# FOURIER TRANSFORM PHOSPHORUS MAGNETIC RESONANCE STUDY OF ATP -- CALCIUM -- G-ACTIN COMPLEX

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## SUMMARY

$^{31}$P-Fourier transform NMR spectroscopy (40.5 MHz) has been employed to investigate the mode of binding of adenosine 5'-triphosphate (ATP) to rabbit muscle G-actin in the presence of calcium in the pH range 6.5 to 10.5. Line width measurements reveal that the nucleotide binds tightest around pH=8.5. Spin lattice ($T_1$) and spin-spin ($T_2$) relaxation times of each of the three phosphorus atoms of bound ATP demonstrate the prime importance of $P_\beta$ and $P_\gamma$ in ATP binding to G-actin through a calcium bridge.

## INTRODUCTION

The binding of nucleoside di- and triphosphates with muscle proteins is of critical importance in the mechanism of muscular contraction. The understanding of the different molecular interactions is rendered extremely difficult in the intact muscle or even in a functional mixture of contractile proteins by the fact that a given nucleotide binds to more than one protein and that the same protein can bind simultaneously more than one nucleotide.

A practical approach, therefore, is to first study the binding of a single nucleotide with a single isolated muscle protein component. The fixation of ATP and related nucleotides to G-actin has already been investigated by various physical and biochemical techniques (1-5). In the present work, phosphorus-31 Fourier transform NMR spectroscopy has been used to obtain struc-

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tural information on the ternary complex ATP--$Ca^{++}$--G-actin.

## MATERIALS AND METHODS

Rabbit muscle G-actin was prepared from the muscle dried acetone powder and purified according to the method of Spudich and Watt (6). The buffer employed throughout the purification scheme is the Spudich Buffer "A", 2 mM Tris-Cl, 0.2 mM ATP, 0.5 mM β-mercaptoethanol and 0.2 mM $CaCl_2$, final pH=8.0. Possible paramagnetic contamination was systematically removed from all buffers and samples by chromatography on Chelex-100 and/or by treatment with dithiazone in $CCl_4$. $^{31}P$-magnetic resonance spectra were obtained at 40.5 MHz on a Varian Associates XL-100 - 15 NMR Spectrometer, equipped with a Transform Technology Fourier transform accessory and Nicolet Model NIC-80 32K-Computer.

## RESULTS AND DISCUSSION

### Line Broadening Measurements

The $^{31}P$-NMR spectrum of ATP, in the absence of protein, is shown in Figure 1. In the proton decoupled spectrum, the upfield doublet is assigned to the $P_\alpha$ phosphorus atom, the downfield doublet to the $P_\gamma$ phosphorus and the triplet is assigned to the $P_\beta$ phosphorus atom. Line widths of 0.3 to 0.5 Hz are easily obtained for all three resonance signals.

The $^{31}P$-NMR spectra of ATP in the presence of active, nucleotide-binding G-actin, under a variety of pH conditions, are shown in Figure 2. Since we are working in a five-fold molar excess of ATP over G-actin (20% of the ATP present is bound to G-actin as calculated from the estimated binding constant, $2.4 \times 10^{+7} M^{-1}$(7)), the dramatic line broadening observed and the appearance of a single signal from each phosphorus atom supports the fast exchange nature of ATP binding to the G-actin surface.

At the pH of maximum line broadening (pH=8.5), the $P_\beta$ and $P_\gamma$ resonance

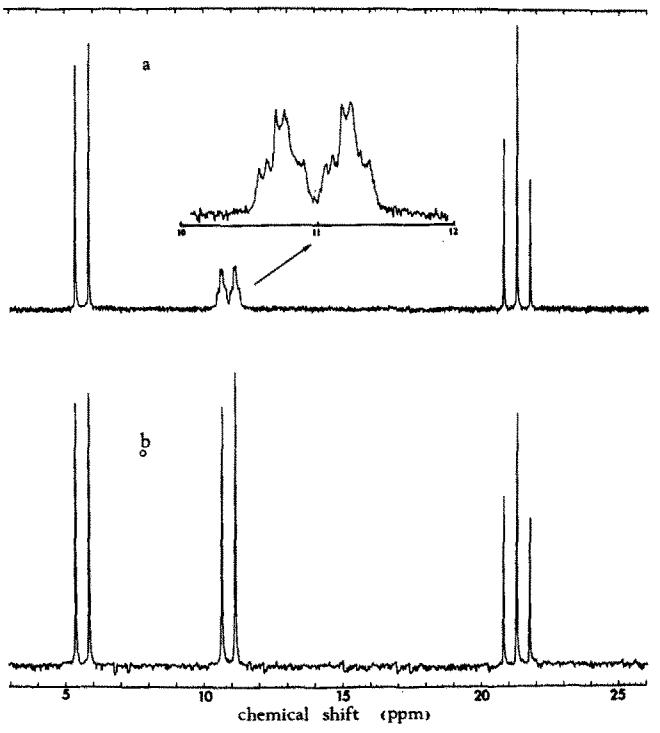


Figure 1. $^{31}P$-NMR spectrum of 0.1 M ATP in $D_2O$ at pH=8.35 and 17°C. Free induction decays were collected at a repetitive rate of 4.0 sec (flip angle=30°) with a sweep width of 1000 Hz using 8K data points. All chemical shifts are recorded upfield from 85% $H_3PO_4$ as external standard. (a) ATP spectrum without proton decoupling, 400 scans. Coupling constants are as follows: $^2J_{P_\beta\ P_\gamma} = {}^2J_{P_\beta\ P_\alpha} = 18.9$ Hz. Insert corresponds to the expanded 10 to 12 ppm region showing the $P_\alpha$ signal with $^3J_{P_\alpha\ H_{5'}} = {}^3J_{P_\alpha\ H_{5''}} = 5.4$ Hz and $^4J_{P_\alpha\ H_{4'}} = 2.4$ Hz. (b) ATP spectrum with heteronuclear proton decoupling, 20 scans. Proton decoupling was achieved with a Varian Spin Decoupler Model V-4421.

signals are broadened to 50 Hz and 40 Hz, respectively, while $P_\alpha$ is broadened to a much smaller extent of 11 Hz. The line width of the $P_\beta$ and $P_\gamma$ resonance signals appear to be sensitive to variations in pH, going through a maximum at pH=8.5 and narrowing to such a degree at the high pH limit (pH=10.5) that triplet and doublet fine structure, respectively, reemerges. Contrasting this

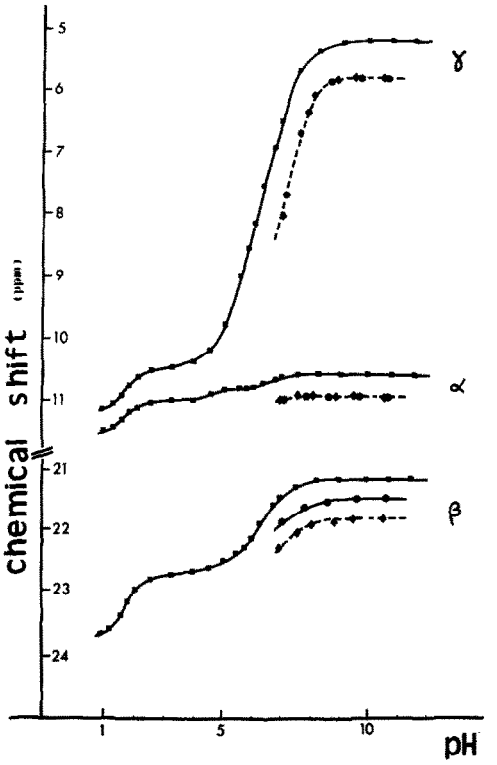


Figure 2. pH titration of the three phosphorus signals of ATP (8K-FID, 20 scans, sweep width=1000 Hz, proton noise decoupling, 17°C) Curves with solid squares: 0.1 M ATP in $D_2O$-Buffer "A". Curves with solid diamonds: 2 mM ATP in $D_2O$-Buffer "A". Curves with solid circles: 2 mM ATP plus 0.4 mM G-actin in $D_2O$-Buffer "A".

behavior is the approximate constancy of line width for the $P_\alpha$ resonance signal throughout the studied range of pH. From the measured line widths, spin-spin relaxation time, $T_2$, values of 20 msec, 1.8 msec and 1.4 msec have been calculated for $P_\alpha$ , $P_\beta$ and $P_\gamma$ , respectively. Thus the differential line broadening and calculated $T_2$ values for the three phosphorus atoms of the bound nucleotide suggest greater involvement of the $P_\beta$ and $P_\gamma$ phosphorus atoms of ATP in binding to the G-actin surface in the presence of $Ca^{++}$.

Chemical Shift Measurements

The different titration curves obtained for the three phosphorus signals of ATP when varying the pH reflects the ionization of the various ionizable groups

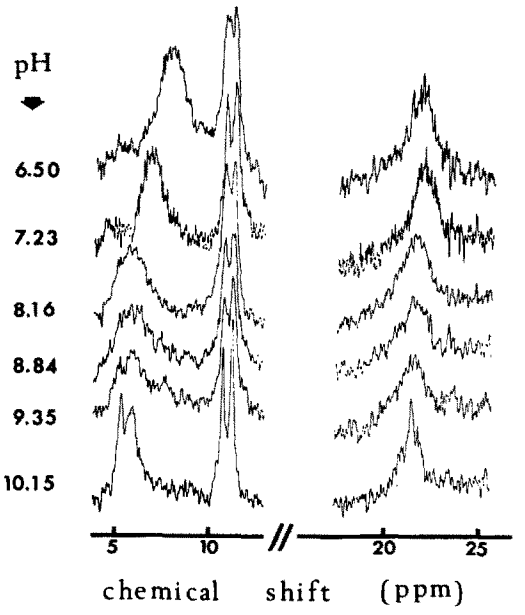


Figure 3. pH effect on the line width of the $^{31}$P-NMR signals of 2 mM ATP in the presence of 0.4 mM G-actin in $D_2O$-Buffer "A" at 17°C, 10,000 scans, sweep width=1000 Hz, 4K-free induction decays.

present in the molecule (Figure 3). The upfield shift of the three signals observed for dilute ATP in Buffer "A" is more sensitive on the $P_\beta$ and $P_\gamma$ than on the $P_\alpha$ and might be attributed to an inter- or intra-molecular complex of $Ca^{++}$ with ATP as described by Cohn and Hughes (8). The probability of the existence of this complex would be enhanced when diluting ATP, given a constant $Ca^{++}$ concentration.

The $P_\alpha$ and $P_\gamma$ titration curves of ATP in Buffer "A" are not significantly affected when G-actin is present (Figure 2), in the pH range studied; i.e., pH=6.5 to pH=10.5. However, the $P_\beta$ signal is shifted downfield by 15 Hz. This change could be attributed to a distortion of the O--$P_\beta$ --O bond angle

upon binding (9) or more likely to the presence of some residual $Mg^{++}$ bound to G-actin in the course of its purification (10).

Thus, with the exception of the small downfield shift noted for the $P_\beta$ resonance, we conclude that negligible chemical shift changes accompany ATP binding to G-actin, at least at the concentration of ATP employed in this study.

Spin-Lattice Relaxation Time ($T_1$) Measurements

The three phosphorus atoms of ATP show similar $T_1$ values in Buffer "A" (Table I). However, the presence of G-actin induces a differential behavior

Table I

Spin lattice relaxation times, $T_1$, of the three phosphorus signals of ATP

| Resonance Signal | Spin lattice Relaxation Time, $T_1$[a] | |
|---|---|---|
| | 2 mM ATP in Buffer "A" pH=8.25 | 2 mM ATP, 0.4 mM G-actin in Buffer "A", pH=8.20 |
| $P_\alpha$ | 1.2 sec | 0.57 sec |
| $P_\beta$ | 1.2 sec | 0.14 sec |
| $P_\gamma$ | 1.2 sec | 0.14 sec |

a. $T_1$ values are measured by the inversion-recovery technique using the repetitive pulse sequence $(180^\circ -- t -- 90^\circ -- T)_n$ where t is a variable delay time between the two pulses and T is a delay calculated to be at least five times the $T_1$ being determined. Best values of $T_1$ were obtained using a $T_1$-calculating computer program processed on an IBM 360 Model 65 Computer.

of the three phosphorus atoms with respect to their $T_1$ values. The $P_\beta$ and $P_\gamma$ exhibit the same short value (0.14 sec) whereas the relaxation of $P_\alpha$ is less affected and is four to five times slower (0.56 sec), indicating a preferential involvement of the two terminal phosphates in the complex.

If the phosphorus nuclear relaxation is dominated by dipolar interactions with protons and if these interactions can be described with a single correlation time, the above values of $T_2$ and $T_1$ can be used to obtain an estimate of relative correlation times, $\tau_c$, for the three phosphorus atoms of ATP in the bound state (11). Such a calculation yields a value of $\tau_c$ for the $\alpha$-phosphorus atom about 60% smaller than for the two terminal phosphorus atoms. Although this estimate is only a crude approximation, due to the number of assumptions involved in the computation (10), nonetheless, the result is in complete agreement with the conclusion based on the separate $T_1$ and $T_2$ measurements of the prime importance of the $P_\beta$ and $P_\gamma$ phosphorus atoms of ATP in binding to the G-actin surface in the presence of calcium.

Further studies, employing proton magnetic resonance techniques, are currently in progress to elucidate any possible involvement of the adenine ring in the binding of nucleotide to the G-actin binding site.

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