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Phosphorus-31 and Proton Fourier Transform NMR. Metal Ion–Ribose Interaction in the Cu^{2+}–Adenine Nucleotide System

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Using the ^{31}P and ^{1}H NMR techniques, the binding site of Cu^{2+} to adenine nucleotide is clearly specified as a function of the pH values. When the pH is strongly basic, neither the N(7) of the base, nor the phosphate groups are still bound. The ribose moiety is then the coordination site of the metallic cation.

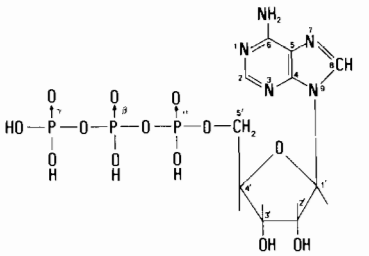
Introduction

The metal ions play a dominant role in the biochemistry of nucleic acids; it is now well known that the metal cations of the 3d-series enhance the rate of dephosphorylation of adenosine-5′-triphosphate (ATP) or adenosine-5′-diphosphate (ADP) [1]. However, this hydrolysis is inhibited when the pH is increased appreciably [2]. This phenomenon was attributed to a modification of the coordination sites of the ligand to the metal [3].

With the complex $Cu(ATP)^{2-}$, present in majority at neutral pH, the N(7) of adenine moiety is directly bound to the metal ion [4]. Moreover ^{31}P NMR studies show that the cation is strongly bound to β and γ phosphate groups [5–7]. There is then formation of a macrocycle implying a folded conformation of ATP. The metal forms a bridge between the base and the phosphate chain. When the pH is over nine, the adenine moiety is no longer bound to the metal [4]. The structure of ATP is then more open and other sites are possible.

Using potentiometric methods to study the adenosine complexes with copper ions, some authors have concluded that there likely exists a metal–ribose interaction at high pH [8–10]. A circular dichroism (CD) study leads to a similar result [11, 12]. However fewer studies have been done with the adenine nucleotides.

In a previous work [13] CD techniques were used to clarify the type of binding between ATP (or ADP) and Cu^{2+} for pH's over 9. At pH 10.5 and 11.5 the CD spectra are characteristic of a metal ion–ribose binding already known for adenosine–Cu^{2+}. But this study did not lead to a conclusion about the eventual existence of an interaction with the phosphate groups for these pH's. In this work the use of ^{31}P and ^{1}H NMR allows us to answer clearly these questions and to confirm our previous conclusions.



Scheme 1: Adenosine-5′-triphosphate (ATP)

Experimental

Materials and Methods

^{1}H NMR spectra were obtained on a CAMECA spectrometer* at 250 MHz. The experimental conditions are: ^{2}D stabilisation, Fourier Transform accumulations (128 scans with 16 K points and Dwell Time of 166 μs).

^{31}P NMR spectra were obtained on a Bruker HX 90 NMR spectrometer* equipped with Fourier Transform and wide band proton decoupling. The experimental conditions are: frequency of 36.43 MHz, ^{2}D stabilisation, complete proton heteronuclear decoupling and Fourier Transform accumulation (1024 scans with 8 K points and Dwell Time of 116 μs).

*Spectrometers of the "Groupement Régional de Mesures Physiques de l'Académie de Nancy-Metz".

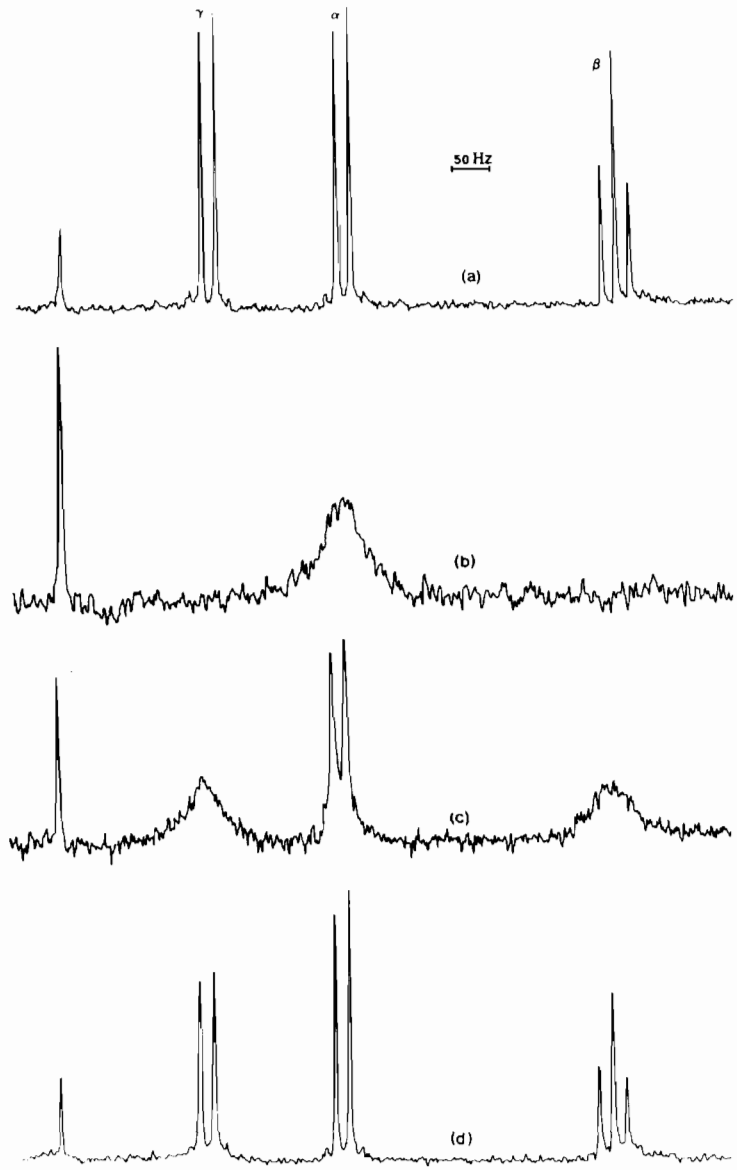


Figure 1. ^{31}P spectra for $NaClO_4$ (0.1 *M*) solutions at 27 °C of ATP alone (5×10^{-2} *M*) at pH 11.45 (or 10.9 or 7.9) (a) and with Cu^{2+} (2×10^{-4} *M*) at pH 7.9 (b), 10.9 (c) and 11.45 (d). D_3PO_4 (0.1 *M* in D_2O) in a capillary tube was used as external standard.

Solutions are prepared in a potentiometer cell at constant temperature (27 °C) and under nitrogen. The pH is measured with a calomel electrode and adjusted by decarbonated NaOH 0.1 N stored under nitrogen. In a typical experiment a solution of the sample to be analysed is prepared by mixing, just before each experiment, of the suitable stock solutions of ligand and metal freshly prepared in $NaClO_4$ 0.1 N. For ^1H NMR spectra H_2O is replaced by D_2O. The pD was calculated by use of the equation pD = pH meter reading +0.4 [14].

The nucleotides were purchased from Calbiochem, San Diego, Calif. and the metal salts were obtained from Fluka A.G., Buchs, Switzerland.

Results

^{31}P NMR Study

ATP in a solution of $NaClO_4$ (0.1 N), with or without Cu^{2+}, was studied at 27 °C and at various pH values between 8 and 11.5. The characteristic spectrum of ATP alone is shown in Figure 1a where the attribution of phosphate groups α, β and γ is indicated [5]. By addition of a very weak concentration of Cu^{2+} to the solution at pH 8, the signals corresponding to P_β and P_γ become progressively wider and disappear in the noise (Figure 1b) for a ratio $[Cu^{2+}]:[ATP]_{total} = 4 \times 10^{-3}$. On the other hand

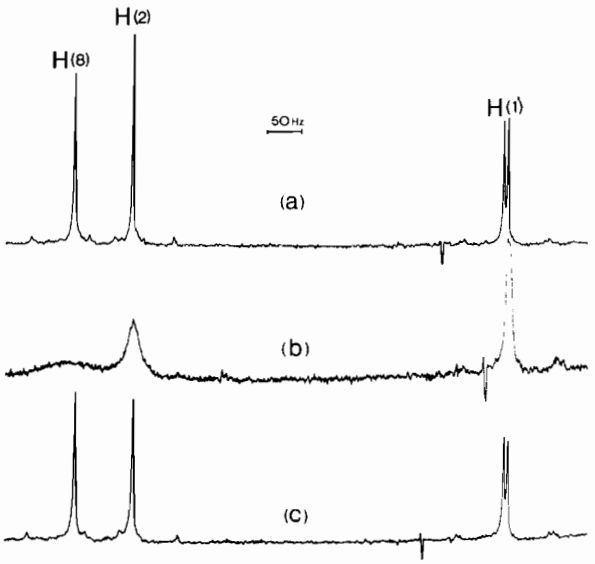


Figure 2. ^1H spectra for $NaClO_4$ (0.1 *M*) solutions at 27 °C of ATP alone (5×10^{-2} *M*) at pD 11.6 (a) and with Cu^{2+} (2×10^{-4} *M*) at pD 7.5 (b) and 12.0 (c).

the signal corresponding to P_α exhibits a small broadening due to a clearly weaker interaction with the paramagnetic cation.

When the pH is increased, these signals again appear progressively. At pH 10.5 (Figure 1c) the P_α signal is very little perturbed while P_β and P_γ again become observable. Figures 1d (spectrum obtained at pH 11.5) and 1a (solution without metal) are practically the same; this result clearly demonstrates that no binding with the phosphate groups persists. In order to verify that no denaturation of the nucleotide occurs, decreasing the pH of the solution has allowed us to again find the spectra b and c of Figure 1.

The same series of experiments was realised with the diphosphorylated nucleotide ADP. Similar results were obtained. As the third phosphate group is missing, only the phosphate groups P_α and P_β are strongly perturbed at pH 8. At pH 11.5 no paramagnetic interaction is observed. 2-2′ bipyridine (bipy) is added to the ATP–Cu^{2+} (or ADP–Cu^{2+}) solutions in order to obtain a final equimolar concentration of bipy and metal ion. At pH 8, 11 and 11.5 the ^{31}P NMR spectra are identical to those of Figure 1a (solution without metal).

Proton NMR Study

^1H NMR allows studying the environment of the protons HC(8), HC(2) and HC(1′) whose signals are separated and easily located [15]. This is not so for the signals of the protons at C(2′), C(3′), C(4′), and C(5′) because these are situated near the signal of HDO which is very widened in the presence of Cu^{2+}.

At pD 7.5 in the presence of a small proportion of metal cations, the proton signals of the adenine ring are more affected than those in C(1′) as shown in Figure 2b (the proton in C(8) is the most perturbed). But when the pD of the solution is increased to 12.0 (with a Cu^{2+} concentration of 2×10^{-4} *M*), the signals of HC(2) and HC(8) (Fig. 2c) narrow. However, the width obtained without the metal cation (Fig. 2a) is not again found since the paramagnetic cations broaden all the signals in a uniform way.

Discussion

Until now the binding sites of metal cations (Cu^{2+}) with adenosine have been precisely determined by CD and NMR [16, 17]. At high pH, a ribose binding has been proposed [8–11]. With ATP, the presence of the phosphorylated chain could modify this type of coordination because it contains phosphate groups which are good chelating agents. A first study of ATP–Cu^{2+} (and ADP–Cu^{2+}) systems by circular dichroism has shown that binding of Cu^{2+} to the ribose moiety exists at high pH's [13]. But is has not allowed us to verify whether the phosphorylated chain still occurred at these pH's. Our present study by ^{31}P and ^1H NMR allows to determine more precisely the structure of the complex obtained at high pH. It should be noted that for quantitative determinations of the binding distances, we must take into account a number of serious criticisms which have been raised against some line broadening studies [18, 19].

Around pH 8, Cu^{2+} is bound chiefly to the β- and γ-phosphate groups [5] as is clearly shown in Figures 1a and 1b. Moreover, the N(7) of the base is also bound to the metal ion [15, 20] (Figs. 2a and 2b). At pH 11.5, the ^{31}P NMR spectrum is practically unmodified by the presence of Cu^{2+}; the phosphate–copper interaction no longer seems to exist.

In a similar manner, the study of ^1H NMR spectra shows that the N(7)–Cu^{2+} binding no longer exists when the pD of the solution increases since the signals of HC(2) and HC(8) are less widened than at pD 7.5. As we have shown, the effect is reversible demonstrating that no deterioration occurs.

The study by circular dichroism indicated that a complex was in fact formed at high pH's [13]. Now if neither the adenylic base nor the phosphorylated chain are bound to Cu^{2+}, the ribose moiety can bind. As Reinnert and Weiss have shown [8], for these high pH values the two hydroxyl groups in 2′ and 3′ are ionised and are then possible binding sites.

During a ^{13}C-NMR study, Weser *et al.* noted a broadening of the signals of the carbon atoms of the sugar moiety, although higher concentrations of Cu^{2+} were required [21]. The C(2′) and C(3′) signals are the most affected. This thus indicates some binding

of the Cu^{2+} to the 2'-OH and 3'-OH groups of the ribose moiety, even for comparatively lower pD values.

Two kinds of complexes were detected by using circular dichroism techniques under equimolar conditions (5×10^{-4} *M*) [13]. For the ratio of concentrations used in our present experiments (very weak proportion of Cu^{2+}), these two types were not observable. However, the question concerning the donor atoms in the coordination sphere of Cu^{2+} arises: either hydroxy complexes, or one Cu^{2+} coordinates to two ribose moieties thus forming two 5-membered chelates. The possibility of forming polymers as has been shown in adenosine–Cu^{2+} systems [22] is improbable with ATP [8]. The formation of the mentioned 1:2 complexes would explain the repulsion of the phosphate groups, and indeed under the experimental conditions $[ATP] \gg [Cu^{2+}]$. Under conditions with $[Cu^{2+}] = [ATP]$, different complexes can be formed.

When pH 11.5 is reached the increase of the dephosphorylation due to the presence of Cu^{2+} is no longer perceptible [2]. It is thus reasonable to believe that this arises at least partially from a modification of the binding sites.

Above pH 8, the ternary ATP–Cu^{2+}–bipy system gives a ^{31}P NMR spectrum identical to that of the binary ATP–bipy system. When the pH is strongly basic, the resemblance of ^{31}P NMR spectra for ternary and binary systems can be explained, as previously, by modification of metal ion binding sites. The ribose moiety is then the only part of ATP bound to Cu^{2+}. This conclusion is confirmed by CD results for equimolar ATP–Cu^{2+}–bipy solutions [23].

For the ternary system, Buisson and Sigel [2] have compiled the different equilibrium constants. Using the se values with the concentration ratio of this paper, the Cu(bipy)ATP^{2-} complex strongly dominates at pH 8. The lack of modification of the ^{31}P NMR spectrum, contrary to the binary system, suggests that no interaction of the metal ion happens. However, at this pH, Cu^{2+} cannot bind the ribose moiety [23] and the observed spectrum results rather from slow chemical exchange of the ATP ligand in the coordination sphere of Cu^{2+} [24]. This agrees with the stability increase formed by Sigel *et al.* [2, 20].

In conclusion, ^{31}P NMR shows that at high pH's the phosphate chain is no longer bound to the metal cations and ^{1}H NMR demonstrates that the binding of N(7) of the base with Cu^{2+} no longer exists. These two results and comparison with those of other techniques therefore show that the ribose residue is coordinated to the Cu^{2+}.

Our results confirm and complete those obtained by CD in our previous work. These techniques are the best to study the nucleotide–metal ion interaction and the simultaneous use of these methods has much potential for a study of biological systems.

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