has been suggested that the purine and pyrimidine bases can also exist in their minor tautomeric imino and lactim forms,[3] the fraction of the minor tautomers, as determined by ir, uv, and thermodynamic measurements, was very small, typically in the order of less than 1%.[4–13] Recently, however, ^{1}H nmr results have indicated that the minor tautomers of cytosine and guanine are present to 15 ± 3% at room temperatures in neutral aqueous solution.[14–16] If the minor tautomers were present to this extent, then abnormal A–C and G–U base pairings would be energetically feasible and this would have great implications on rates of mutation in genetic replication. Evidence for the existence of abnormal G–U pairing was subsequently presented.[17]

Since Lee, *et al.*, were unable to locate the proton resonances of the minor tautomers,[14–16] we initially attempted to detect these tautomers of cytosine and guanine by examining their proton and ^{13}C resonances. No additional resonances other than those due to the normal amino or lactam form were detected. Furthermore, the line width of GH8 (~2.5 Hz) which we observed at 20° in neutral H_2O solution[18] was significantly smaller than the value of ~10 Hz reported by Lee and Chan[16] under similar conditions in D_2O solvent. We thought this difference of GH8 line width could be due to a deuterium effect, and, if this were the case, it could provide an interesting aspect to the tautomeric exchange problem. We repeated these measurements in D_2O and in H_2O solvents and found the GH8 line width was the same in both solvents, ~2 Hz. In view of this discrepancy and the important biological implication of their proposal, we have reexamined the pD and temperature dependence of the line widths of cytosine CH5 and guanine GH8 proton resonances. In a preliminary note we presented experimenal evidence to indicate that the line-width broadening of CH5 and GH8 proton resonances of cytosine and guanine is not the result of interconversion between tautomeric structures of these nucleic acid bases, but rather it can be caused by paramagnetic metal contaminations.[19] Additional ^{1}H nmr evidence and an analysis of the pD and temperature dependence of the GH8 and CH5 line widths data are now presented and explained with respect to the presence of paramagnetic Cu^{2+} ions. The existence of the abnormal G–U pairing is also discussed in light of the present experimental results and other pertinent evidence obtained by ^{1}H nmr and circular dichroism studies.

Experimental Section

Materials. For this study we used 5′-CMP and 2′-GMP, the same compounds used by the previous authors in their detailed studies.[14–16] 2′-GMP was obtained from Sigma Chemical Co. (Lot 90C-7532) and P-L Biochemical (Lot 1196). 5′-CMP was obtained from Miles Laboratories (Lot 15-2-573) and Calbiochem (Lot 010251). All samples were purified with an analytical grade Dowex 50W-X8 column obtained from Bio-Rad as AG50W-X8, 100–200 mesh (Lot 10198). The column material was washed with EDTA and extensively with double distilled H_2O. The purity of all samples and solvents was checked by atomic emission spectroscopy using a 3.4-m grating Jarrell-Ash atomic emission spectrometer.

Methods. For the pmr experiments, the samples were prepared by weighing and dissolving the lyophilized material in either double distilled water or in D_2O supplied by the Columbia Organic Chemicals. The pH or pD of the solution was measured with a Radiom-

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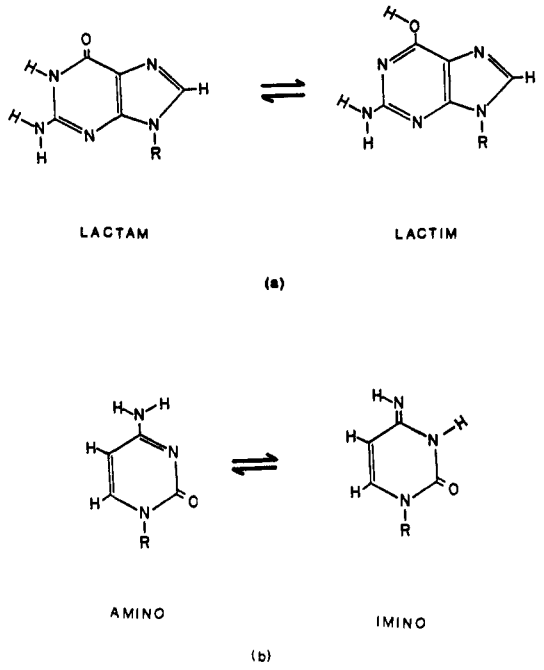

Figure 1. The tautomers of (a) guanine and (b) cytosine.

eter Model 26 pH meter and adjusted with HCl and NaOH or with DCl and NaOD. The value of pD is the pH meter reading plus 0.4 (the standard correction).[20] The 100-MHz ^{1}H nmr spectra of these compounds at several concentrations were taken as a function of pH/pD and temperature. The CH5, GH8, and the H1′ proton resonances were located according to published assignments.[15,16,21,22] The error in the measurements of the line widths, unless otherwise specified, is ±0.5 Hz.

In the variable pD/pH experiments, the temperature of the sample was the probe temperature of the Varian HA-100, 30 ± 1°. In the variable temperature experiments, the sample temperature was controlled to ±1° by means of a variable-temperature controller and was measured using the methanol or ethylene glycol standard and charts which were supplied by Varian. A Varian C-1024 computer of average transient was used to enhance the signal-to-noise ratio.

Results

One of the important observations which led Lee, *et al.*, to suspect the interconversion between the major and minor tautomers of guanine and of cytosine is that the line width of GH8 and CH5 proton resonances is pD dependent.[14-16] At 30° and pD ~5.0, the reported maximum line widths of CH5 and GH8 resonances at 100 MHz were about 5 and 9 Hz, respectively. We have examined the pD dependence of the line widths of CH5 and GH8 and the results obtained at 30° and 100 MHz are shown in Table I for purified 2′-GMP and 5′-CMP. The GH8 line width was found to be pD independent (*ca.* 1.6 to 1.8 Hz) over the range of concentrations (*ca.* 0.02 to 0.1 *M*) and pD (*ca.* 1 to 11) studied. Similar results were also obtained for the CH5 resonance which was also found to be pD independent, with a constant line width of 1.4 ± 0.1 Hz. In addition to the variations of GH8 and CH5 line widths as a function of pD, the previous authors also used the variation of the line widths and of their integrated intensities as a function of temperature to support their suggestions that minor tautomers of these nucleic acid bases were present to the extent of 15 ± 3%.[14-16] We have attempted to repeat these experiments with purified samples of 2′-GMP and 5′-CMP and found GH8 and CH5 line widths and their integrated intensities remained constant throughout the temperature range studied (between −10 and 30°).[19] Our experimental observations do not confirm those reported previously and therefore raise serious questions about the existence of 15% minor tautomers in cytosine and guanine.

Table I. The pD and Concentration Dependence of GH8 and CH5 Line Widths[a] at 30° and 100 MHz

	2′-GMP Concentration, *M*			5′-CMP Concentration, *M*	
pD	0.02	0.05	0.01	0.05	0.1
1.0	1.6[b]	1.8[b]	1.8[b]	1.3	1.4
2.0	1.7	1.7	1.8	1.4	1.4
4.0	1.7	1.7	1.8	1.4	1.5
5.0	1.6[b]	1.6[b]	1.6	1.5	1.5
6.0	1.6[b]	1.6[b]	1.7[b]	1.5	1.4
7.0	1.8	1.8	1.8	1.3	1.5
8.4	1.8	1.6	1.7	1.5	1.4
11.0	1.7[b]	1.6	1.8[b]	1.3	1.4

[a] All values are accurate to ±0.1 Hz for 2′-GMP and ±0.2 Hz for 5′-CMP. [b] Identical results were obtained in H_2O solvent.

In order to account for this discrepancy, we have prepared samples which reproduce the pD dependence of the GH8 and CH5 proton resonance data at 100 MHz and 30° reported by the previous authors. Since the 2′-GMP sample supplied by Sigma contained impurities of iron and copper and gave a GH8 line width of ~30 Hz, we purposely added small amounts of this crude 2′-GMP to the purified sample until the line width of GH8 was increased from 1.8 to 8.5 Hz at pD 5.0. The pD of this sample was then changed and the line width of the GH8 determined. The results of this experiment are shown in Figure 2, and they are seen to be identical with those reported previously.

To elucidate the possible specific cause of pD dependence of line-width broadening of GH8, known concentrations of paramagnetic Fe^{3+} and Cu^{2+} ions were selectively added to samples of purified 2′-GMP. The results are shown in Figure 2; in both cases they are virtually identical with those reported previously. In order to reproduce the maximum line width of ~8.5 Hz at pD 5, either 2×10^{-3} *M* Fe^{3+} or 2×10^{-5} *M* Cu^{2+} was required. The pD dependence profile reported by Lee and Chan was best reproduced by the presence of 2×10^{-5} *M* paramagnetic Cu^{2+} ion. We also find that the temperature dependence of the GH8 line width in neutral aqueous solution reported by the previous authors can be reproduced by adding 2×10^{-5} *M* Cu^{2+} to the purified 2′-GMP sample and these results are presented in Figure 3. Similar results on the pD and temperature dependence of the CH5 line-width broadening were obtained as shown in Figures 2 and 3 with 3.2×10^{-6} *M* Cu^{2+} in 5′-CMP.

Discussion

Although it is not possible to evaluate the amount of minor tautomers in purified samples of 2′-GMP and 5′-CMP from the data presented in the Results section, the absence of CH5 and GH8 proton line-width broadening under various conditions of pD, temperature,

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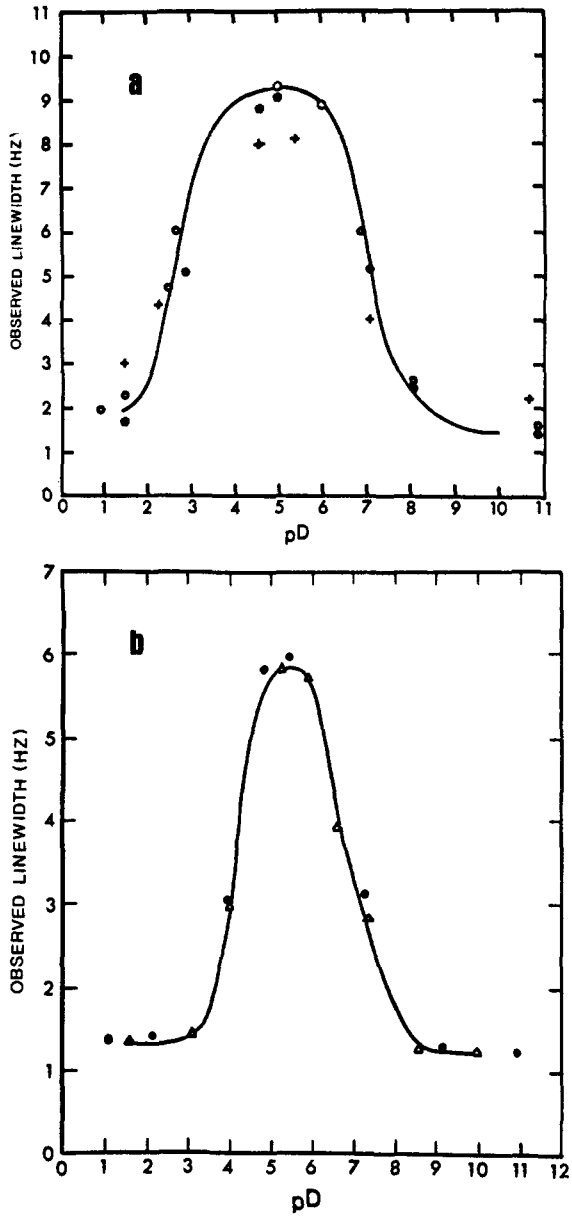

Figure 2. The pD dependence of GH8 and CH5 line widths at 100 MHz and 30°. (a) GH8: solid line, taken from Lee and Chan;[15] (○) crude Sigma 2′-GMP + purified 2′-GMP, 0.1 *M* 2′-GMP; (+) 1×10^{-3} *M* Fe^{3+}, 0.05 *M* purified 2′-GMP; (●) 2×10^{-5} *M* Cu^{2+}, 0.05 *M* purified 2′-GMP. (b) CH5 (△) taken from Lee, Prestegard, and Chan;[15] (●) 3.2×10^{-6} *M* Cu^{2+}, 0.1 *M* purified 5′-CMP.

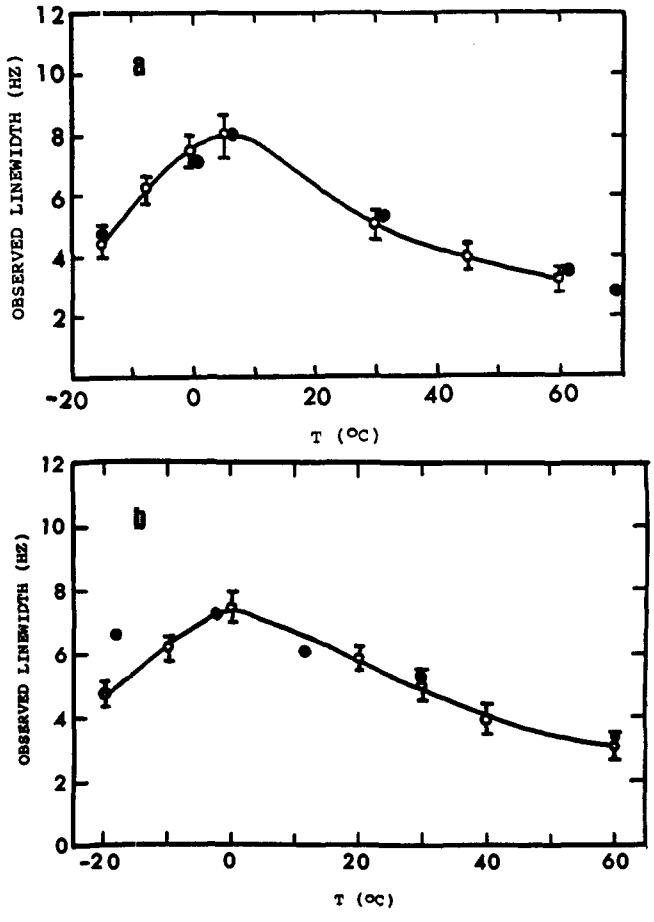

Figure 3. The temperature dependence of GH8 and CH5 line widths at 100 MHz. (a) GH8 (○) taken from Lee and Chan;[16] (●) 2×10^{-5} *M* Cu^{2+}, 0.05 *M* purified 2′-GMP in 5 *M* NaCl at pD 7. (b) CH5 (○) taken from Lee, Prestegard, and Chan;[15] (●) 3.2×10^{-6} *M* Cu^{2+}, 0.1 *M* purified 5′-CMP in 5 *M* NaCl at pD 5.6.

and concentration removes much of the support for the previous contention that minor tautomers are present to the extent of 15 ± 3%.[14-16] Since much of the previously reported data was reproduced by the addition of paramagnetic Cu^{2+} ions, this suggests that paramagnetic (such as Cu^{2+}, for example) contaminations may not have been completely removed in the earlier work. In what follows we shall present an analysis of our experimental results of the GH8 and CH5 line widths in the presence of paramagnetic Cu^{2+} ions and show how previous observations of line-width variations in 2′-GMP and 5′-CMP and their derivatives can be explained.

pD-Dependence Studies. (a) 2′-GMP and 5′-CMP. Experimentally it is observed in the present and previous studies that the GH8 line width has a value of 8 to 9 Hz at pD 5 ± 1.5 and decreased when the pD was either increased or decreased.[14-16] In our experiments the observed GH8 line-width broadening can be explained by the binding of the paramagnetic Cu^{2+} ion at the N_7 site. It has been shown previously that when Cu^{2+} binds to a ligand the protons nearest that binding site are relaxed more rapidly by the paramagnetic ions and their nmr line widths are thus broadened.[23,24] At lower pD's protonation of N_7 occurs (pK_a = 2.3) and this competes with the Cu^{2+} ions for the ligand and a gradual narrowing of the line width was observed. Finally, at pD < 2, a line width of 1.8 Hz was observed. In the high pD range (pD > 7) the binding of Cu^{2+} to N_7 is decreased apparently due to an increase in the proportion of the Cu^{2+} ions involved in copper hydroxide formation; until about pD 11 most of the copper is in the copper hydroxide complex and no line-width broadening is observed.

Similarly, the fact that the maximum CH5 line width of 6 Hz is observed at pD 5–6 and decreases when the pD either increased or decreased can be explained by Cu^{2+} binding at the N_3 site. At the lower pD values, the N_3 site can be protonated (pK_a = 4.3), and at pD < 2 a line width of 1.4 Hz was observed. At the

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higher pD's (pD > 7) the binding of Cu^{2+} to N_3 is decreased presumably due to increased proportions of the Cu^{2+} ions that are involved in copper hydroxide formation, as in the case of 2′-GMP.

On close examination of Figure 2 it is seen that the GH8 has a different pD profile than CH5, and one might suspect that the pD dependence of 2′-GMP and 5′-CMP is different. Since the pK_a of N_7 of 2′-GMP is lower than the pK_a of N_3 of 5′-CMP, and presumably the formation of nucleotide–copper complex is favored above their pK_a's and below pD 6.4, the observed different pD profiles for GH8 and CH5 could merely reflect the difference in pK_a's of 2′-GMP and 5′-CMP and the pD-dependence profiles of these compounds are caused by the same mechanism.

Our results are in agreement with the extensive work of Eichhorn and coworkers[25-28] on the binding of copper and other divalent metals to nucleic acids. They have already shown that copper, at the concentration used in the present study, broadens the GH8 and CH5 resonances preferentially.[25] It is generally agreed that GN_7 and CN_3 sites are involved in metal binding and consequently the GH8 and CH5 line widths are broadened.[25,29]

(b) Other Guanine and Cytosine Derivatives. It was reported that the line width of GH8 and CH5 of other guanine and cytosine derivatives[14-16] which we did not examine in the present study also exhibited the pD-dependence behavior of 2′-GMP and 5′-CMP. Although we do not know the exact source of line-width broadening, it is quite possible they too can be explained by the same reasons presented in the previous section a. For example, in 2-*N*,*N*-dimethylaminoethyleneguanosine and the dinucleotide ApG, the GH8 line width was preferentially broadened since the N_7 site is available for metal binding. The preferential broadening of the GH8 line width in ApG over AH2 and AH8 line width has been observed previously in a Mn^{2+} binding study.[29] However, in other guanine and cytosine derivatives, only sharp GH8 and CH5 resonances were observed throughout the entire pD range. This could be due to a lower level of paramagnetic metal contamination or the fact that the GN_7 and CN_3 sites were blocked and/or could be sterically hindered and rendered unavailable for metal binding. For example, the binding site in 3-methylcytidine is blocked and in 4-*N*,*N*-dimethylcytosine and 6-methoxypurine riboside the CN_3 and GN_7 binding sites are probably sterically hindered.

Temperature-Dependence Studies. It is observed that the GH8 and CH5 line widths, at a given pD, vary as a function of temperature.[14-16,19] These line widths reached a maximum value at a particular temperature and decreased when either the temperature is increased or decreased. These results can be taken to indicate that chemical exchange is taking place and the observed line width is the result of averaging between the free, diamagnetic and the bound, paramagnetic states. A rigorous treatment of paramagnetic broadening has been given by Swift and Connick[30] and a qualitative treatment was given recently by Glassman, *et al.*,[31] in their study of metal binding to CTP and ATP. Although no attempt is made here to obtain a detailed kinetic analysis of the temperature dependence of the GH8 and CH5 line-width data, it appears that some simple, qualitative statements are sufficient to explain the observations.

In a solution containing nucleotides (2′-GMP or 5′-CMP) and Cu^{2+} ions there are paramagnetic Cu^{2+}–nucleotide complexes and free diamagnetic nucleotide molecules. In the absence of exchange the GH8 or CH5 line width of the free nucleotides is relatively sharp. The proton resonances GH8 and CH5 of the complexed state cannot be observed because their signals are generally too broad and they are present in very low concentrations (*ca.* 10^{-5} to 10^{-6} *M*). If the exchange of nucleotides between the free and the complexed states is allowed, the line width of the GH8 and CH5 will be affected. In the slow exchange limit the proton line width will be increasingly broadened as the rate of exchange increases by increasing temperature. As the rate of exchange between the complexed and the free nucleotides increases (by increasing temperature) and reaches some intermediate exchange limit, the line width will reach a maximum value. Finally, on increasing the temperature further, the GH8 and CH5 line widths will again sharpen as the fast exchange limit is reached.

Experimentally it is observed that the GH8 line width of 2′-GMP increases with increasing temperature between −20 and 8°. Similar results were also obtained for the CH5 line width of 5′-CMP between −20 and 0°. These results indicate, within this temperature range, that the exchange rate could be in the slow to intermediate exchange limit (on the nmr time scale). Between 8 and 60° the GH8 line width decreases with increasing temperature and this result could be taken to indicate the exchange rate is in the intermediate to fast exchange limits. Similar results were also obtained for the CH5 line width of 5′-CMP in the temperature range of 0 to 60°.

EDTA Effect. It was previously noted[14-16] that the broadened GH8 and CH5 line width can be sharpened by the addition of $\sim 10^{-6}$ *M* EDTA. If paramagnetic metals such as copper were present to the extent of $\sim 10^{-6}$ *M*, adding EDTA would certainly be sufficient to collapse the GH8 and CH5 line width since EDTA is an extremely good chelating agent (with a copper binding constant of $10^{17.4}$). Since other chelating agents such as ethylenediamine have a complex constant of about $10^{10.5}$, it is not surprising that ethylenediamine is not nearly as efficient as EDTA in removing paramagnetic metal contaminations. The previous authors argued that EDTA merely served as a catalyst for the tautomeric exchange and not a chelating agent; however, it is difficult to understand why EDTA is more efficient than ethylenediamine in catalyzing the proposed tautomeric exchange of guanine and cytosine.

G–U Base Pairing. Subsequent to the suggestion

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that there is 16 ± 3% minor tautomer of guanine in aqueous solution,[16] Chan and coworkers reported that they have obtained ^1H nmr evidence indicating the existence of abnormal G–U base pairing.[17] By increasing the fraction of water in the guanosine–uridine solution dissolved in a DMSO–H_2O mixture, they observed line-width broadening of the GN_1H and UN_3H resonances. However, by increasing the fraction of water in either guanosine or uridine alone, little line-width broadening was observed.[17] This is in contradiction with the observation[32] where the GN_1 or UN_3 line width in guanosine or uridine alone increases in the same order of magnitude as those reported in their study of guanosine–uridine dissolved in a DMSO–H_2O mixture.

Recently Raszka and Kaplan reported ^1H nmr results indicating the interaction of GMP with UMP involved hydrogen bonding[33] and might be taken as evidence of G–U base pairing. It is also important to note that there is no evidence of G–U base pairing in poly G–U in a circular dichroism study.[34] If the lactim tautomer of guanosine were present to ~16% and abnormal G–U base pairing were energetically feasible, the existence of G–U pairing should have been detected in the CD study. In view of this result and the results presented in the previous section that the existence of a minor tautomer of guanine to the extent of ~16% is unlikely, the evidence of G–U base pairing must be taken with caution.

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Conclusion

New experimental evidence is presented showing that the GH8 and CH5 line-width broadening phenomena do not appear in purified samples of 2′-GMP and 5′-CMP and that the unusual line-width broadening of these resonances observed in the ^1H nmr spectra can be caused by paramagnetic Cu^{2+} ions. The pD dependence of these line widths in the presence of paramagnetic Cu^{2+} ions can be understood by metal binding or protonation at the GN_7 and CN_3 sites at the appropriate pD. The temperature dependence can be explained by the different rate of chemical exchange between the free nucleotide and the Cu^{2+}–nucleotide complex. We conclude therefore that, while the minor tautomers of guanine and cytosine certainly exist, there is no strong evidence to indicate they are present to the extent of 15 ± 3%. Furthermore, the conclusion reached by the previous authors about the existence of G–U base pairing must be viewed with caution since conflicting ^1H nmr evidence was reported and no G–U base pairing was observed in a recent study of the circular dichroism of poly GU.

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Peptide Steric Effects on the Kinetics of Copper(II)–Tripeptide Reactions

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Abstract: Variation of the residues from glycyl to L-leucyl or L-alanyl in copper tripeptide complexes of the type $Cu(H_{-2}\text{ tripeptide})^-$ causes a significant decrease in the rates of nucleophilic attack on these complexes. A L-leucyl residue in the middle position (GLG) exhibits the greatest steric effect for the removal of copper from the tripeptide complex by the reaction of triethylenetetramine (trien). The rate constant for this process is reduced by a factor of 200 compared to the triglycine complex, $Cu(H_{-2}GGG)^-$. A similar effect is observed for the GGL tripeptide while the substitution of L-leucine for glycine at the amine terminal (LGG) is relatively ineffective in reducing the rate of the trien reaction. The reactions of ethylenediamine (en) and $EDTA^{4-}$ with the $Cu(H_{-2}\text{tripeptide})^-$ complexes exhibit similar steric effects and in some instances [$Cu(H_{-1}$tripeptide)en] mixed complexes are observed. The relative rates of the reactions of the different tripeptides with en and trien support a mechanism in which the initial attack is at the carboxylate end with the rate step being the cleavage of the Cu–N(peptide) bond adjacent to the carboxylate terminal. However, in contrast to the nucleophilic reactions, the rate constants for the general acid catalyzed transfer of copper(II) from $Cu(H_{-2}\text{tripeptide})^-$ to $CuEDTA^{2-}$ show relatively little dependence on the structure of the tripeptide, the variation being less than a factor of 3 in all cases. The absence of steric effects for the acid rate constants indicates that for these reactions the rate-determining step is the proton transfer to the deprotonated peptide group rather than cleavage of the Cu–N(peptide) bond.

Previous work has demonstrated the existence of two paths for the replacement of triglycine from copper–triglycine. In general, ligands containing primary or secondary amine groups react rapidly *via* a nucleophilic path[1] while the reactions with tertiary nitrogen containing ligands are slower and are indirect, tending

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