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^{31}P-NMR Spectra of AP_4

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Summary. ^{31}P-NMR spectra were obtained from adenosine-5′-tetraphosphate under a variety of conditions and the four discernible resonances assigned. The pK-value of the metal-free compound was determined to be 6.4, the pK-value of the Mg^{2+} complex to be 5.3. The dissociation constant for the $AP_4 \cdot Mg^{2+}$ complex was estimated to be 10^{-4} *M* from the downfield shift of the resonances assigned to the γ- and δ-phosphorus nuclei. The binding of a second metal ion can also be followed by NMR; the dissociation constant for this ternary complex is several orders of magnitude larger than that for the binary complex.

Key words: ^{31}P-NMR spectra – AP_4 – Resonances

Introduction

In the last 20 years, i.e. since the introduction of Fourier Transform Spectroscopy, NMR has become a standard method in biochemical research (Wüthrich 1976; Jardetzky and Roberts 1981). The relatively recent introduction of instrumentation allowing the use of large volume samples has broadened the range of applications so that the method is no longer limited to in vitro measurements but can also be applied directly to living organisms. This has led, in the case of ^{31}P-NMR, to the characterization of important metabolic processes, such as phosphate transfer between molecules, such as nucleotides, sugar phosphates, and creatine phosphate in vivo (Hoult et al. 1974; Gadian et al. 1979; Clonek et al. 1981). For such studies, it is important that the metabolites in question should be thoroughly characterised in vitro with respect to their NMR properties.

In addition to the well-known nucleoside mono-, di-, and triphosphates, nucleoside polyphosphates with longer phosphate chains also appear to play an important role in living systems. Polyphosphates have been easily detected in

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yeast using ^{31}P-NMR (e.g., Navon et al. 1978). Similarly, based on ^{31}P-NMR measurements in rat muscle, it has been suggested that adenosine tetraphosphate (AP_4) is present in these cells in addition to the well-known mono-, di-, and triphosphates (Keidel et al. 1984). Since the ^{31}P-NMR properties of AP_4 have not yet been reported in detail, we have investigated these with the object of assigning the individual resonances and describing the dependence of the spectrum on pH both in the presence and in the absence of Mg^{2+}.

Materials and Methods

AP_4 was prepared as described (Feldhaus et al. 1975). NMR spectra were obtained using a Bruker CXP 360 spectrometer at a ^{31}P frequency of 147.8 MHz. Homonuclear double-resonance experiments were performed using a gated-decoupling procedure with the BSV 3 synthesizer-driven frequency unit. Protons were not decoupled. Ten-millimeter sample tubes (Wilmad, Buena, NJ, USA) were used. Typical parameters were: pulse angle 75°; spectral width 4,500 Hz; computer memory 16 k; quadrature detection mode ca. 600 accumulations per spectrum (4,000 for the decoupling experiments). Eighty-five percent H_3PO_4 was used as external standard.

All measurements were done in a solution containing KCl (50 m*M*), Pipes (50 m*M*) and EDTA (0.1 m*M*) with AP_4 at a concentration of 10 m*M*. In addition, the solution contained ca. 20% D_2O to provide a signal for the lock channel.

The pH-dependence of the chemical shift was determined using the least squares method according to

$$\delta = \delta_{AH} + (\delta_{A^-} - \delta_{AH}) \frac{10^{pH-pK}}{1 + 10^{pH-pK}}$$

using a Simplex curve-fitting procedure with δ_{AH}, δ_{A^-} and pK as free parameters.

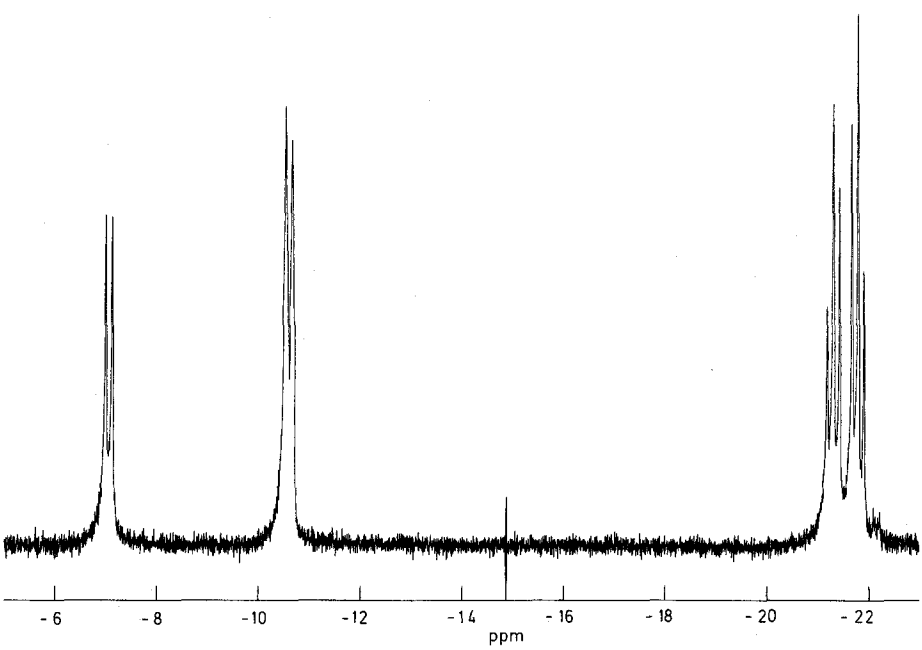

Fig. 1. The ^{31}P-NMR spectrum of 10 m*M* AP_4, pH 7, 50 m*M* KCl, 50 mM PIPES, temperature: 298 K

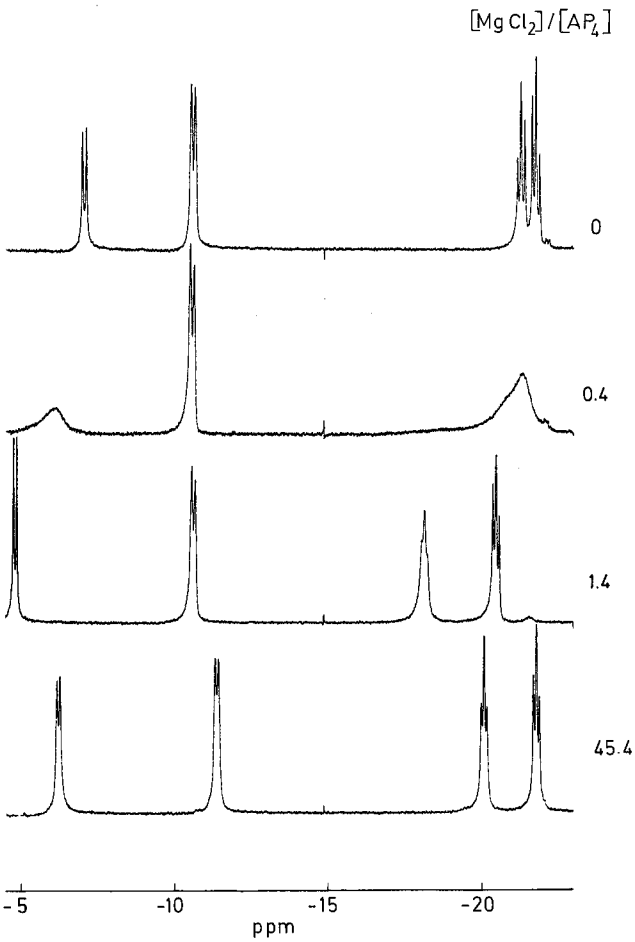

Fig. 2. The ^{31}P-NMR spectrum of AP_4 at various $MgCl_2$-concentrations. Other conditions as in Fig. 1

Results and Discussion

The ^{31}P spectrum of AP_4 at pH 7 is shown in Fig. 1. From the established assignment of resonances in the spectrum of ATP, we can assign two of the four peaks immediately, i.e. the α-phosphate at -10.4 ppm and the terminal (δ-)phosphate at -5.0 ppm (ATP: α-phosphate at -10.9 ppm, γ-phosphate at -6.0 ppm). Analogous to ATP, these resonances are both doublets, due to coupling to the single neighboring phosphorus nucleus. Furthermore, the α-phosphate peak is broadened further, again as in ATP, due to the imperfectly resolved coupling to the neighboring CH_2 group. These features confirm the assignments.

The assignment of the remaining two resonances is less trivial. The resonances of both nuclei are apparent triplets, due to coupling to their neighboring phosphorus nuclei, which are chemically almost equivalent. Spin-decoupling of

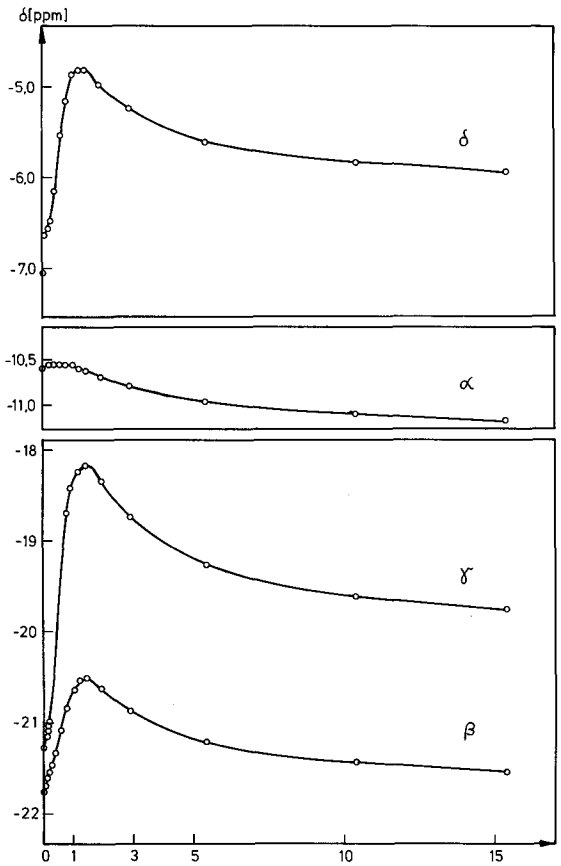

Fig. 3. Chemical shifts of the resonances as a function of the relative $MgCl_2$-concentration

the δ-phosphorus was used to assign the peaks. This led to collapse of the multiplet at lower magnetic field to a doublet, indicating that this resonance (i.e., at -21.5 ppm) arises from the γ-phosphorus. A further decoupling experiment confirmed the spin-coupling of the remaining two resonances.

The influence of Mg^{2+} on the ^{31}P-NMR spectrum at constant pH (7.0) was also investigated. Figure 2 shows the spectrum at various Mg^{2+} concentrations. The main effect of increasing the Mg^{2+} concentration to be equimolar with AP_4 is a shift of both the γ- and δ-resonances to lower field. At intermediate concentrations of Mg^{2+}, both the γ- and δ-resonances are strongly broadened, and this effect can be ascribed to exchange between the species $AP_4 \cdot Mg^{2+}$ and $AP_4 + Mg^{2+}$ (exchange broadening). Since it is apparently an intermediate exchange process, the rate of exchange can be estimated to be about $300 \cdot s^{-1}$. The dissociation constant for the $AP_4 \cdot Mg^{2+}$ complex can be estimated to be about $10^4 \cdot M^{-1}$.

Further increase of the Mg^{2+} concentration above that of the AP_4 leads to a shift of the resonances of the two terminal phosphorus nuclei to a higher field, i.e., in the direction of the free nucleotide (Fig. 3). This shift is probably caused

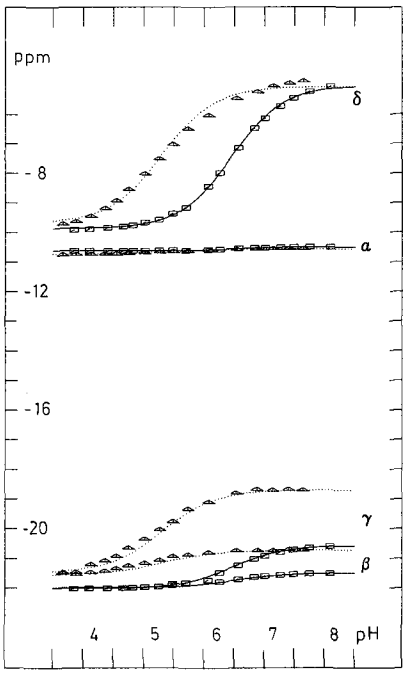

Fig. 4. Dependence of the chemical shifts of the ^{31}P-resonances of AP_4 on pH in metal-free solution (■) and in the presence of Mg (△). Conditions as in Fig. 1: 11 m*M* $MgCl_2$

Table 1. Chemical shifts of the ^{31}P-resonances of AP_4 in metal-free solution

Resonance	δ_{AH}	δ_{A^-}	
α	−10.62	−10.50	(10.9)
β	−21.98	−21.50	(21.3)
γ	−22.02	−20.56	
δ	−9.87	−5.02	(6.0)

pK = 6.45 (6.7); values in brackets are data for ATP (Jaffe and Cohn 1978)

by the binding of a second metal ion to the phosphate chain. The dissociation constant for this complex is, however, much higher than that of the complex with one metal ion, and only a small line broadening is caused, indicating a faster rate of exchange than for the first complex.

The influence of pH on the chemical shifts of the resonance was examined for both the $AP_4 \cdot Mg^{2+}$ complex and the free nucleotide. As in the case of ATP, there is a shift of the δ-resonance to higher field at lower pH values. A shift in the same direction of the γ-resonance can also be recognized. A pK-value of 6.45 can be determined from the titration data. The line broadening effect, which can be seen most easily for the δ-phosphorus nucleus, is caused by a chemical exchange between the protonated and deprotonated forms of AP_4.

The Mg^{2+} complex of AP_4 behaves qualitatively in a similar manner. An upfield shift of both the δ- and γ-resonances is observed on protonation. Again, this is similar to the known behavior of $Mg^{2+} \cdot ATP$. A pK value of 5.3 was esti-

Resonance	δ_{AH}	δ_{A^-}	
α	−10.74	−10.56	(10.7)
β	−21.56	−20.73	(19.1)
γ	−21.48	−18.70	
δ	−9.68	−5.07	(5.5)

pK = 5.30 (5.3); values in brackets are data for ATP (Jaffe and Cohn 1978)

Table 2. Chemical shifts of the ^{31}P-resonances of AP_4 in the presence of Mg^{2+} [$(Mg^{2+}) = 1.1 \times (AP_4)$]

mated for the $Mg^{2+} \cdot$ ATP complex. Figure 4 and Tables 1 and 2 serve to summarize the results of the titration experiments.

The measured J-coupling constants (16–19 Hz) were in the same range as for ATP and ADP.

The results of our investigations with AP_4 show that its ^{31}P-NMR properties are those to be expected from the simplest extrapolation of data from similar substances, in particular ATP and ADP. The spectral properties are particularly similar to those of ATP. Thus, it will be difficult to differentiate between ATP and AP_4 using NMR spectra alone, at least under experimental conditions which do not allow collection of high resolution data or in relatively complicated mixtures of phosphate compounds.

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