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Effects of Manganous Ion on the Phosphorus-31 Nuclear Magnetic Resonance Spectrum of Adenosine Triphosphate Bound to Nitrated G-Actin: Proximity of Divalent Metal Ion and Nucleotide Binding Sites†

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ABSTRACT: G-Actin has one high-affinity binding site for ATP and one high-affinity binding site for divalent metal ions such as Ca^{2+}, Mg^{2+}, or Mn^{2+}. ^{31}P NMR has been used to study the high-affinity ATP binding site of a relatively nonpolymerizable selectively nitrated derivative of G-actin. When paramagnetic manganous ion was added to nitrated G-actin, the line widths of the resonances for the α-, β-, and γ-phosphates of the bound ATP did not increase substantially. However, the areas of the resonances of all three phosphates decreased with increasing concentration of manganous ion. This decrease in area paralleled a decrease in tightly bound calcium displaced by the manganous ion. Manganese-induced polymerization of the nitrated G-actin was found to be a relatively minor process in these experiments. The ^{31}P NMR results are consistent with very slow exchange between the Ca^{2+}·ATP·nitrated G-actin complex and the Mn^{2+}·ATP·nitrated G-actin complex. Thus, the areas of the observed resonances, which represent the Ca^{2+}·ATP·nitrated G-actin complex, vary as a function of the population of this complex, but the line widths are not affected by exchange with the Mn·ATP·nitrated G-actin complex. The line widths of the ^{31}P NMR resonances of the bound ATP in the Mn·ATP·nitrated G-actin complex are too broad to be detected (greater than 400 Hz) due to the paramagnetic effect of the tightly bound manganous ion. This indicates that the high-affinity metal ion binding site on G-actin (occupied by manganous ion) must be less than 10 Å from the ATP binding site.

G-Actin is a globular protein of molecular weight 42 300 (Elzinga et al., 1973) that plays an essential role in the contractile event of muscle cells as well as in various motility processes of nonmuscle cells. G-Actin has three distinct classes of divalent metal ion binding sites. There is one high-affinity binding site primarily for divalent metal ions ($K_D \simeq 10^{-5}$ M)[1] which is required for protein stability (Bárány et al., 1962; Martonosi et al., 1964; Kasai et al., 1965; Frieden et al., 1980). A second class of five to seven lower affinity binding sites ($K_D \simeq 10^{-3}$ M) are implicated in the polymerization of G- to F-actin (Martonosi et al., 1964; Oosawa & Kasai, 1971). Finally, there are low-affinity sites ($K_D \simeq 10^{-2}$ M) which appear to be involved in the formation of paracrystals from F-actin filaments (Hanson, 1973; Strzelecka-Golaszewska et al., 1978). Various studies have shown that several different divalent cations can bind interchangeably to the same high-affinity metal ion binding site, particularly Ca^{2+}, Mg^{2+}, and Mn^{2+} (Drabikowski & Strzelecka-Golaszewska, 1963; Kasai & Oosawa, 1968).

G-Actin requires bond nucleotide, usually ATP, to maintain its structural integrity (Laki et al., 1950; Straub & Feuer, 1950). ATP binds in a 1:1 molar ratio with G-actin, with a dissociation constant of about 10^{-10} M (Engel et al., 1977). It has long been known that the binding of ATP to its binding site on G-actin and the binding of divalent metal ion to the high-affinity metal binding site affect each other (Tonomura & Yoshimura, 1962; Strohman & Samarodin, 1962; Kuehl & Gergely, 1969). Treatment of G-actin with EDTA or Dowex 50 to remove divalent metal ion from the high-affinity site results in a dramatic increase in the dissociation rate constant for ATP leaving G-actin and subsequent irreversible denaturation of the protein (Strzelecka-Golaszewska & Drabikowski, 1967; Strzelecka-Golaszewska et al., 1974; Waechter & Engel, 1977). Alternately, the abstraction of bound ATP by Dowex causes a more rapid dissociation of divalent metal ion from the high-affinity site and again protein denaturation (Bárány et al., 1962; Strzelecka-Golaszewska & Drabikowski, 1967). This parallel dissociation of divalent metal ion and ATP indicates that the ATP binding site and high-affinity metal ion binding site interact strongly but does not prove that these two sites are in fact geographically close to each other on the G-actin molecule. The binding constants for ATP to actin in the presence of divalent cation are about 1000 times stronger than in the absence of cation (West, 1971). In the absence of cation, both ADP and ATP have similar binding constants, while in the presence of bound divalent cation, ATP binds

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[1] Abbreviations: K_D, dissociation constant; ESR, electron spin resonance; k_{-1}, dissociation rate constant; Tris, tris(hydroxymethyl)aminomethane; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; EDTA, ethylenediaminetetraacetic acid.

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much more strongly than ADP. This suggests that the G-actin-bound divalent cation binds directly to the β- and γ-phosphates of ATP. Fluorescence quenching of actin-bound ϵ-ATP by Mn^{2+} bound to the high-affinity site of G-actin is as great as the quenching in the free Mn^{2+}–nucleotide complex (Loscalzo & Reed, 1967); this indicates that these two sites on G-actin are indeed close together and suggests that the nucleotide and divalent cation bind to actin as a metal–nucleotide complex. However, the nucleotide and high-affinity metal binding sites could be separated by tryptic or chymotryptic cleavage (Jacobsen & Rosenbusch, 1976). Recent ^{1}H NMR studies of G-actin (Barden et al., 1980) found no detectable broadening effects of bound paramagnetic lanthanide ions on resonances believed to correspond to protons of bound "mobilized" ATP and concluded that the two sites must be more than 16 Å apart.

By use of ^{31}P NMR, it is possible to monitor the microenvironment of the phosphates of ATP bound to G-actin directly. Jardetzky and co-workers (Nelson et al., 1974; Cozzone et al., 1974) studied the ^{31}P NMR resonances of ATP in the presence of G-actin. Recently, Brauer & Sykes (1981a) were able to study the G-actin-bound ATP resonances specifically, under conditions of slow exchange of ATP between its free and bound sites. G-Actin can be reacted with tetranitromethane to form a derivative in which only one residue (Tyr-69) is nitrated (Elzinga, 1971). This modification inhibits to some extent the polymerization of G-actin without appreciably altering the protein conformation (Elzinga, 1971; Chantler & Gratzer, 1975) or ^{31}P NMR spectrum (Brauer & Sykes, 1981b). The relaxation mechanisms of the ^{31}P NMR resonances of ATP bound to the nitrated derivative of G-actin have been delineated to obtain structural information about the Ca^{2+}·ATP·nitrated G-actin complex (Brauer & Sykes, 1981b). It should be noted that the nitration of a tyrosine residue creates a new metal ion binding site (Marinetti et al., 1975, 1976). However, nitrotyrosine binds metal ion rather weakly, comparable to the affinities of the low-affinity sites of G-actin ($K \simeq 10^{-2}$ M) (Marinetti et al., 1977).

Mn^{2+} is a divalent metal ion very similar to Ca^{2+} and Mg^{2+}, except that it is paramagnetic. Mn^{2+} has been used as an ESR probe of the structure of actin on its own (Loscalzo & Reed, 1976) and as a perturbant of other spin labels on actin (Cooke & Duke, 1971; Burley et al., 1972; Barden et al., 1980). It binds tightly (apparent dissociation constant of 2.6 μM) to the one high–affinity divalent cation binding site and has approximately five more weak binding sites (apparent dissociation constant of 0.6 mM) (Loscalzo & Reed, 1976). The unpaired spin of Mn^{2+} also has dramatic effects on the relaxation rates of ^{31}P resonances in the Mn·ATP complex (Cohn & Hughes, 1962; Sternlicht et al., 1965; Brown et al., 1973). The purpose of this study was to see if Mn^{2+} has dramatic effects on the spin–spin relaxation rates of ^{31}P resonances in the Mn·ATP·nitrated G-actin complex as a means of determining the distance between the metal ion binding site and the nucleotide binding site of G-actin.

Materials and Methods

Actin was prepared from rabbit skeletal muscle by the method of Spudich & Watt (1971). G-Actin, after dialysis against 2 mM Tris, 0.2 mM $CaCl_2$, 0.5 mM dithiothreitol, and 0.2 mM ATP at pH 7.8, 4 °C, was centrifuged at 80000g for 5 h, and the supernatant was nitrated with tetranitromethane by the method of Elzinga (1971). Concentrations of nitrated G-actin were determined at 280 nm. The extinction coefficient for nitrated G-actin at pH 7.8 was determined by the Coomassie blue method (Bradford, 1976), using unmodified G-actin as a standard. For nitrated G-actin, an extinction coefficient of $\epsilon^{1\%}_{280nm} = 12.5 \pm 0.5$ was determined, based on a value of $\epsilon^{1\%}_{280nm} = 11.1$ for unmodified G-actin (Johnson & Taylor, 1978). Nitrated G-actin was concentrated and prepared for ^{31}P NMR analysis as previously described (Brauer & Sykes, 1981b). ^{31}P NMR spectra (109.29 MHz) were taken at 4 °C, using a spectral width of ±2500 Hz, an acquisition time of 0.409 s, a repetition rate of 2.0 s between scans, and a pulse width of 22 μs (78° pulse angle) (Brauer & Sykes, 1981a).

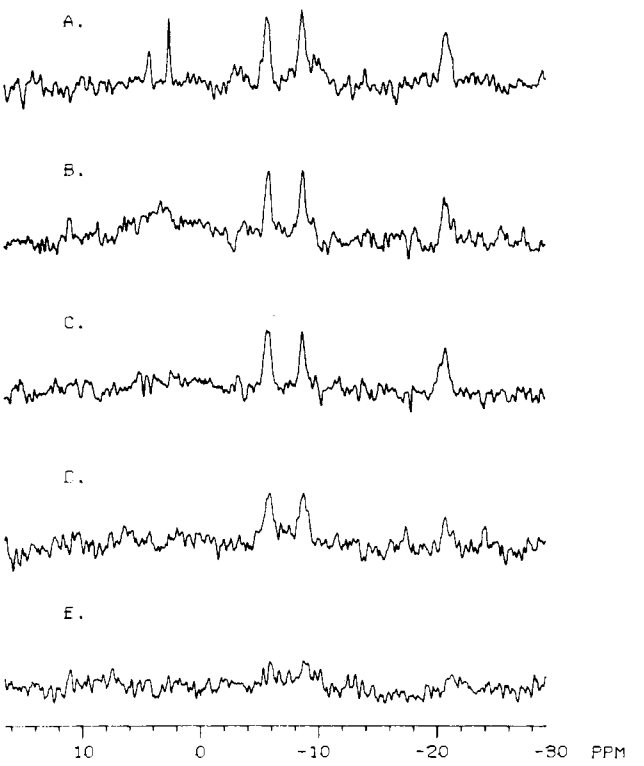

FIGURE 1: Effects of Mn^{2+} on the ^{31}P NMR spectrum of nitrated G-actin. 109.29-MHz ^{31}P NMR spectra were taken of nitrated G-actin (0.88 mM) in 1 mM Tris, 0.1 mM $CaCl_2$, and 0.25 mM dithiothreitol in 50% deuterium oxide at 4 °C, pH 7.8. Exponential multiplication of the free-induction decay was used to decrease spectral noise, resulting in 20-Hz line broadening. Molar ratios of Mn^{2+} to nitrated G-actin were as follows: (A) 0; (B) 0.11; (C) 0.32; (D) 0.59; (E) 1.08.

Manganese chloride (reagent grade) was purchased from Mallinckrodt Chemicals. For the titration of nitrated G-actin with Mn^{2+}, aliquots of $MnCl_2$ were added gradually with stirring to the protein solution at pH 7.8, 4 °C, and samples were then analyzed by ^{31}P NMR. For determination of the concentration of tightly bound Ca^{2+} in the presence of added Mn^{2+}, unbound divalent metal ions and divalent metal ions bound to the low-affinity sites on G-actin were first removed by a 2-min exposure to AG 50W-X8 resin (Bio-Rad Laboratories) equilibrated in 1 mM Tris, pH 7.8 at 4 °C, according to the method of Strzelecka-Golaszewska & Drabikowski (1967). The resin was then centrifuged at 8000g for 1 min, and an aliquot of the supernatant was analyzed for Ca^{2+} by atomic absorption spectroscopy (Willis, 1961).

Results

The effects of increasing concentrations of Mn^{2+} on the ^{31}P NMR spectrum of nitrated G-actin are shown in Figure 1. Figure 1A shows the spectrum of nitrated G-actin in the absence of Mn^{2+}. The two sharp resonances at +4.2 and +2.5 ppm (from 85% H_3PO_4) are assigned to free AMP and inorganic phosphate, respectively. The three broad resonances

Table I: Effects of Mn^{2+} on the Line Widths and Chemical Shifts of Nitrated G-Actin-Bound ATP Resonances

molar ratio of Mn^{2+} to nitrated G-actin	$\Delta\nu$ (Hz)[a]			δ [b]		
	γP	αP	βP	γP	αP	βP
0	48	48	48	−5.64	−8.66	−20.66
0.11	48	43	51	−5.71	−8.68	−20.63
0.32	60	38	60	−5.69	−8.70	−20.81
0.59	68	53	44	−5.73	−8.70	−20.70
1.08	44	50	58	−5.84	−8.73	−20.86
1.08 + AG 50W-X8	40	35	75	−5.76	−8.61	−20.77

[a] Line widths corrected for exponential multiplication of the free induction decay. All line widths are ±10 Hz. [b] Chemical shifts in parts per million relative to 85% H_3PO_4.

Table II: Effects of Mn^{2+} on the Areas of the Nitrated G-Actin-Bound ATP Resonances

molar ratio of Mn^{2+} to nitrated G-actin	area[a]			
	γP	αP	βP	total
0	117	129	94	340
0.11	113	127	77	317
0.32	117	94	80	291
0.59	106	95	67	268
1.08	32	45	42	119
1.08 + AG 50W-X8 resin	31	39	14	84

[a] Areas are in arbitrary units ± 20 units.

at −5.8, −8.7, and −20.8 ppm have been assigned to the γ-, α-, and β-phosphates of nitrated G-actin-bound ATP (Brauer & Sykes, 1981b). With the addition of 0.11 mol of Mn^{2+} per mol of nitrated G-actin (Figure 1B), the resonances of free AMP and inorganic phosphate have been broadened almost beyond detection. However, there is very little effect of the Mn^{2+} on the line widths of the bound ATP resonances (see Table I). As the molar ratio of Mn^{2+} to nitrated G-actin is raised to 0.32, 0.59, and 1.08 (Figure 1C–E, respectively), one can see that the line widths of the bound ATP resonances are relatively constant (Table I). However, the intensities[2] and hence the areas of the resonances decrease dramatically with increasing levels of Mn^{2+} (Table II). At a molar ratio of 2.0 Mn^{2+} to nitrated G-actin, no bound ATP resonances could be seen.

It is possible to remove divalent cation from the lower affinity ($K_D \simeq 10^{-3}$ M) binding sites of G-actin (while leaving the high-affinity site occupied) by using a short exposure time to cation-exchange resins (Strzelecka-Golaszewska & Drabikowski, 1967). When the NMR sample containing 1.08 Mn^{2+} per nitrated G-actin (Figure 1E) was subjected to a 2-min exposure to AG 50W-X8 resin and centrifuged to pellet the resin and the supernatant again analyzed by ^{31}P NMR, no increase in the areas of the bound ATP resonances were seen (see Tables I and II). This indicates that the decrease in the areas of the bound ATP resonances is not due to Mn^{2+} bound to the lower affinity sites of actin or due to unbound Mn^{2+} in the aqueous medium.

[2] In comparing the areas of the resonances, we are assuming that the T_1 values of the bound ATP resonances of the Ca·ATP·nitrated G-actin complex do not change with the addition of Mn^{2+}. Any effect of Mn^{2+} on the T_1 values would result in an increase in the resonance areas because of the rapid repetition rate between scans (2 s) relative to the T_1 values of the bound resonances (3–4 s) (Brauer & Sykes, 1981b). Thus the dramatic decrease in resonance areas cannot be due to a T_1 effect.

Table III: Effects of Mn^{2+} on the Amount of Ca^{2+} Bound to High-Affinity Binding Site of Nitrated G-Actin

molar ratio of Mn^{2+} added to nitrated G-actin	concn of tightly bound Ca^{2+} (mM)	molar ratio of tightly bound Ca^{2+} to nitrated G-actin
0	0.80	0.92
0.22	0.65	0.75
0.42	0.64	0.73
0.61	0.64	0.73
0.79	0.50	0.57
0.95	0.45	0.51
1.11	0.36	0.41

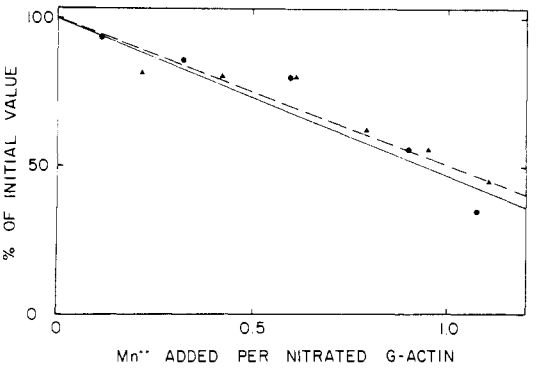

FIGURE 2: Effects of Mn^{2+} on the areas of bound ATP resonances and on the tightly bound Ca^{2+} in nitrated G-actin. For purposes of comparison, both the areas of the bound ATP resonances and the concentrations of tightly bound Ca^{2+} are plotted as percentages of those values in the absence of Mn^{2+}. (−●−) Combined areas of the γ-, α-, and β-phosphate resonances of nitrated G-actin-bound ATP. (--▲--) Tightly bound Ca^{2+} in nitrated G-actin.

Part of the nitrated G-actin sample used in obtaining Figure 1A was set aside for Ca^{2+} binding studies to monitor the displacement of Ca^{2+} from its high-affinity metal binding site by Mn^{2+}. Mn^{2+} was added in increasing amounts to aliquots of this sample and equilibrated for 15 min at 4 °C. Then each aliquot was subjected to a 2-min exposure to AG 50W-X8 resin to remove free and weakly bound Ca^{2+} or Mn^{2+} and centrifuged, and the supernatant was analyzed for Ca^{2+} by using atomic absorption spectroscopy. The addition of Mn^{2+} resulted in a definite decrease in tightly bound Ca^{2+} (see Table III), indicating that Mn^{2+} does in fact displace Ca^{2+} from its high-affinity binding site.

The percentage of initial tightly bound Ca^{2+} as a function of the molar ratio of Mn^{2+} to nitrated G-actin is plotted in Figure 2. The displacement of Ca^{2+} by Mn^{2+} is consistent with a ratio of binding constants of Mn^{2+} and Ca^{2+} to the high-affinity site of nitrated G-actin of about 1.0 ± 0.5. The association constant for Mn^{2+} binding to G-actin at pH 8.0, 0 °C, has been estimated to be 0.6 to 1.0, using the association constant of Ca^{2+} as unity (Oosawa & Kasai, 1971). At pH 7.6, 0 °C, the binding of Mn^{2+} was found to be 1.33 times stronger than that of Ca^{2+} to G-actin (Strazelecka-Golaszewska, 1973). Thus, the ratio of binding constants of Mn^{2+} and Ca^{2+} to nitrated G-actin is within the range of values reported for binding to G-actin.

In Figure 2, the percentage of initial total area of bound ATP resonances as a function of the molar ratio of Mn^{2+} to nitrated G-actin is plotted. The decrease in the total area of the bound γ, α, and β ^{31}P resonances parallels the decrease in Ca^{2+} bound to the high-affinity site of the protein.

One possible mechanism for the decrease in the areas of the bound ATP resonances is polymerization of the nitrated G-actin in the presence of Mn^{2+}. Polymeric F-actin is larger than

monomeric G-actin with a correspondingly longer correlation time and thus would have very broad, virtually undetectable bound resonances. A sample of nitrated G-actin plus Mn^{2+} in a molar ratio of 1.08 Mn^{2+} per protein (same sample as was used to obtain the NMR spectrum in Figure 1E) was subjected to ultracentrifugation at 80000g for 4 h at 4 °C to check for the presence of polymerized nitrated F-actin as the result of added Mn^{2+}. Ninety-two percent of the initial protein, as judged by optical density measurements, was still present in the supernatant after ultracentrifugation, indicating that very little Mn^{2+}-induced polymerization had occurred in the course of these experiments. Also, the polymerization of the G-actin·ATP complex is invariably accompanied by ATP hydrolysis to ADP (Oosawa & Kasai, 1971). A sample of Mn^{2+} plus nitrated G-actin in a 0.95:1.0 molar ratio was denatured at 4 °C in 8 M urea and 10 mM EDTA to check for Mn^{2+}-induced ATP hydrolysis. The ^{31}P NMR spectrum of this sample was taken as before, except for a repetition rate of one scan per 10 s. The spectrum showed 72% ATP, 19% ADP, and 9% AMP, again indicating that polymerization was not a major factor in the decrease of the areas of the bound ATP resonances. The value of 72% ATP is likely a lower limit, since some ATP hydrolysis occurs during concentration and pH adjustment of the nitrated G-actin before any Mn^{2+} is added to the protein.

Discussion

The addition of Mn^{2+} to nitrated G-actin did not result in a significant change in chemical shifts or an increase in the line widths of the bound ATP resonances. The major effects of Mn^{2+} were to decrease the areas of the three phosphate resonances which paralleled the decrease in the amount of tightly bound Ca^{2+}. To explain these effects, let us consider the simple two-site exchange between the Ca·ATP·nitrated G-actin complex (designated site A) and the Mn·ATP·nitrated G-actin complex (designated site B) [see Sykes & Scott (1972); McConnell, 1958]. Each site is characterized by a chemical shift (ω) in radians per second, a line width ($\Delta\nu$) in hertz, a population P, and a lifetime τ. The rate of exchange between the two sites is given by $\tau_{ex}^{-1} = (\tau_A + \tau_B)/(\tau_A\tau_B)$. For $P_A = P_B$, $\tau_{ex}^{-1} = 2k_{-1}$, where k_{-1} is the rate constant for conversion of A to B.

The chemical shift and line width for a resonance at site A can be determined from the spectrum of Ca^{2+}·ATP·nitrated G-actin before the addition of any Mn^{2+}, i.e., $P_A = 1.0$ (Figure 1A). As Mn^{2+} is added, it displaces Ca^{2+} from the high-affinity binding site within the actin protein. This results in an increase in the fractional occupancy of site B, i.e., P_B increases, and the fractional occupancy of site A decreases ($P_A = 1 - P_B$). Theoretical line shapes were calculated (Sutherland, 1971) for two-site exchange situations where P_A was varied from 1.00 to 0 (see Figure 3). The line widths of resonances at sites A and B were assumed to be 50 and 400 Hz, respectively. Cases were considered where the resonances at sites A and B were 200 Hz apart (Figure 3A) and where there was no chemical shift difference between the two sites (Figure 3B). The effects of the rate of exchange between sites A and B were also considered. The spectra on the left represent the slow-exchange limit ($k_{-1} \leq 10^{-2}$ s^{-1}), while the specta on the right represent the fast-exchange limit ($k_{-1} \geq 10^{4}$ s^{-1}). The central column of spectra represents the intermediate-exchange case ($k_{-1} = 10^{2}$ s^{-1}).

It is clear from Figure 3 that in both the intermediate-exchange and fast-exchange cases, the line width of the observed resonance increases as P_A decreases. In Figure 3A, where there is a chemical shift difference between sites A and B, the

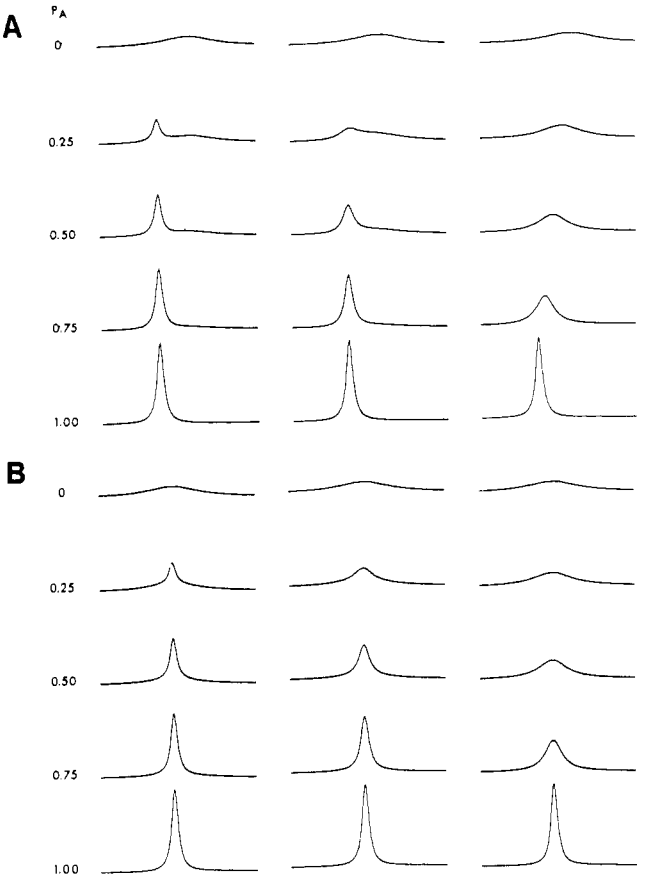

FIGURE 3: Theoretical line-shape analysis for simple two-site exchange. Theoretical curves were determined for a phosphate group of ATP undergoing exchange between site A (the Ca·ATP·nitrated G-actin complex) and site B (the Mn·ATP·nitrated G-actin complex). The resonance at site A has a line width of 50 Hz ($T_{2A} = 0.0064$ s), and at site B the line width is 400 Hz ($T_{2B} = 0.0008$ s). In part A, the resonances for sites A and B are 200 Hz apart ($2\pi|\omega_A - \omega_B| = 200$ Hz) and in part B, the two sites have the same chemical shift ($2\pi|\omega_A - \omega_B| = 0$). Spectra on the left represent the slow-exchange limit ($k_{-1} \leq 10^{-2}$ s^{-1}), spectra on the right represent the fast-exchange limit ($k_{-1} \geq 10^{4}$ s^{-1}), and spectra in the middle represent intermediate exchange ($k_{-1} = 10^{2}$ s^{-1}). From bottom to top, the fractional occupancy (P_A) was varied from 1.00 (site A fully occupied, i.e., zero Mn^{2+}) to 0 (site A unoccupied, i.e., high Mn^{2+} relative to Ca^{2+} concentration). Each spectrum is 1000 Hz wide.

chemical shift of the observed resonance also shifts as P_A decreases. Only in the slow-exchange limit do the theoretical spectra mimic the observed effects of Mn^{2+} on the bound ATP resonances of nitrated G-actin. The observed resonance in both the theoretical and experimental spectra does not change line width or chemical shift, but its height and apparent area do decrease as P_A decreases. In the experimental spectra, the resonance for site B was too broad to be detected over instrumental noise, so one cannot determine whether the slow exchange case in Figure 3A ($\omega_A \neq \omega_B$) or Figure 3B ($\omega_A = \omega_B$) best describes the experimental results.

Literature values for the rates of exchange of divalent metal ions from the high-affinity site of G-actin are quite compatible with a slow-exchange limit situation. The loss of Mn^{2+} from G-actin at pH 8.5, 4 °C, was determined to have a half-life of >24 s or a dissociation rate constant $k_{-1} < 3 \times 10^{-2}$ s^{-1} (Loscalzo et al., 1975). Half-lives of Ca^{2+} dissociating from G-actin at 21 °C were 80 min at pH 8.0 ($k_{-1} = 1.4 \times 10^{-4}$ s^{-1}) and 155 min at pH 7.0 ($k_{-1} = 7.5 \times 10^{-5}$ s^{-1}) (Strzelecka-Golaszewska & Drabikowski, 1967).

The ^{31}P resonances of the bound ATP in site B are too broad to be detected because of their interaction with the para-

magnetic Mn^{2+} bound to the nitrated G-actin. The line broadening induced by Mn^{2+} is described by (Solomon, 1955; Bloembergen, 1957)

$$\frac{1}{T_{2B}} = \frac{1}{15}\left[\frac{S(S+1)\gamma_p^2 g^2 \beta^2}{r^6}\right]\left(4\tau_c + \frac{3\tau_c}{1+\omega_p^2\tau_c^2} + \frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right) + \frac{1}{3}\left[\frac{S(S+1)A^2}{\hbar^2}\right]\left(\tau_e + \frac{\tau_e}{1+\omega_s^2\tau_e^2}\right)$$

where $1/T_{2B}$ is the contribution of paramagnetic relaxation on the spin–spin relaxation rates of the bound ^{31}P nuclei, S is the electron spin quantum number, γ_p is the gyromagnetic ratio for the ^{31}P nucleus, g is the electronic g factor, β is the Bohr magneton, ω is the Larmor frequency, A is the hyperfine coupling constant, τ_c and τ_e are correlation times for the dipolar and scalar interactions, respectively, and r is the distance between the ^{31}P nuclei of the bound ATP and the bound Mn^{2+}. γ_p is 1.083×10^4 rad s^{-1} G^{-1} for the ^{31}P nucleus. For Mn^{2+}, $S = {}^5/_2$, $g \simeq 2.00$, and $\beta = 9.284 \times 10^{-21}$ erg G^{-1}. τ_c can be determined from $\tau_c^{-1} = \tau_M^{-1} + \tau_R^{-1} + \tau_S^{-1}$, where τ_M is the residence time of a molecule in the first coordination sphere of the Mn^{2+}, τ_R is the rotational correlation time of the Mn^{2+}·ATP·nitrated G-actin complex, and τ_S is the electron spin–lattice relaxation time. We know that Mn^{2+} exchanges relatively slowly from its tightly bound site (see above), i.e., $\tau_M^{-1} = k_{-1} = 10^{-3}$–$10^{-5}$ s^{-1}. Thus, we can neglect τ_M^{-1}. The rotational correlaton time τ_R of the Ca^{2+}·ATP·nitrated G-actin complex has been determined to be 40–44 ns (Brauer & Sykes, 1981b). A value for τ_S of about 3×10^{-9} s has been determined for Mn^{2+} in aqueous solution (Swift & Connick, 1962). For our actin system, the best estimate of τ_S has been determined from proton relaxation rate studies of Mn^{2+}·F-actin at pH 8.0, 3 °C, to be 4.4×10^{-9} s (Loscalzo & Reed, 1976). For the purposes of our calculations, we will assume $\tau_c \simeq \tau_S = 4.4 \times 10^{-9}$ s. We cannot determine $1/T_{2B}$ from our experiments, because the resonances were too broad to be detected. From spectral simulation studies, we determined that a resonance with the same area as a bound ATP resonance in Figure 1A would be detectable (signal to noise ratio greater than one) if its line width was less than 400 Hz. Since no resonances could be found for the Mn·ATP·nitrated G-actin complex, the line widths of these resonances must be greater than 400 Hz. From this lower limit of 400 Hz, we can estimate an upper limit for r, the distance between the ATP and divalent metal ion sites on actin. Let us assume for the moment that at this upper limit value for r the direct through-bond scalar interactions between Mn^{2+} and ^{31}P nuclei will be negligible, i.e., $A \simeq 0$. On the basis of the various values and assumptions stated above, the distance r between the tightly bound divalent metal ion site and the bound ATP site on nitrated G-actin cannot be more than 8.4 Å.

The assumption that scalar interactions between the bound Mn^{2+} and the ^{31}P nuclei of bound ATP are negligible ($A \simeq 0$) is almost certainly valid for a distance $r = 8.4$ Å. This is because scalar coupling requires a finite electron spin density at the ^{31}P nucleus. This interaction is probably not important unless the ^{31}P nuclei are involved in direct chemical coupling with the Mn^{2+} such as the participation of the phosphates of ATP in the first coordination sphere of Mn^{2+}. The hyperfine coupling constant $A/\hbar$ for $LiMnPO_4$ crystals was found to be 3.3×10^6 Hz (May, 1963), and for the Mn^{2+}·ATP complex $A/\hbar$ was found to be $>2.0 \times 10^6$ Hz (Sternlicht et al., 1965). For the Mn^{2+}·ATP complex, the distance between the Mn^{2+} and the ^{31}P nuclei of the phosphates in the first coordination sphere of the metal is about 3.3 Å (Sundaralingam, 1969). Thus, the assumption that $A \simeq 0$ almost certainly is valid if $r = 8.4$ Å, but if $r \leq 4$ Å, scalar interactions probably become very important.

Before we can conclude that the high-affinity metal ion binding site and the nucleotide binding site on G-actin are <8.4 Å apart, we must consider other possible mechanisms which could account for the loss of bound ATP resonances upon addition of Mn^{2+}. Mn^{2+}-induced polymerization of the nitrated G-actin was shown to be minimal, since very little nitrated F-actin could be pelleted upon ultracentrifugation and because most of the bound ATP remained unhydrolyzed through the course of the experiments. Decrease in the areas of bound ATP resonances due to the presence of Mn^{2+} free in solution or bound to the low-affinity sites on G-actin was also ruled out, since AG 50W-X8 treatment did not result in any return of the bound ATP resonance to their former areas. Also, these experiments were done at low concentrations of Mn^{2+} such that occupancy of any one weak binding site by Mn^{2+} would be minimal. Thus, neither Mn^{2+}-induced polymerization nor Mn^{2+} binding to low-affinity binding sites on G-actin can account for the loss of the bound ATP resonance areas. Only the paramagnetic broadening effects of Mn^{2+} at the high-affinity metal binding site within 8.4 Å of the phosphates of the bound ATP can account for the effects on the ^{31}P NMR spectrum of nitrated G-actin described in this paper.

Our conclusion regarding the close proximity of the ATP and divalent cation sites is in agreement with the main body of literature (Tonomura & Yoshimura, 1962; Strohman & Samarodin, 1962; Bárány et al., 1962; Strzelecka-Golaszewska & Drabikowski, 1967; West, 1971; Strzelecka-Golaszewska et al., 1974; Loscalzo & Reed, 1976; Waechter & Engel, 1977) which finds that the two sites affect one another and are close together and that in all likelihood the tightly bound divalent cation is complexed to the phosphates of the bound ATP. The results of Jacobson & Rosenbusch (1976) in which the nucleotide and divalent cation sites can be proteolytically separated are not incompatible with the model presented above. The divalent metal ion may be partly complexed with several amino acid residues on the N-terminal loop (residues 1–67 are removed by chymotrypsin and residues 1–68 are removed by trypsin) and may be further complexed by one or more of the phosphates of ATP bound to the protease-resistant core. (Ca^{2+}, Mg^{2+}, and Mn^{2+} accommodate six ligands into their coordination sphere.) Thus, trypsin and chymotrypsin digestion may result in the removal of residues essential to divalent cation binding and leave the nucleotide site intact, even though the two sites may be physically close together. The work of Barden et al. (1980) indicates that the divalent cation and nucleotide binding sites are more than 16 Å apart. This is based in part on the lack of any effect on Mn^{2+} bound at the high-affinity metal ion site on the ESR spectrum of actin-bound nucleotide spin label. However, if the Mn^{2+} were complexed to the β-phosphate of bound ATP, it could easily be greater than 20 Å from the nitroxide spin label on the adenine ring. Thus, one could easily have a situation where these two paramagnetic centers would be too far apart to affect one another and yet still have the bound ATP very close to the bound divalent cation. ^1H NMR data are also presented, showing that various paramagnetic lanthanides bound at the high-affinity metal ion binding site cause an "increase in the mobility of the bound ATP", but with no paramagnetic shifting or broadening of the H(2), H(8), or H(1′) proton of the bound ATP. However, the premise that 1 mol of a lanthanide per

mol of G-actin binds selectively to the high-affinity metal ion binding site has been strongly questioned (Ferri & Grazi, 1981). Fluorescence studies of Tb^{3+} binding to G-actin indicate that the lanthanides do not bind to the high-affinity site. (When Tb^{3+} was incorporated into the high-affinity site through the use of sonication, the G-actin structure was destroyed.) Rather, these ions bind to six low-affinity cation binding sites, forming microcrystalline aggregates even at low protein concentrations and fairly low ratios of lanthanide to protein (>0.26 mol/mol) (Ferri & Grazi, 1981; Burtnick, 1982a,b). Barden & Dos Remedios (1979) themselves present data which indicates that 1.0 mol of Gd^{3+} per mol of G-actin (in excess of free ATP) displaces only about 30% of the $^{45}Ca^{2+}$ bound to the high-affinity divalent cation site. Thus, it is possible that the addition of the various lanthanides used in the ^1H NMR studies could bind to the low-affinity sites of G-actin, form microcrystalline aggregates having ^1H NMR resonances too broad to be detected, and leave only those resonances for G-actin to which lanthanides have not bound.

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