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Proton Nuclear Magnetic Resonance and Electron Paramagnetic Resonance Studies on Skeletal Muscle Actin Indicate That the Metal and Nucleotide Binding Sites Are Separate†

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ABSTRACT: The distance separating the high-affinity binding sites on actin for a divalent metal ion and nucleotide was evaluated by using high-resolution proton NMR and EPR spectroscopy. Replacement of the Ca^{2+} or Mg^{2+} bound to the high-affinity divalent cation site of G-actin by trivalent lanthanide ions such as La^{3+}, Eu^{3+}, or Gd^{3+} results in an increase in the mobility of the bound ATP as observed in the NMR spectra of G-actin monomers. Little difference was observed between the spectra obtained in the presence of the diamagnetic La^{3+} control and the paramagnetic ions Eu^{3+} and Gd^{3+} which respectively shift and broaden the proton resonances of amino acids in the vicinity of the binding site. Analysis of the NMR spectra indicates that the metal and nucleotide binding sites are separated by a distance of at least 16 Å. In the past, the metal and ATP have been widely assumed to bind as a complex. Further verification that the two sites on actin are physically separated was obtained by using an ATP analogue with a nitroxide spin-label bound at the 6′ position of the purine ring. An estimate of the distance was made between the site containing the ATP analogue and the paramagnetic ion, Mn^{2+}, bound to the cation binding site. These EPR experiments were not affected by the state of polymerization of the actin. The data obtained by using this technique support the conclusion stated above, namely, that the cation and nucleotide sites on either G- or F-actin are well separated.

Skeletal muscle actin has long been known to have a single, high-affinity cation binding site (Martonosi et al., 1964). The site is normally occuped by Mg^{2+} in vivo (Weber et al., 1969) because of the high concentration of this ion in muscle cells. It is readily occupied by Ca^{2+} in vitro (association constant $\sim 10^5$ M^{-1}) as well as a large variety of other divalent cations (Strzelecka-Golaszewska, 1973a,b; Strzelecka-Golaszewska et al., 1978). Recently, it was reported that the lanthanide ion Gd^{3+} displaces the high-affinity Ca^{2+} from its locus on the actin monomer and does so on a stoichiometric basis (Barden & dos Remedios, 1979). Further addition of Gd^{3+} progressively displaces Ca^{2+} from the five low-affinity binding sites (assuming a molecular weight of 42 300; Collins & Elzinga, 1975), thereby displacing all the Ca^{2+} from the actin monomer. The total number of Ca^{2+} binding sites is exactly equal to the number of moles of Gd^{3+} which can bind per mole of actin (Barden & dos Remedios, 1979). It is now well established in the literature that the lanthanide ions occupy Ca^{2+} binding sites in proteins [e.g., see Luk (1971), Matthews & Weaver (1974), Gomez et al. (1974), Moews & Kretsinger (1975), Levine et al. (1977), Epstein et al. (1977), Sowadski et al. (1978), Birnbaum & Sykes (1978), and Cave et al. (1979)]. For general reviews concerning the use of lanthanides as calcium probes in biological systems, see Reuben (1975), Williams (1976), Martin & Richardson (1979), and dos Remedios (1980).

G-Actin has a single site for a nucleotide which preferentially binds ATP with a binding constant of 10^{10} M^{-1} (Engel et al., 1977). G-Actin rapidly denatures in the absence of bound cation and nucleotide (Kasai et al., 1965; Strzelecka-Golaszewska & Drabikowski, 1967). The metal (Barany et al., 1962; Kasai & Oosawa, 1968) and ATP (Martonosi et al., 1960) exchange rapidly in G-actin (of the order of minutes; Strzelecka-Golaszewska et al., 1974), but only slowly in F-actin (of the order of hours).

It has been widely suggested that the divalent cation participates in the binding of the ATP moiety (Martonosi & Gouvea, 1961; Strohman & Samorodin, 1962; Barany et al., 1962; Tonomura & Yoshimura, 1962; Strzelecka-Golaszewska & Drabikowski, 1967). Moreover, the affinity of actin for ATP increases when the high-affinity cation binding site is occupied (Strzelecka-Golaszewska, 1973a,b). Kuehl & Gergely (1969) have demonstrated that the rate of exchange of ATP in G-actin is inversely proportional to the Ca^{2+} concentration. This observation led to the suggestion that the exchange of ATP occurs through those molecules of G-actin which are temporarily free of bound Ca^{2+}. Martonosi et al. (1964) found no proton liberation from actin-bound ATP on addition of Mg^{2+}, and this seemed to indicate that the phosphate side chain of ATP was linked to actin in a way which prevented the Mg^{2+}-induced proton release. These authors suggested that the likely binding site for the divalent cation (Ca^{2+}) is the triphosphate chain of ATP. This conclusion is consistent with the observation (Strzelecka-Golaszewska et al., 1974) that the nucleotide and cation were removed simultaneously from EDTA-denatured actin. More direct evidence for the binding of nucleotide and divalent cation at the same site in actin was obtained from measurements of fluorescence quenching of Mn-ϵ-ATP- and Mg-ϵ-ATP-actin complexes at pH 8.5 (Loscalzo & Reed, 1976).

There is little evidence in the literature to suggest that the metal and nucleotide binding sites are indeed separate. Jacobson & Rosenbusch (1976) observed a large (35 000 dalton), protease-resistant fragment of actin which remained after digestion with either trypsin or chymotrypsin. This fragment apparently has its nucleotide binding site little changed by the proteolysis while the high-affinity metal binding site is apparently lost. Apart from this report, there appear to be no

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other studies which suggest that the two sites are physically separate. Moreover, there are apparently no quantitative estimates of the distance between these sites.

Materials and Methods

Protein Preparation. Actin was extracted from rabbit muscle acetone powder (Carsten & Mommaerts, 1963) and purified according to the method of Spudich & Watt (1971), except for minor modifications reported previously (dos Remedios & Barden, 1977). Protein concentrations were determined from the absorbance at 290 nm with an extinction coefficient ($E_{290}^{0.1\%}$) for G-actin of 0.63 and 0.67 M^{-1} cm^{-1} for F-actin (Cooke, 1975).

EPR[1] *Measurements.* The spin-labeled ATP analogue (NSL) was prepared by the method of Cooke & Duke (1971). EPR spectra were obtained from G-actin samples containing either Mg^{2+} or Mn^{2+} by using a Varian E-3 EPR spectrometer at a frequency of 9.5 GHz at 22–25 °C with a scan time of 4 min and a range of 0.01 T. Free nucleotides were first removed by the addition of 0.1 volume of Dowex X-1 (Cl^-), followed by sedimentation of the resin. Either Mn^{2+} or Mg^{2+} was incorporated into actin by homogenizing F-actin pellets in 5 mM Tris with either 0.2 mM Mn^{2+} or 0.2 mM Mg^{2+} at pH 7.0. Actin concentrations were adjusted to 1.0 mg/mL (24 μM). Depolymerized actin was polymerized by adding 0.1 M KCl with stirring at 0 °C for 2 h. F-Actin was then sedimented at 100000*g* for 4 h. The resulting pellet was then resuspended in 5 mM Tris, 0.2 mM Mn^{2+} or Mg^{2+}, and 0.1–0.2 mM NSL at pH 7.0 and exhaustively dialyzed to convert the actin back to the monomeric form at a concentration of 24 μM. The Dowex X-1 treatment was repeated to remove all but the high-affinity bound nucleotide. Spectra were then measured on Mn–NSL–G-actin and the results were compared.

NMR Experiments. ^1H NMR spectra were obtained on a Bruker HX-270 spectrometer operated in the Fourier transform mode. G-Actin samples were prepared from F-actin pellets containing either Mg^{2+} or Ca^{2+}. Following homogenization in the extraction buffer, the actin was exhaustively dialyzed for 48 h against 0.1 mM ATP, 0.5 mM β-mercaptoethanol, 2.0 mM NaN_3, and 2.0 mM Pipes at pH 6.9. The concentration of G-actin was increased to 150 μM by using an Amicon 52 concentrator with PM 10 membranes. H_2O was replaced with D_2O by using a 10 × 1 cm column of Sephadex G-15 superfine preequilibrated with D_2O. The G-actin samples at a concentration of 100 μM and pH 6.9 (uncorrected meter reading in D_2O) were then placed in 10 mm diameter NMR tubes. No free cation or nucleotide was present. G-Actin concentration was kept below 200 μM at all times to avoid spontaneous polymerization. The bound divalent cation is readily replaced by any of the trivalent lanthanide ions such as Gd^{3+} (Barden & dos Remedios, 1979). Spectra from samples of Gd–actin, Eu–actin, and La–actin were obtained for ratios of added lanthanide ion to actin from 1:20 to 1:1. Each sample contained dioxane as an internal standard for the determination of chemical shifts. However, all shifts are referenced to TSS. Dioxane (0.1 mM) was also used as an internal standard for intensity measurements. Spectra for intensity measurements were accumulated by using sufficiently long pulse delays to avoid saturation of the dioxane resonance. All spectra were obtained with suppression of the residual HOD peak by gated irradiation.

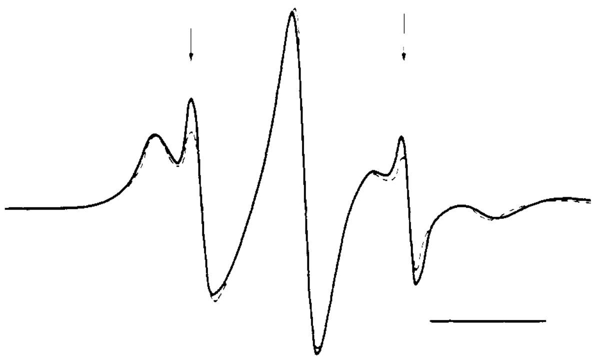

FIGURE 1: EPR spectra of NSL attached to monomeric G-actin at a temperature of 296 K. The nitroxide spin-label has iodoacetamide reactivity and is covalently attached to the 6′ of ATP to give the nucleotide spin-label (NSL). The solid curve contains bound Mn^{2+}; the dashed curve contains bound Mg^{2+}. The arrows indicate the contribution from a small quantity of free spin-label. In the Mg–NSL–G-actin spectrum, there is less free spin-label present and consequently there is a slight reduction in the signal amplitude. The spectra demonstrate that the NSL signal is not significantly altered by the presence of the paramagnetic cation, Mn^{2+}. Bar = 0.002 T.

Results

Binding Measurements. The amount of both Mn^{2+} and NSL in the actin samples was determined by denaturing the actin in 8 M urea at 50 °C for 10 min. The concentrations were then determined by comparison of the spectra for the free Mn^{2+} and NSL with spectra of known standard concentration. The Mn^{2+} was incorporated into 90% of the single high-affinity cation sites while NSL was incorporated into ~50% of the nucleotide sites.

EPR Spectra of the Mn– and Mg–NSL–Actin Complex. As shown in Figure 1, the spectra of Mn–NSL–G-actin and Mg–NSL–G-actin do not differ. In separate experiments the spectrum of the Mn^{2+} was recorded in the presence of NSL. The amplitude of the NSL spectrum was decreased by the presence of the paramagnetic ion Mn^{2+}. Actin samples were polymerized by addition of either 0.1 M KCl or 2.0 mM $MgCl_2$. Either procedure caused a slight broadening of the spectrum. No differences were observed in the resulting F-actin spectra, regardless of the method of polymerization using either Mg–actin or Mn–actin. From the Mn^{2+} EPR spectrum, it was determined that ~50% of the bound Mn^{2+} was exchanged in the presence of an 80-fold excess of Mg^{2+}, when $MgCl_2$ was used as the polymerizing agent.

^1H NMR Spectra of Lanthanide–G-Actin Complexes. NMR Spectrum of G-Actin. G-actin appears to spontaneously polymerize if the actin concentration is not kept below 0.2 mM (8 mg/mL) in a low ionic strength buffer (containing 0.1 mM ATP, 0.5 mM β-mercaptoehanol, 2.0 mM NaN_3, and 2.0 mM Pipes, pH 6.9). Any polymerization or aggregation of actin would result in a broadening of the spectrum beyond detection. Indeed, such broadening commenced when the Gd/actin ratio exceeded 1:1. In the region of the spectrum where the protons of aromatic amino acid side chains resonate, samples of 0.1 mM G-actin scanned in 5 mm diameter tubes resulted in a signal-to-noise ratio that was lower than the threshold required for accumulation and enhancement of data. However, by use of 10 mm diameter tubes, satisfactory spectra were obtained after 4000 pulses for each sample. Since the proton resonances were already broad, due to the large (42 300) molecular weight of the protein and the consequent slow tumbling in solution, the increase in the line width arising from the use of the larger tube size was not a major consideration. Figure 2 illustrates

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[1] Abbreviations used: ^1H NMR, proton nuclear magnetic resonance; EPR, electron paramagnetic resonance; NSL, nucleotide spin-label; Pipes, piperazine-*N,N′*-(2-ethanesulfonic acid); HOD, hydrogen-oxygen-deuterium; TSS, trimethylsilylpropanesulfonic acid.

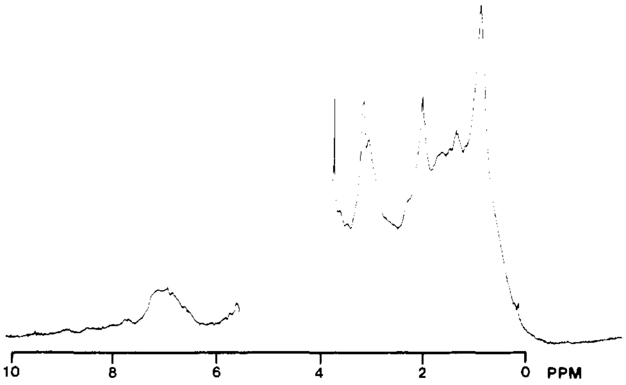

FIGURE 2: 270-MHz ^{1}H NMR spectrum of a 0.1 mM solution of G-actin in D_2O at a temperature of 291 K. All free nucleotide and divalent metal cations were removed from the sample. The residual HOD peak has been excluded from the plot. Experimental conditions were as follows: 10 mm diameter sample tube; pulse delay of 2 s which was sufficient to elimate any saturation of the dioxane resonance; spectral width of 3600 Hz. Note the absence of nucleotide proton resonances which are clearly resolved in the aromatic region of Figure 3.

the ^{1}H NMR spectrum of G-actin at pH 6.9 containing bound Ca^{2+}, in the absence of lanthanide ions. An estimate could be made of the proportion of aromatic protons in actin contributing to the aromatic peak by increasing the delay between pulses to ensure that no saturation of the dioxane peak occurred. Approximately 25% of the aromatic protons contribute while ~80% of the methyl and methylene protons are observed in the aliphatic region of the spectrum. The reduction in intensity, particularly apparent in the aromatic region, may well arise from the use of gated irradiation to remove the residual HOD peak. Under such conditions, selective suppression of broad components of a protein spectrum have been observed (Akasaka et al., 1978). The above intensity losses could not be accounted for by partial actin polymerization since examination of the NMR samples showed no evidence of F-actin formation as judged by viscosity, electron microscopy, and birefringence. No resonances from the H(2), H(8), or H(1′) protons of ATP could be seen. In the presence of any excess ATP, sharp resonances due to these protons were superimposed upon the spectra. These resonances of free ATP were neither shifted nor broadened in the presence of actin. Exchange between free and bound sites is therefore slow on the NMR time scale. This is consistent with the measured rate of ATP exchange (10^{-2} s^{-1}; Strzelecka-Golaszewska et al., 1974).

Lanthanide–G-Actin Complexes. Calcium was titrated into a sample of G-actin (in the absence of any excess ATP) up to an equimolar ratio with actin. The results in Figure 3a indicate that the addition of one Ca^{2+} per actin has no observable effect on the spectrum. However, when diamagnetic La^{3+} is added to actin up to a 1:1 ratio, there is a profound effect. Figure 3b shows the aromatic region in which the H(2), H(1′), and H(8) peaks of ATP are prominent. We note that these peaks cannot arise from free ATP since the H(2) resonance is shifted downfield by ~0.1 ppm relative to its position in a solution of ATP. The H(8) and H(1′) proton resonances have the same chemical shifts as in free ATP.

The H(1′) peak is difficult to observe routinely because of the relatively large water peak that was present in several samples. However, in those experiments where the residual water peak was small, the increases in intensity of the H(2) and H(8) peaks were accompanied by an equivalent increase in the H(1′) peak. These data may be interpreted as indicating a change in the conformation of G-actin when a trivalent

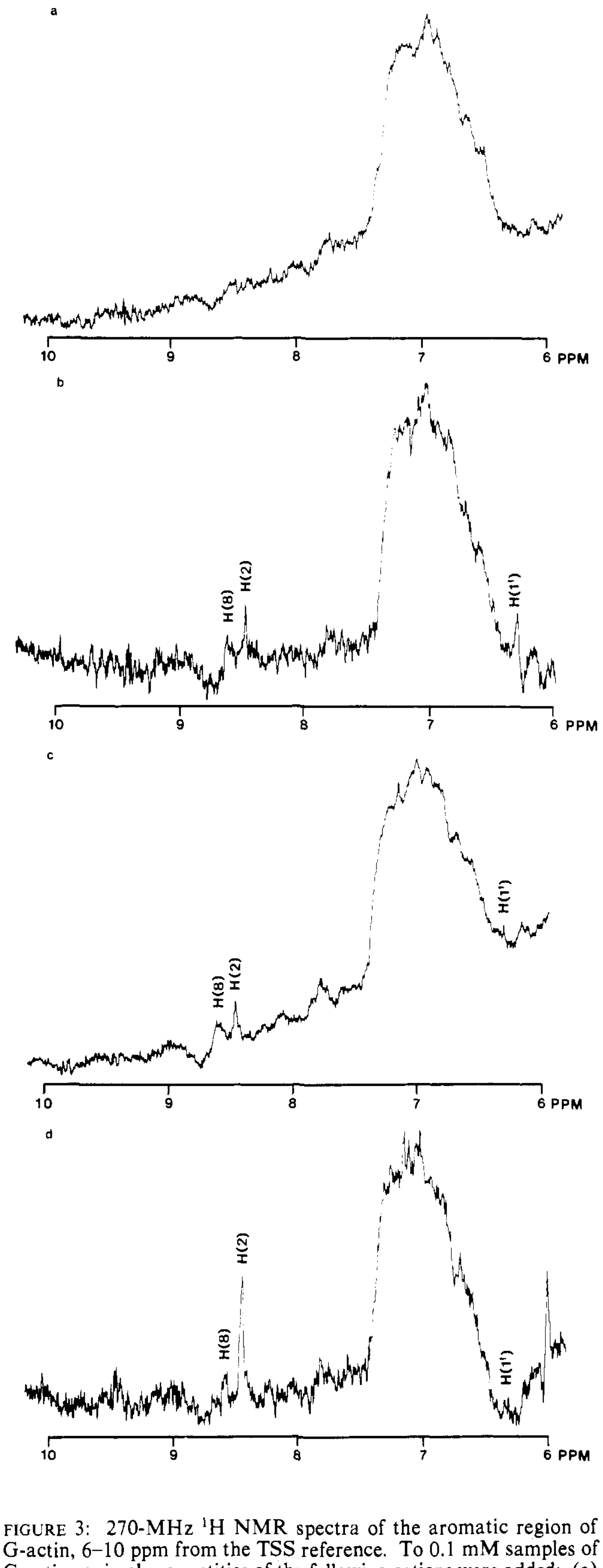

FIGURE 3: 270-MHz ^{1}H NMR spectra of the aromatic region of G-actin, 6–10 ppm from the TSS reference. To 0.1 mM samples of G-actin equimolar quantities of the following cations were added: (a) 0.1 mM Ca^{2+}; (b) 0.1 mM La^{3+}; (c) 0.1 mM Eu^{3+}; (d) 0.1 mM Gd^{3+}. Other conditions were the same as in the legend to Figure 2 except that the Eu–actin and La–actin samples were partially saturated since the pulse delay was reduced to 0.5 s. The intensities of the ATP peaks are thus reduced in these two samples. The addition of a divalent cation such as Ca^{2+} does not mobilize the ATP. In the lanthanide–actin samples, despite the appearance of sharp ATP resonances, the nucleotide remains entirely inaccessible to the anion-exchange resin Dowex 1 (Cl^-).

lanthanide ion displaces the divalent cation from its high-affinity binding site. Parts c and d of Figure 3 show spectra obtained after equimolar quantities of Eu^{3+} and Gd^{3+}, respectively, have been bound to actin. Normally, Eu^{3+} functions as a shift reagent, shifting the resonances of protons in the vicinity of the binding site while Gd^{3+} broadens these resonances. However, little difference is observed between the spectra obtained with the diamagnetic La^{3+} acting as a control (neither shifting nor broadening resonances in the spectra) and the two paramagnetic lanthanide ions. The resonances of protein groups in the vicinity of the lanthanide binding site thus appear to be absent from the spectrum. All three lanthanide ions increase the mobility of the bound ATP as evidenced by sharpening of the H(2) and H(8) resonances. Even after mobilization as a result of lanthanide binding, no ATP was removed by brief treatment with the anion-exchange resin, Dowex 1 (Cl^-). The spectrophotometric detection limit of the free ATP level was 0.2 μM (0.2% of the total bound ATP). It should also be pointed out that once the molar ratio of lanthanide ion to actin is equal to or greater than 1:1, the actin aggregates and that at higher (5 or 6) molar ratios, the actin forms crystalline aggregates (dos Remedios & Dickens, 1978; dos Remedios et al., 1980a,b; Barden & dos Remedios, 1980).

The intensities of the ATP proton resonances in the spectra of actin in the presence of varying concentrations of Gd^{3+} were measured by comparing their areas with that of the resonance of 0.1 mM dioxane. In these experiments the delay between successive pulses was made sufficiently long to prevent saturation of the dioxane peak. Increasing the concentration of added Gd^{3+} from 0.01 to 0.1 mM produced a directly proportional increase in the area of the H(2) peak of ATP. When a stoichiometric amount of Gd^{3+} had been added (0.1 mM), the area of the H(2) peak indicated that 0.1 mM ATP was observed in the NMR spectrum. Addition of further Gd^{3+} aggregated the protein but the intensity of the H(2) proton resonance remained constant. These results show clearly that one molecule of ATP is mobilized per Gd–actin monomer.

Discussion

In the absence of lanthanides, no resonances can be observed for the H(2), H(8), or H(1′) protons of ATP in the NMR spectrum of G-actin. This indicates that the nucleotide is tightly bound to the protein such that the correlation time for the dipolar relaxation processes will be the rotational tumbling time of the protein [$\tau_R \sim 1.8 \times 10^{-8}$ s calculated from the Stokes–Einstein equation (Dwek 1973) for a globular protein of molecular weight 42 000]. The rate of ATP exchange between free and bound environments (Strzelecka-Golaszewska et al., 1974) is much too slow ($\sim 10^{-2}$ s^{-1}) to affect the resonance widths; thus in G-actin, the nucleotide is bound tightly and rigidly to the protein.

The dramatic appearance of sharp resonances arising from the H(2), H(8), and H(1′) protons of ATP upon binding La^{3+}, Eu^{3+}, or Gd^{3+} clearly reflects a conformational change leading to rapid internal motions of the adenine and ribose rings of the nucleotide. It is difficult to quantitate the increase in motional freedom of the adenine ring, but the narrowness of the H(2) resonance in particular suggests that the correlation time, τ_c, for dipolar relaxation will now be shorter than $\sim 10^{-9}$ s. One interpretation of these data is that the ATP binds to the actin monomer by the triphosphate side chain rather than the ribose or purine rings. Despite this mobilization, ATP remains firmly bound to the protein and cannot be removed by treatment with anion-exchange resin. The present results give no indication of the extent of the protein conformational changes affecting the nucleotide binding site. We cannot as yet determine whether it is the nucleotide alone which experiences increased mobility (and hence is likely to be located at the surface of the protein) or whether the changes also affect a small surrounding region of the protein itself. However, others have shown by UV difference (Higashi & Oosawa, 1965) and fluorescence (Cheung et al., 1971) spectroscopy that actin can undergo conformational changes during polymerization and during metal binding (Strzelecka-Golaszewska et al., 1974).

The complete absence of perturbations of the sharp ATP resonances by the paramagnetic lanthanide ions, Gd^{3+} (broadening) and Eu^{3+} (shift), is particularly significant. Titration of ATP with paramagnetic ions such as Gd^{3+} (Tanswell et al., 1975) or Mn^{2+} (Cohn & Hughes, 1962; Sternlicht et al., 1965) causes the ATP proton resonances to be broadened beyond detection. Even when the nucleotide is bound to the protein phosphoglycerate kinase (Tanswell et al., 1976) or contained within its storage complex in chromaffin granules (Daniels et al., 1978), these metal ions produce significant increases in resonance line widths. The complete failure of Gd^{3+} to broaden the resonances of actin-bound ATP therefore indicates that the metal does not bind near to the nucleotide. The distance between the lanthanide binding site and the H(2) and H(8) protons of the ATP is probably greater than ~ 16 Å, calculated by using the Solomon–Bloembergen equations (Solomon, 1955; Bloembergen, 1957), assuming that the correlation time for the localized motions of the nucleotide is shorter than $\sim 10^{-9}$ s.

The ^1H NMR evidence that the metal and nucleotide binding sites are widely separated is supported by the EPR data. The theory of the interaction of two paramagnetic ions, both of which are bound to a macromolecule, has been developed by Leigh (1970) and states that the decrease in the amplitude of the spectrum falls off as the sixth power of the distance separating the paramagnetic ion and the nitroxide spin-label. Taylor et al. (1969) have employed this system to measure Mn^{2+}–spin-label distances in creatine kinase. Assuming that the relaxation time for Mn^{2+} bound to actin is similar to that bound to creatine kinase, one can calculate a lower limit for the distance between the spin-label and Mn^{2+} on actin. This distance must be greater than 12 Å for instances where no effect is observed.

The ^1H NMR and nitroxide spin-label EPR data both lead to the following conclusions: (1) the metal cations do not bind to the actin-bound nucleotide (ATP) and (2) the high-affinity nucleotide and high-affinity cation binding sites are separated by a distance greater than 16 Å.

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