Binding of Copper(II) and Manganese(II) to 9-Methylpurine Studied by Nuclear Magnetic Resonance and Potentiometric Titration

NILS AGE FRØYSTEIN and EINAR SLETTEN
Department of Chemistry, University of Bergen, 5000 Bergen, Norway
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Abstract

The binding of Cu(II) and Mn(II) to 9-methylpurine has been examined by proton NMR and potentiometric titration. The spin–lattice relaxation rates of ring protons H2, H6 and H8, used as input to the Solomon–Bloembergen equation, give a binding pattern which qualitatively agrees with crystallographic data. At neutral pH Cu(II) and Mn(II) binding occurs at both N1 and N7 with a preference for N7 (66%). At acidic pH, where N1 is the predominant protonation site, a further shift towards N7 binding is observed. These results are not in complete agreement with those based on comparison of N1/N7 basicity.

Introduction

NMR paramagnetic relaxation rates have often been used to elucidate molecular geometries in solutions of bioinorganic and biological systems [1, 2]. The multisite binding behaviour of nucleic acids and their monomeric constituents towards cations both in solution and in the solid state has been the subject of extensive studies [3–6]. Several of these studies seem to focus on an apparent discrepancy between the solid state and solution structure. Fazakerley *et al.* [1] have used relaxation rates in combination with the well-known Solomon–Bloembergen equation to determine binding sites for Cu(II) and Mn(II) in N(9)-substituted purine bases. The results obtained are not compatible with structural information obtained in the solid state [3]. The most obvious discrepancy being the proposal of chelate formation between the C6 substituent and N7.

Obviously, the preference for certain binding sites depends on pH, which has to be monitored carefully. The solid state structures relevant to the present study were all crystallized from water at neutral or slightly acidic solutions [7–9].

For 9-substituted 6-oxopurines with $pK_a \approx 9$ at N1, predominantly N7 coordinated species are obtained at neutral pH. The corresponding 6-aminopurines have $pK_a \approx 4$ at N1, leaving both N1 and N7 available for metal coordination at neutral or slightly acidic pH. The distribution between N1/N7 binding depends to a certain degree on the intrinsic donor properties of the ligating atom. By comparing stability constants of N1 and N7 type ligands *versus* pK_a values, Martin [6] concludes that, e.g. for adenosine with most metal ions, N1 coordination dominates at $pH > 3$. However, several other factors are of importance; e.g. the stereoselectivity of metal ions depending on preferred coordination geometry; denticity of competing ligands; type of anion in solution; stacking interaction; and the formation of intra- and inter-complex hydrogen bonding.

Steric requirements certainly play an essential role, as shown by Arpalahti and Ottoila [5]. Introduction of methyl substituents in 9-methylpurine decreases the stability of the metal ion complexes even though the electropositive inductive effects of methyl groups increase the basicity of the ligand atoms. In all known crystal structures of 6,9-substituted purine–metal complexes, octahedral geometries are only found for N7 coordination. In those cases where both N1 and N7 binding take place, invariably tetrahedral, square-planar or linear geometries have been observed. The only known structure with octahedral coordination simultaneously at N1 and N7 is the copper complex of 9-methylpurine where the 6-position is free [9]. In order to compare crystal and solution structures we have carried out NMR studies of 9-methylpurine complexation in aqueous solution. The ligand has three well-resolved single protons which are suitable as NMR probes for determining binding sites. Potentiometric titration experiments have been performed to determine stability constants.

Experimental

Materials

9-Methylpurine (9-MePu) was prepared by treating the free purine with dimethyl sulphate in acetone [10]. Separation of the isomeric mixtures was obtained by using a DOWEX-50WX2-200 ion exchange

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resin loaded with Mg(II) ions. The resin was of standard grade and was washed several times by 2 M HCl and 2 M NaOH and finally with distilled water. Purine and the ion exchange resin were obtained from Sigma Chemical Company.

Titrimetric Measurements

Potentiometric titrations were carried out with an automatic titrator (Metrohm 636) equipped with a Ross-type pH electrode, model 81-02, obtained from Orion Research. Stability constants for the 9-MePu–Cu(II) complexation were determined by titrating an acidic solution of 0.01 M 9-MePu, 0.05 M $Cu(NO_3)_2$ and 0.15 M KNO_3 with 0.1 M NaOH. The total volume was 100 ml and 0.2 ml aliquots of base were added to give pH values in the range 2.0–6.0. The stability constants were calculated with the computer program SUPERQUAD [11]. As input to the program for determining precision in K, errors in volume and pH were estimated at 0.001 ml and 0.002 pH units respectively.

NMR Measurements

^{1}H NMR data were obtained at 100 and 200 MHz with Bruker CXP-100 and CXP-200 instruments operating in the pulse/Fourier transform mode. The free induction decay over a 2000 Hz bandwidth contains 8 K data points. For shift data a pulse length of 2.4 μs, corresponding to a 45° flip angle, was used. To improve the signal-to-noise ratio an exponential multiplication was applied to the FID, adding 0.2 Hz to the line width. Prior to Fourier transformation, 8 K zero filling was added. Spin–lattice relaxation times (T_1) were determined using a 180°–τ–90° pulse sequence with typically 12 τ-values. T_1-values were obtained by fitting the peak intensities to a single exponential decay curve by a three-parameter least-squares program. Spin–spin relaxation times (T_2) were derived from measured line widths at half height.

9-MePu and the metal salts were prepared in D_2O (99.8%). Chemical shifts were measured relative to internal tetramethylammonium nitrate, but are reported relative to the methyl resonance of DSS (2,2-dimethyl-2-silapentane-5-sulphonate). DCl and NaOD were used to adjust pH values, which are given as pD by adding 0.4 units to the meter reading. The titration experiments were performed by incremental addition from micropipettes. The pH was measured with a Radiometer PHM64 Research instrument equipped with an Ingold microcombination electrode, except for the data at 200 MHz, where a Beckmann pH-Meter H5 with a Cole-Parmer electrode was used.

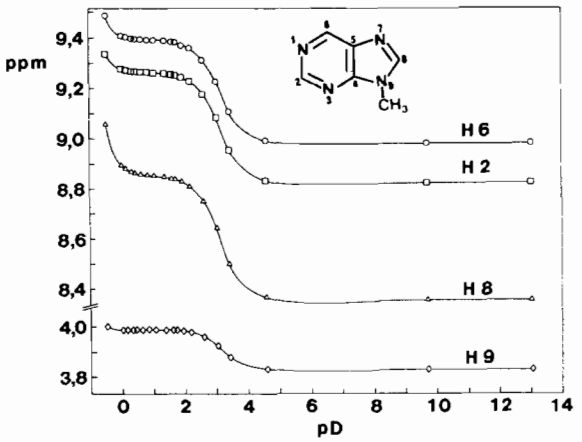

Fig. 1. Chemical shifts of 9-methylpurine (0.05 M) in D_2O *vs.* pD at 298 K (Bruker CXP-100). The ppm scale is downfield from DSS.

Results and Discussion

Protonation of 9-MePu

^{1}H NMR spectra were measured as a function of pH at 0.025 M and 298 K. At this low concentration, less than 8% of the 9-MePu exists as dimers and higher aggregates [12]. The titration curves in Fig. 1 indicate two protonation steps in the measured region, corresponding to $pK_{a1} = 3.16$ and $pK_{a2} \approx -1$, the latter being a rough estimate. The corresponding pK_{a1} value obtained in H_2O by potentiometric titration is 2.69; the difference of 0.47 may be explained by isotope effects [13].

The site of protonation in 9-MePu is less obvious from an inspection of the titration curves. Qualitatively, at least, the effect of protonation on the chemical shifts indicates that all the basic sites (N1, N3, N7) are involved. Based on ^{13}C–H satellite spectra of purine in aqueous solution, Read and Goldstein arrived at a distribution of 47, 24 and 29% between N1, N3 and N7 + N9 respectively [14]. However, later work [15, 16] based on ^{15}N-shift data clearly points towards exclusive N1 protonation for the mono cation. Protonation of N1 in purines is found to cause relatively large changes in the geometry of the pyrimidine ring, while the imidazole part is left unaffected. From this background it is difficult to rationalize why N1 protonation produces almost identical shift changes for H2 and H8. In numerous papers on purine complexes of diamagnetic metal ions, chemical shifts of ring protons have been used to determine predominant binding sites. Such data should be treated with caution in view of the experience with protonation equilibria.

Changes in coupling constants, on the other hand, seem to support the idea of N1 protonation. The H2 and H6 peaks exhibit a splitting of 0.3–0.4 Hz at

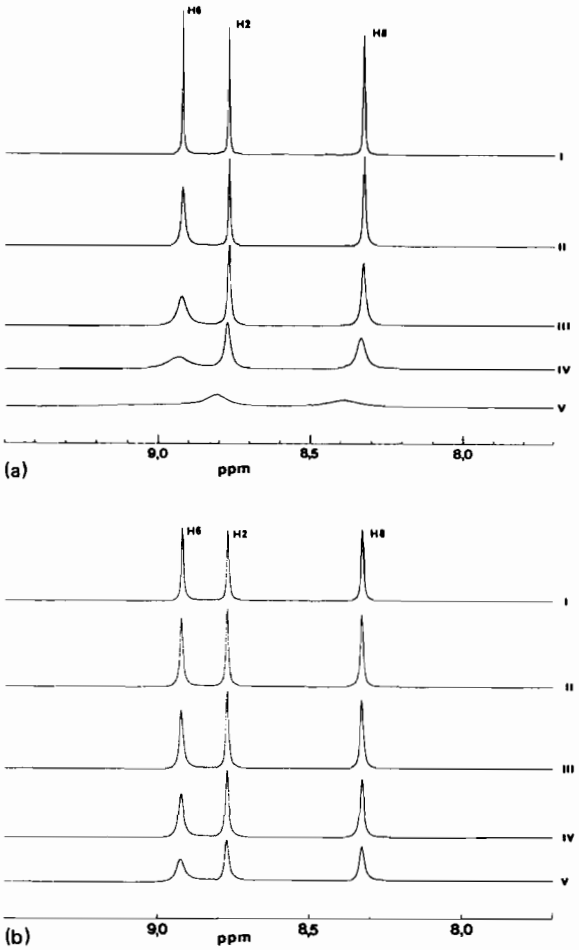

Fig. 2. ^{1}H NMR spectra of 9-methylpurine (0.05 M) in D_2O at 200 MHz (289 K, pD = 6.70), I–V. (a): $Cu(II)_{conc.}$ = 0, 0.99×10^{-5}, 1.97×10^{-5}, 3.9×10^{-5} and 13.4×10^{-5} M. (b): $Mn(II)_{conc.}$ = 0, 2.17×10^{-5}, 4.3×10^{-5}, 8.4×10^{-5} and 18.6×10^{-5} M.

pD = 1.50, not detectable at pD = 6.7. This $J_{2,6}$ coupling is evidently promoted by N1 protonation. A corresponding splitting is not observed for H8. The same type of splitting pattern observed in unsubstituted purine has been explained by Read and Goldstein [14] as due to decreased quadropolar line broadening at H2 and H6 when N1 is protonated. However, in 9-MePu the overall line width slightly increases with decreasing pH, indicating a true pH-dependent coupling mechanism.

T_1 values for the mono cation are significantly longer than those for the neutral molecule. This effect could be explained by invoking a decreased ability of charged molecules to form stacks in solution. However, in the range of concentrations used, the fraction of neutral molecules forming aggregates is too low to significantly influence T_1 measurements. The relaxation mechanisms involved are basically related to changes in electronic structure and rotational correlation times, the latter being affected by hydrophobic interaction between water molecules and neutral/charged species. Changes in electronic structure in the molecule by protonation should manifest themselves by affecting the ring protons differently. Comparing T_1 values for neutral and charged 9-MePu, the changes for H2, H6 and H8 are 5.6, 5.8 and 3.9 s respectively, supporting the idea of N1 protonation.

The theoretical mechanism relating chemical shift, coupling constants and relaxation times to particular protonation sites in small molecules is not well understood at present. Several factors are involved requiring a systematic investigation to elucidate the basic principles.

Metal Binding to 9-MePu

The paramagnetic ions Cu(II) and Mn(II) selectively broaden the ring proton resonances of 9-MePu, as shown in Fig. 2. At neutral pH, the order of broadening is H8 > H6 > H2. As first pointed out by Espersen and Martin [17], geometric information derived from line broadening studies are less reliable, since T_2 usually is dominated by scalar interaction. However, in several cases assignments of binding sites of paramagnetic ions based on T_2 measurements have proved to be in qualitative agreement with those derived from T_1 data [18].

The origin of spin–lattice relaxation time (T_1) enhancement caused by paramagnetic metal ions is assumed to be a dipolar coupling between the unpaired electrons on the metal and a nucleus on the ligand. Through-bonds scalar interaction is almost always negligible for T_1 [17]. Thus the Solomon–Bloembergen equation [18] for the inverse spin–lattice relaxation time for nuclei of ligands bound to paramagnetic ions can be used to obtain geometric information:

$$T_{1P}^{-1} = \frac{6}{15} pq\gamma_I^2\beta^2 S(S+1)\tau_c r^{-6} \quad (1)$$

T_{1P} is the induced paramagnetic enhancement, p the ratio of molar concentration for paramagnetic metal ion to ligand, q the average number of ligands bound in an identical way, τ_c the correlation time modulating the dipolar interaction, and r the distance between the paramagnetic ion and the measured nucleus. Since τ_c is difficult to determine accurately, only relative distances between a paramagnetic ion and ligand nuclei can be calculated:

$$(T_{1P}^{-1})_A/(T_{1P}^{-1})_B = (r_B/r_A)^6 \quad (2)$$

The results of copper titration of 9-MePu *versus* T_{1P}^{-1} at neutral and acidic pH are shown in Fig. 3. The ring proton resonances are shown to exhibit selective T_{1P}^{-1} response in the same order as shown in the line broadening spectra. The methyl signal shows no significant interaction with Cu(II) at

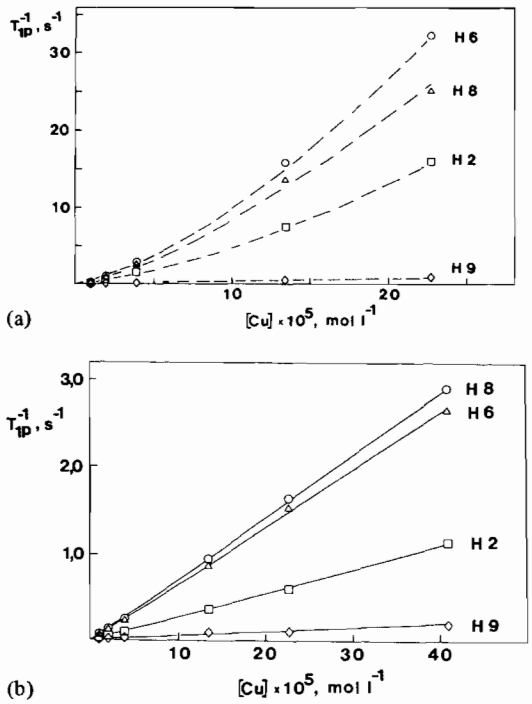

Fig. 3. Paramagnetic induced spin–lattice relaxation *vs.* copper ion concentration at 298 K. (a): pD = 6.70; (b): pD = 1.50.

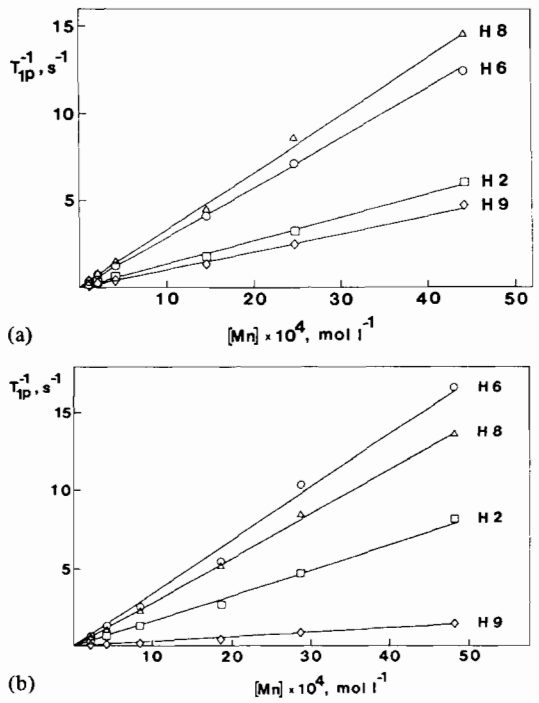

Fig. 4. Paramagnetic induced spin–lattice relaxation *vs.* manganese ion concentration at 298 K. (a): pD = 6.70. (b): pD = 1.50.

neutral pH but, at low pH and especially with Mn(II), a marked paramagnetic influence is observed.

In the crystal structure of Cu(II)–9-MePu obtained from neutral aqueous solution, octahedral Cu complexes are shown to form a chain structure composed of Cu–N1–Cu–N7 bridges [9]. The simultaneous binding at N1 and N7 of 6-coordinated Cu(II) ions is sterically feasible since the 6-position is unsubstituted.

In the concentration range studied at pD = 6.70, the T_{1P}^{-1} *versus* added $CuCl_2$ shows a non-linear response. Two parallel titrations with ligand concentration 0.05 M and a third run with half this concentration gave virtually the same result. In contrast, the experiment run at low pH shows perfect linearity. Also the Mn(II) titrations both at neutral and low pH appear to give linear response (Fig. 4). At a metal-to-ligand ratio of 1:250 the fraction of mononuclear species in solution is predominant. However, the shift from non-linear to linear plots by lowering the pH may be explained by invoking a minor fraction of binuclear N1/N7 coordinated species at neutral pH. At low pH with N1 being the main protonation site, binuclear N1/N7 complexes are less likely to be present in solution. Mn(II) complexes of 9-MePu are far less stable than the corresponding Cu(II) complex, the pK values being 0.2 and 1.88 respectively [20]. Thus any binuclear Mn(II) complexes are not expected to be present causing non-linear response.

The main question to be addressed in this paper is the distribution of metal ions between the potential binding sites (N1, N3, N7). Applying the T_{1P}^{-1} ratios derived above, eqn. (2), one has to assume isotropic tumbling rates for the protons involved. As a first approximation the purine ring may fulfill this requirement. The methyl group, on the other hand, obviously has additional rotational motion and should not be included in any calculations of internuclear distances. Earlier work on purines based on the SB-equation did not discriminate between methyl and ring protons [1].

Based on the large amount of data both in the solid state and in solution on N9-substituted purine complexes, N3 is not a likely candidate for metal binding. Thus a simplified expression giving the relation between T_{1P}^{-1} and mole fractions of metal attached to the base may be used, where k is a collec-

$$T_{1P,H_i}^{-1} = k\left(\frac{XN_1}{r_{1-i}^{6}} + \frac{X_{N7}}{r_{7-i}^{6}}\right) \qquad (3)$$

tion of fundamental physical constants together with a common correlation time τ_c, X_{N1} and X_{N7} the mole fraction of metal ions at N1 and N7 respectively, and r_{j-i} the distance between the metal ion bonded to nitrogen atom N_j (j = 1, 7) and proton H_i (i = 2, 6, 8). The equation is valid under conditions of fast chemical exchange, which have been demon-

strated by the change in T_{1P} with temperature. The Cu–H distances were calculated from crystal structure coordinates [9]. The same values were also used for Mn(II). Fitting the three T_{1P,H_i}^{-1} equations to the data gave, for Cu(II), 66% N7 binding at neutral pH and 76% at low pH. The results for Mn(II) complexation were not significantly different.

These results are, of course, subject to large errors, but we feel that the qualitative bonding picture is correct. The use of T_1 data in binding studies must always take into account a certain deviation from the point-dipole approximation. In the derivation above, the paramagnetic spin density is assumed to reside on the metal centre. Any charge delocalized to the ligand may affect proton relaxation. The reason why the method is after all useful may be explained by the fact that if N7 binding increases the spin density at C8, a corresponding increase is produced at C2 by N1 binding. Consequently, the net result of a minor scalar interaction may not drastically influence the derivation based exclusively on dipolar effects. This may also explain why line broadening data may after all give useful geometric information.

The level of precision in this type of investigation will inherently be low. It is totally unwarranted to attempt distance determinations down to an accuracy of 0.1 Å in order to distinguish between e.g. monodentate and chelate binding in 6-substituted purines [1]. In order to improve the method significantly, quantum mechanical calculations on transition metal complexes should be performed to obtain realistic ligand charge distributions.

The NMR data presented are in excellent agreement with work on stability constants of complexes of a variety of methylated purines [10, 20]. The complexing ability of 9-methylpurine as compared to the 2, 9 and 8, 9 ligands shows that the C8 methyl group retards the complex formation slightly more than the C2 bonded methyl group, in agreement with the NMR results. A similar study of 9-methyladenine compared to 2, 9 and 8, 9 substitution shows that the C8 methyl has a large influence and the C2 methyl has no effect on the stability constant. This very convincingly demonstrates N7 as the predominant binding site for Cu(II), in accordance with the crystallographic data on 9-methyladenine complexation [3, 4].

Martin and coworkers provide an indirect line of evidence based on basicity of potential donor atoms in order to predict binding sites [6]. In the outset they discard most of the crystallographic data as being obtained in too acidic media. For Cu–adenosine, for example, they propose a 50/50 N1/N7 distribution at neutral pH, shifted towards predominant N1 coordination at lower pH. This bonding scheme does not fit the experimental evidence obtained from stability constant measurement and crystal data on 9-substituted adenine analogues. In our view the complexing ability of the N1/N7 sites appears to be more sensitive to the steric requirement of the metal ion and the adjacent substituents than to the basicity of the binding sites.

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