The Role of Coordinated Water in Metal Ion–Adenine Ring Binding in Complexes of Adenosine Triphosphate*

G. P. P. Kuntz, Thomas A. Glassman, Cecil Cooper, and T. J. Swift†

ABSTRACT: Both ^{17}O resonance at 5.2 MHz and proton resonance at 60 MHz were used to study the shifting of water nuclear magnetic resonance signals in Co^{2+}–ATP and Co^{2+}–CTP solutions. In both cases, Co^{2+}–ATP is two-thirds as effective as Co^{2+}–CTP in shifting the solvent water signal. In contrast, an earlier study of the water proton relaxation rates done at 15 MHz showed approximately equal relaxation for Co^{2+}–ATP and Co^{2+}–CTP solutions. This dependence on the mode of observation is consistent with a model for the Co^{2+}–ATP complex that contains a water molecule simultaneously coordinated to the phosphate-bound metal and hydrogen bonded to the adenine ring.

For the metal–ATP complexes of Co^{2+}, Mn^{2+}, and Ni^{2+} in aqueous solutions, we have proposed that a water molecule is simultaneously coordinated to the phosphate-bound metal ion and hydrogen bonded to N-7 of the adenine ring (Figure 1). By comparing the transverse relaxation rates (T_2^{-1}) of the bulk water protons in solutions of metal–ATP complexes with those metal–CTP complexes we were able to "count" the number of rapidly exchanging water molecules bound to the metal in the complex (Glassman *et al.*, 1971).

The Ni^{2+}–ATP complex was approximately two-thirds as effective at relaxing the bulk water proton as the Ni^{2+}–CTP complex. The most probable explanation for this is that either Ni^{2+} forms a direct Ni^{2+}–nitrogen bond at N-7 and displaces a water molecule from the coordination sphere of the metal or that the Ni^{2+}–ring bond occurs via a water molecule that exchanges too slowly to make a significant contribution to the relaxation of bulk water. The possibility of a direct bond was ruled out by showing that the change in chemical shift of H-8 of the Ni^{2+}–ATP complex is an order of magnitude less than the change in shift of H-8 in a complex in which Ni^{2+} is directly bonded to N-7 of the adenine ring, the bis(acetylacetonato)nickel(II)–adenosine adduct (Glassman *et al.*, 1971).

The relaxation times of bulk water protons in Co^{2+}–ATP and Mn^{2+}–ATP complexes were identical with those in solutions of the corresponding CTP complexes. Since the ATP complexes were shown to have a much greater metal ion–ring interaction than the corresponding CTP complexes and since this ring interaction is not accompanied by any decrease in the metal ion–water interaction, it was concluded that Co^{2+} and Mn^{2+} are bound to the phosphates of ATP and simultaneously are bound to the adenine ring in some outer sphere fashion. It was also suggested that the outer sphere complex involves a bridging water molecule but because the solvent exchanges rates with Mn^{2+} and Co^{2+} are much faster than those with Ni^{2+}, the slower exchanging water molecule could not be detected.

Figure 2 shows the effects of paramagnetic ions on solvent line widths and shifts as a function of reciprocal temperature. The physical and theoretical bases for the regions of Figure 2 are described by Glassman *et al.* (1971) and in references cited by them. Region a and the lower part of region b occur when there is either no exchange or slow exchange between the coordinated solvent and bulk solvent, point c occurs when the signals for the two species coalesce and region e occurs when exchange is very rapid and the shifts and widths have become the weighted averages of the two species. For the water proton relaxation studies at 15 MHz, the exchange rates of all water molecules coordinated to metal ions in Mn^{2+}–CTP, Co^{2+}–CTP, Ni^{2+}–CTP, Mn^{2+}–ATP, and Co^{2+}–ATP complexes are in region e, the weighted-average region. For Ni^{2+}–ATP, two water molecules are in the fast-exchange region e and the third water molecule is in the slow-exchange region, a or b. In order to test the hypothesis that a slowly exchanging water molecule is involved in the metal–ATP complex, it would be necessary to study the Ni^{2+} complexes at a temperature sufficiently high so that the slowly exchanging water molecule enters the fast exchange region e and to study the Mn^{2+} and Co^{2+} complexes at a temperature low enough so that the slowly exchanging water molecule would not be in the fast-exchange region e. The relaxation time of the bulk water protons in the Ni^{2+}–ATP solution would be equal to those of Ni^{2+}–CTP solutions at high temperatures and the relaxation rates of the bulk water protons in Co^{2+}–ATP and Mn^{2+}–ATP solutions would be less than those of the CTP solutions at sufficiently low temperatures. Unfortunately the temperature range experimentally available is not large enough to accomplish this.

There are two ways to change the position of a system on the temperature profile curve. The first is to vary the frequency (Swift and Connick, 1962). For any two nuclear magnetic resonance (nmr) signals arising from chemically nonequivalent nuclei the chemical shift of one with respect to the other is directly proportional to the radiofrequency employed. In the presence of exchange, a higher frequency necessitates a higher exchange rate to achieve signal coalescence. Thus region b of the temperature profile curve is extended to higher temperature by increasing the frequency. The original comparison of Co^{2+}–ATP and Co^{2+}–CTP was performed at 15 MHz. Based on an activation energy of 7 kcal for the solvent exchange process (Swift and Connick, 1962), it can be calculated that at 60-MHz region b will extend to about 40° higher than region b at 15 MHz. A comparison of the water proton shifts at 60 MHz might show the possible difference between Co^{2+}–ATP and

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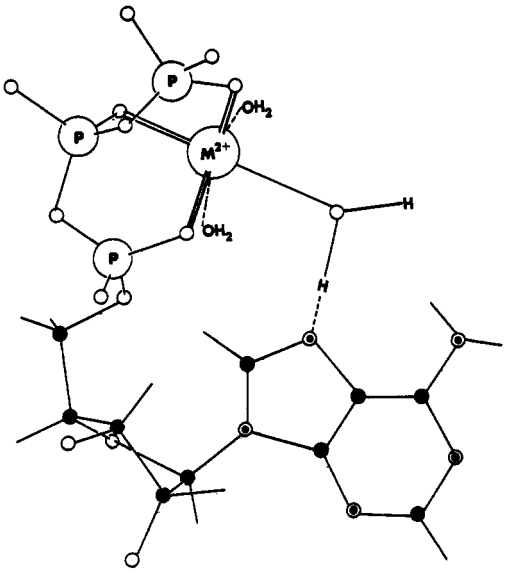

FIGURE 1: Proposed structure of complexes of ATP with Mn^{2+}, Co^{2+}, and Ni^{2+}.

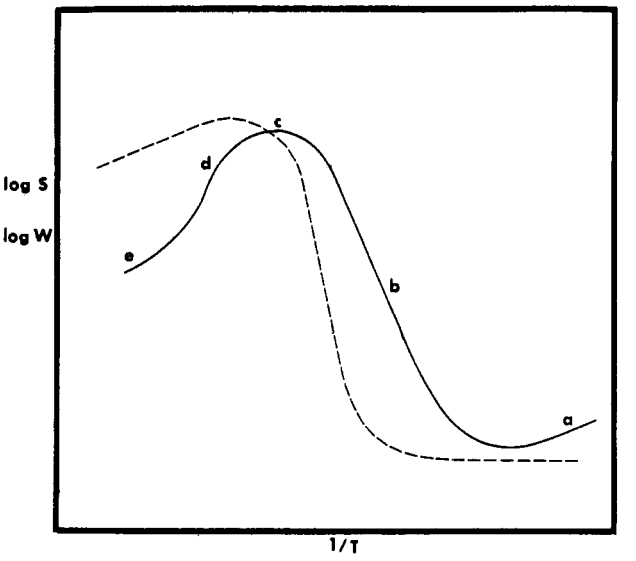

FIGURE 2: Hypothetical plot of solvent line broadening log (W) (—) and line shift, log (S) (---) *vs.* $1/T$ for a solution of a paramagnetic ion.

Co^{2+}–CTP discussed above. Of the metal ions, Co^{2+} and Mn^{2+}, the former is more appropriate for this type of study because the rate of exchange of water in complexes of Mn^{2+} is much more rapid than the rate for complexes of Co^{2+}; as a result region b is more likely to be accessible with Co^{2+}.

The second means for changing the position of a system on the temperature profile curve is to record ^{17}O rather than proton nuclear magnetic resonance spectra. Swift and Connick (1962) have shown that ^{17}O resonance is a more sensitive tool than proton resonance for the study of solvent exchange. In general, with the same paramagnetic ion, the shift to line broadening ratio for ^{17}O resonance is significantly larger than for proton resonance. The reason for this is that the metal ion is bound directly to the oxygen and therefore the transfer of "unpaired spin density" to the oxygen is greater than to the protons. This leads to enlargement of regions b and d. Since the ^{17}O shift is typically more than an order of magnitude larger than the proton shift, the ^{17}O study accomplishes what raising the frequency does in the proton study. Because Mn^{2+} produces greater ^{17}O line broadening than shifting (Swift and Connick, 1962), this ion cannot be used to compare chemical shifts. A comparison of the ^{17}O shifting effects of the Co^{2+}–ATP and Co^{2+}–CTP might be expected to yield results comparable to those obtained for Ni^{2+} by proton resonance.

As can be seen in Figure 2, a study of chemical shifts is much less complicated than a study of line broadenings. The shift reflects the rate of exchange only in region b and does so more sharply with temperature than does the line broadening. The line broadening curve is sensitive to exchange rate both in region b and d and only reaches the weighted-average value in region e, while the shift curve effectively reaches the weighted-average value at point c. For this reason, the most meaningful measurement is of the bulk shifts of the metal–nucleotide complexes rather than the line broadening (or T_2 relaxation rate).

Experimental Section

Oxygen resonance signals were recorded on a Varian VF-16 wide-line spectrometer equipped with the standard temperature control. A 10-mm OD tube contained the sample and the pure ^{17}O water reference was contained in a concentric 3-mm OD tube.

Water proton chemical shifts were measured on a Varian A-60A spectrometer with tetramethylammonium ion as an internal standard.

Lyophilized samples of the metal ion–nucleotide complexes were prepared by the procedure previously outlined and dissolved in water containing 10 atom percent excess ^{17}O. The $[^{17}O]H_2O$ was obtained from ICN. The solutions were adjusted to pH 5.5. This pH was selected because there are no complications from ring protonation or metal hydroxide formation.

Results

Figure 3 shows the water proton shift at 60 MHz produced by Co^{2+}–CTP and Co^{2+}–ATP as a function of reciprocal temperature. Solutions of 0.5 M Co^{2+}–ATP and Co^{2+}–CTP were used so that temperatures well below 0° could be attained. The top of region b, where there is a decrease in shift at the lowest temperature (Figure 2), is particularly apparent. The curve for the Co^{2+}–ATP is displaced from that of Co^{2+}–CTP

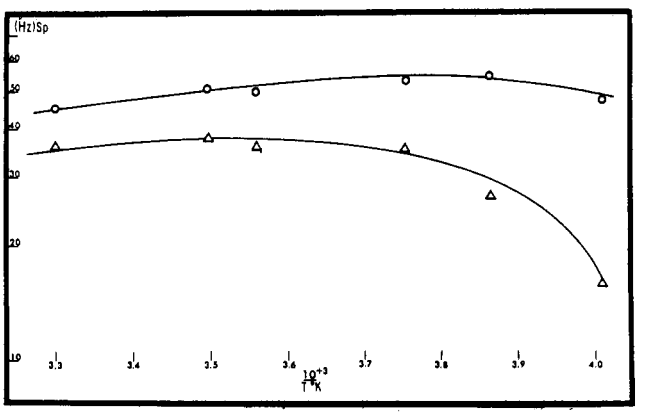

FIGURE 3: Chemical shift (Sp) of the water proton resonance signal at 60 MHz as a function of reciprocal temperature. Solutions contained 0.50 M Co^{2+}–ATP (△) and 0.50 M Co^{2+}–CTP (○).

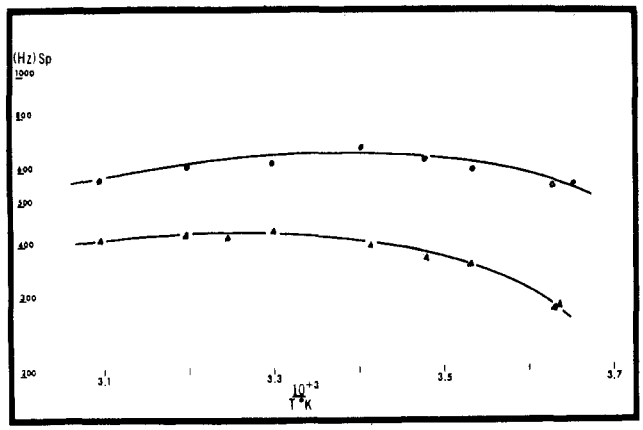

FIGURE 4: Chemical shift (Sp) of the water oxygen resonance signal at 5.2 MHz as a function of reciprocal temperature. Solutions contained 0.11 M Co^{2+}–ATP (△) and 0.11 M Co^{2+}–CTP (○).

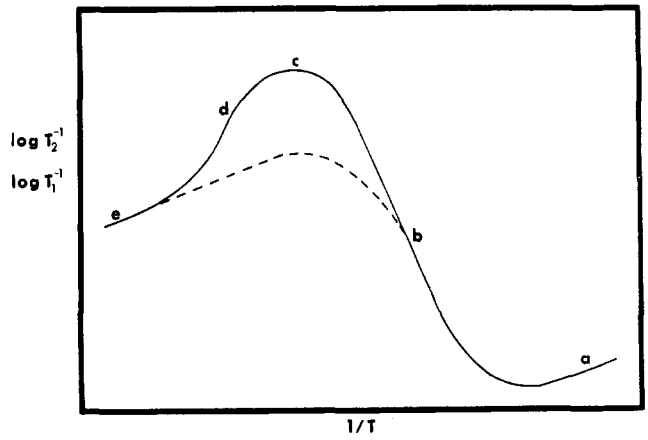

FIGURE 6: Hypothetical plot of log T_2^{-1} (—) and log T_1^{-1} (---) *vs.* $1/T$ for the solvent signal in a paramagnetic ion solution.

with region b extending to higher temperatures for Co^{2+}–ATP. For this reason a comparison of the relative shifting effectiveness must be made at corresponding points on the two temperature profile curves. The best place on the temperature profile to do this is at the maximum shift which corresponds to point c. Note that the maximum shift for Co^{2+}–CTP is nearly 50% greater than for Co^{2+}–ATP (54 Hz *vs.* 37 Hz). There was no significant difference in the ratio of the shifting of Co^{2+}–CTP to Co^{2+}–ATP between pH 5.5 and 6.6 at 60 MHz. This indicates that possible incomplete complexing of the nucleotide to the metal at pH 5.5 did not cause any problems in interpretation of the data.

Figure 4 shows the comparison of water ^{17}O shifts produced by Co^{2+}–ATP and Co^{2+}–CTP as a function of reciprocal temperature. Once again the top of region b, where there is a decrease in shift at the lowest temperature, is readily apparent. As in the case of the water proton shifts at 60 HMz, the curve for Co^{2+}–ATP is displaced with respect to Co^{2+}–CTP. When the corresponding points (coalescence point c) are compared, the striking feature is that Co^{2+}–CTP is approximately 50% more effective in shifting the ^{17}O signal than is Co^{2+}–ATP (640 Hz *vs.* 430 Hz).

Figure 5 shows the spectra of Co^{2+}–CTP at 15° and Co^{2+}–ATP at 34°. The temperatures selected were near the points of maximum shift.

Discussion

From Figures 3 and 4, it is clear that when either ^{17}O resonance at 5.2 MHz or proton resonance at 60 MHz is used to study the shifting of water nmr signals, Co^{2+}–CTP is 50% more effective than Co^{2+}–ATP. This should be contrasted with the study of proton relaxation efficiencies at 15 MHz which showed that Co^{2+}–ATP is 0.94 times as effective as Co^{2+}–CTP (Glassman *et al.*, 1971).

This raises a question concerning the validity of comparing the previous proton T_2 relaxation rates at 15 MHz with the current studies at 60 MHz. A comparison of proton T_2 relaxation rates is only valid if the measurements are performed at a temperature at which the system is uncomplicated by exchange rates and is in the weighted-average region. The curve of log (S) *vs.* T^{-1} reaches the weighted-average value at point c while the curve of log (W) *vs.* T^{-1} reaches the weighted-average value in region e. For relaxation rates, region e is readily distinguished from region d by comparing T_2^{-1}, the transverse relaxation rate with T_1^{-1}, the longitudinal relaxation rate. Figure 6 shows typical temperature profile curves of T_2^{-1} and T_1^{-1}. Two features are apparent; first there is no region d in the T_1^{-1} profile; and second, in region e, T_2^{-1} is equal to T_1^{-1}. The equality of T_1^{-1} and T_2^{-1} is therefore sufficient to show that the system is in region e. In the 15-MHz study previously reported, T_1^{-1} and T_2^{-1} were found to be equal, within experimental uncertainty at all temperatures with both Co^{2+}–ATP and Co^{2+}–CTP.

The inability of the Co^{2+}–ATP complex to shift the water signal as much as the Co^{2+}–CTP complex might be explained by the presence of one less coordinated water molecule in the Co^{2+}–ATP complex because of direct Co^{2+}–ring binding. This explanation is not tenable when the proton nmr studies at 15 MHz are considered (Glassman *et al.*, 1971). No matter what nucleus is employed or what frequency is used, the Co^{2+}–CTP complex would maintain the same degree of shifting effectiveness with respect to Co^{2+}–ATP if it contained one more water molecule than the Co^{2+}–ATP complex, *i.e.*, it would be impossible to change the result by changing the mode of observation.

The best explanation for these observations is that in the Co^{2+}–CTP complex there are three bonds to the phosphates and three to exchanging water molecules. In contrast, in the Co^{2+}–ATP complex there are three bonds between the Co^{2+} and the phosphates, two to freely exchanging water molecules and one to a slowly exchanging water molecule also bound to the adenine ring (Figure 1). If this is the case then it is to be expected that changing the relative positions of these systems on the temperature profile curve by using different experi-

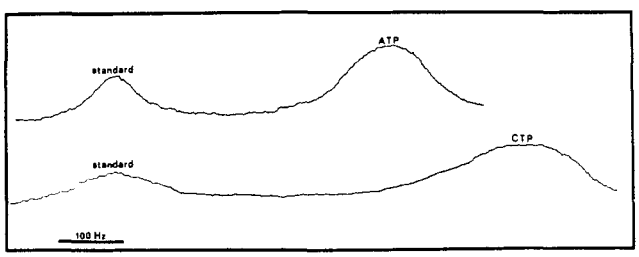

FIGURE 5: $[^{17}O]H_2O$ magnetic resonance spectra of Co^{2+}–CTP at 15° and Co^{2+}–ATP at 34°. The standard water peaks vary in line width because of the different temperatures used in the two cases (Swift and Connick, 1962).

mental conditions will show different degrees of shifting effectiveness. These results therefore, provide strong support for the model (Figure 1) previously proposed (Glassman *et al.*, 1971).

Acknowledgment

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Mathematical Models for Interacting Groups in Nuclear Magnetic Resonance Titration Curves

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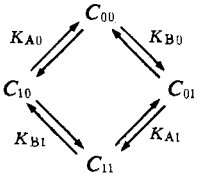
ABSTRACT: Mathematical models were developed for the interpretation of nuclear magnetic resonance titration curves of chemical shift *vs.* pH with emphasis on the case of two interacting titrating groups. The equations were applied by means of computer curve fitting to data obtained for the imidazole C-2 and C-4 proton resonances of L-histidine, *N*-acetyl-L-histidine, L-histidine methyl ester, and several L-histidine dipeptides. Inflections are observed in these titration curves due to the effects of the neighboring titrating amino and carboxyl groups. The curve-fitting procedure provided accurate values for the ionization constants of the imidazole and the interacting titrating groups, even when the pK values were sufficiently close that the inflections had merged to produce an asymmetric curve. Application of these procedures to the titration curves of L-histidyl-L-histidine indicated that the asymmetry noted in the high pH region of one of the curves is due to the effect of an adjacent amino group and not to an interaction between the two imidazole rings.

Proton magnetic resonance spectroscopy has been used to determine the pK values of the imidazole rings of individual histidine residues in peptides and proteins (Bradbury and Scheraga, 1966; Roberts *et al.*, 1969; Cohen, 1969, 1971). The values obtained give information about the local environments of these residues, and may be used to study the effects of ligands and other perturbants (Meadows *et al.*, 1969; Ruterjans and Witzel, 1969). In several cases the titration curves of imidazole proton chemical shift as a function of pH have deviated from the theoretical curve describing a simple proton association equilibrium (Ruterjans and Witzel, 1969; Cohen *et al.*, 1970a; King and Bradbury, 1971; Ruterjans and Pongs, 1971). These deviations represent interactions between the imidazole residues and other charged groups within the molecule.

We present here a general mathematical treatment of such nuclear magnetic resonance (nmr) titration curves, using computer curve fitting for calculating pK values for interacting groups. These methods facilitate identification of interacting groups and thus may prove useful as a "probe" of the environment around histidine residues in proteins.

Theoretical Section. A. GENERAL FORMULA FOR INTERACTING SITES. We assume that two sites, called A and B, can interact in two ways. First, the state of each site, *i.e.*, occupied or unoccupied by some ligand, may influence the equilibrium constant of the other site for that ligand. Second, the state of each site may influence the chemical shift of the other site. The overall state of the two-site systems may be described by four concentrations: C_{00} = concentration with both sites unoccupied; C_{10} = concentration with only site A occupied; C_{01} = concentration with only site B occupied; C_{11} = concentration with both sites occupied. This may be illustrated as follows (Edsall *et al.*, 1958),



Assuming hydrogen to be the sole occupant of the sites, we define the equilibrium constants as follows, where H is the concentration of hydrogen ions

$$K_{A0} = C_{10}/C_{00}H, \quad K_{B0} = C_{01}/C_{00}H, \quad K_{A1} = C_{11}/C_{01}H \quad K_{B1} = C_{11}/C_{10}H \tag{1}$$

from which it follows that

$$K_{A0}K_{B1} = K_{A1}K_{B0}$$

The equilibrium constants are identified by two subscripts:

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