^{19}F NMR Study of the Myosin and Tropomyosin Binding Sites on Actin†

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ABSTRACT: Actin was labeled with pentafluorophenyl isothiocyanate at Lys-61. The label was sufficiently small not to affect the rate or extent of actin polymerization unlike the much larger fluorescein 5-isothiocyanate which completely inhibits actin polymerization [Burtnick, L. D. (1984) *Biochim. Biophys. Acta 791*, 57–62]. Furthermore, the label resonances in the 376.3-MHz ^{19}F NMR spectrum were unaffected by actin polymerization. However, the binding of the relaxing protein tropomyosin resulted in the fluorinated Lys-61 resonances broadening out beyond detection due to a substantial increase in the effective correlation time of the label. Similarly, the binding of myosin subfragment 1 to F-actin resulted in the dramatic broadening of the labeled Lys-61 resonances. Thus, Lys-61 on actin appears to be closely associated with the binding sites for both tropomyosin and myosin, suggesting that both these proteins can compete for the same site on actin. The other region of actin known to be involved in myosin binding, Cys-10, was found to be more remote from the actin–actin interfaces than Lys-61. Labels on Cys-10 exhibited substantially greater mobility than fluorescein 5-isothiocyanate attached to Lys-61 which appeared to be held down on the surface of the actin monomer. This may sterically hinder the actin–actin interaction about 1 nm from the tropomyosin/myosin binding site.

Actin is ubiquitous in eukaryotic cells, and it is involved in a large number of different cellular processes such as the maintenance of cell shape, axonal transport, cytoplasmic streaming, and cell division (Korn, 1982). Filamentous myosin and filamentous or F-actin together form the essential component of the contractile process in muscle. The thin filaments, which also contain the regulatory proteins tropomyosin and troponin, slide past the thick myosin filaments. Tropomyosin is located within the grooves on each side of the actin helix. Actin in its monomeric form (G-actin) is a globular protein unable to bind to myosin. The tropomyosin–troponin complex regulates the cyclic interaction of the myosin heads with sites on actin in the thin filaments either through direct steric blocking or else through a long-range effect on the myosin ATPase activity (Adelstein & Eisenberg, 1980).

The region 53–69 on actin, specifically involving residues Tyr-53 (Bender et al., 1976), Lys-61 (Burtnick, 1984), and Tyr-69 (Chantler & Gratzer, 1975), has been implicated in an actin–actin binding site as have the regions 87–113, 168–226, 283–291, and 374 [see Hambly et al. (1986) for a review]. The acidic residues 1–4 and 11 on actin have been cross-linked to the myosin heavy chain while the residues 360–363 have been cross-linked to myosin alkali light chain 1 (Sutoh, 1982a,b, 1983). However, Chen et al. (1985a,b) questioned these results after analyzing the actomyosin cross-linking reaction. Mejean et al. (1986) have found that residues 1–7 of actin do not bind to myosin on the basis of enzyme-linked immunosorbent assays. Miller et al. (1987) demonstrated that actin could bind both myosin and an antibody directed against residues 1–7, similarly indicating that the myosin site did not directly involve the acidic N-terminal residues. However, these authors revealed that the binding constant of the antibody to actin was reduced 5-fold in the presence of myosin subfragment 1 (S-1). Mejean et al. (1986, 1987) concluded that the myosin site more directly involved residues 18–28. A recent NMR study (Barden et al., 1989) has revealed that the ^{19}F resonance of the very small CF_3Hg label attached to Cys-10 on actin is completely broadened by the binding of myosin S-1. Thus, Cys-10 must be very close to the myosin binding site.

The actin residues involved in binding tropomyosin are less well established (Hambly et al., 1986). Two regions have been suggested involving 70–86 and 340–375 (Johnson & Stockmal, 1982). Tropomyosin binding to actin remained unaffected by the presence of antibodies bound either to the N-terminal residues 1–7 and 18–28 or to 374 (Mejean et al., 1987). These authors concluded that the tropomyosin and myosin binding sites on actin do not overlap.

Lys-61 is a surface residue accessible to a wide range of enzymes (Jacobson & Rosenbusch, 1976; Hambly et al., 1986). The residue has been specifically labeled with the fluorescent probe fluorescein 5-isothiocyanate (FITC) by Burtnick (1984). Following labeling with FITC and the separation of all the nonlabeled polymerizable actin, the FITC–actin becomes unpolymerizable except in the presence of phalloidin (Miki, 1987). FITC–actin in the absence of phalloidin is unable to bind myosin, again provided that all the unlabeled actin is first removed from the sample (Miki et al., 1987a).

In this paper, an investigation was made of the mobility of the FITC label on Lys-61 with a view to determining how this large label blocks actin polymerization. A much smaller probe has been attached to Lys-61, pentafluorophenyl isothiocyanate (PFPITC). Unlike FITC–actin, the PFPITC–actin remains fully polymerizable and is able to bind myosin as well as the regulatory protein tropomyosin. The mobility of probes attached to the other myosin binding site at Cys-10 was undertaken for a comparison with the mobility of FITC attached to Lys-61. ^{19}F NMR spectroscopy has been used to examine the influence of actin–actin, actin–tropomyosin, and actin–myosin binding on the ^{19}F NMR resonances as an aid in determining some of the residues involved in these various binding sites on actin.

Materials and Methods

Protein Preparation. Actin was prepared with very high yield by using the method of Barden et al. (1986a) from rabbit muscle acetone powder. The final actin dialysate contained 0.2 mM $CaCl_2$, 0.2 mM ATP, 1 mM β-mercaptoethanol, 1

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mM NaN_3, and 2 mM sodium borate at pH 8.5. The G-actin was centrifuged at 100000*g* for 1.5 h. The supernatant was diluted to 3–4 mg/mL in the above buffer and the temperature raised from 4 to 20 °C. Labeling of Lys-61 was performed essentially according to the method of Burtnick (1984) but with some modifications (Miki, 1987; Miki et al., 1987a). Pentafluorophenyl isothiocyanate (PFPITC) was obtained from Fluka, and both FITC (isomer I) and 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (1,5-IAEDANS) were obtained from Molecular Probes. The isothiocyanate group (NCS) is able to react with both sulfhydryl and amine groups, though the reactivity with amine groups is strongly favored above pH 7.5 (Means & Feeney, 1971). Disulfide bond formation is relatively weak and is readily cleaved with β-mercaptoethanol or dithiothreitol. To label amines, and in this case specifically Lys-61, the pH was kept high, and a disulfide reducing agent was present.

FITC–actin was prepared as described previously (Miki et al., 1987a) with care taken to remove all unlabeled and thus polymerizable actin. PFPITC–actin was prepared by adding a 15–35-fold molar excess of PFPITC to actin over 1 h in several aliquots with constant stirring in a solution consisting of 0.2 mM $CaCl_2$, 1 mM NaN_3, 1 mM β-mercaptoethanol, 0.2 mM ATP, and 2 mM sodium borate and with the pH maintained at 8.5 for 3–6 h. The labeled actin remained fully polymerizable (reduced viscosity of 1.0), unlike FITC–actin (reduced viscosity of 0). Thus, the PFPITC–actin was polymerized in 0.1 M KCl, 1.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1 mM NaN_3, and 20 mM sodium phosphate, pH 7.0. After 2 h, the actin was centrifuged at 100000*g* for 1.5 h to remove the bulk of excess label. The pellets of labeled F-actin were resuspended in 0.2 mM $CaCl_2$, 1 mM ATP, 1 mM NaN_3, and 2 mM sodium phosphate, pH 8.0, and then dialyzed exhaustively against the same buffer except for a lower concentration of ATP (0.1 mM) to remove any small remaining quantity of unbound label.

Actin was made unpolymerizable by modification of Tyr-53 with 5-diazonium(1*H*)tetrazole (DHT) (Bender et al., 1976). All unmodified actin was removed (Miki et al., 1987b), and then Cys-10 on the DHT–actin was labeled with AEDANS according to the method of Barden et al. (1986b).

The synthesis of [*m*-(trifluoromethyl)phenyl]mercuric bromide proceeded from the symmetrical bis[[*m*-(trifluoromethyl)phenyl]mercury] compound by cleavage with mercuric bromide. *N*-Butyllithium solution (Merck) was standardized (4.5 M) with 2,5-dimethoxybenzyl alcohol (Winkel et al., 1980). Distilled ether (200 mL) was dried by benzophenone ketal and placed in a two-neck round-bottom flask. 3-Bromo-1,1,1-(trifluoromethyl)benzene (10 g, 44 mmol) was added, stirred, and cooled to −70 °C under a nitrogen atmosphere. *N*-Butyllithium (33 mL, 50 mmol) was added against a nitrogen counterflow, and the temperature was allowed to return to 25 °C. Mercuric bromide (4.02 g, 14.8 mmol) was added, which produced a slight reflux. After being stirred for 1 h, the solution was refluxed overnight. The opaque white solution was cooled and filtered through Celite before removal of the ether, and the crude product was recrystallized from ethyl acetate/ether. This produced fine white crystals of the bis[[*m*-(trifluoromethyl)phenyl]mercury]. The yield was 51% and the melting point 175–176 °C. An ^{19}F NMR spectrum was recorded. The resonance was found at 11.56 ppm downfield of trifluoroacetic acid (TFA). Following the method for the preparation of (pentafluorophenyl)mercury(II) bromide (Chambers et al., 1962), 3.65 g or 7.43 mmol of the diaryl compound was dissolved in 100 mL of methanol distilled from magnesium. Equimolar mercuric bromide (2.68 g, 7.43 mmol) was added; the apparatus was placed under nitrogen and refluxed overnight. The clear solution was cooled and filtered prior to the removal of methanol by vacuum distillation. Recrystallization of the crude product from carbon tetrachloride yielded large white crystals which were filtered and dried (4.79 g, 13.3 mmol or 81% yield) with a melting point of 178–179 °C. A ^{19}F NMR spectrum was recorded, and the CF_3 resonance was found at 15.06 ppm downfield of TFA.

Actin was labeled with [*m*-(trifluoromethyl)phenyl]mercury (TFMPM) at Cys-10 after first reacting the actin with *N*-ethylmaleimide (NEM) to block Cys-374. G-Actin at 30 μM in 2 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.1 mM ATP, and 1 mM NaN_3 at pH 8.0 was reacted with 0.12 mM NEM for 45 min. The reaction was terminated by adding a 100-fold molar excess of β-mercaptoethanol over NEM and the actin polymerized with 0.1 M KCl, 2 mM $MgCl_2$, 1 mM NaN_3, and 20 mM sodium phosphate at pH 8.0 for 1 h. The F-actin was centrifuged at 80000*g* for 1.5 h, and the washed pellets were redissolved and dialyzed exhaustively against the G-actin buffer. A stoichiometric amount of TFMPM bromide was added to the NEM–G-actin and stirred overnight in the dark at 4 °C. The solution was centrifuged at 80000*g* for 1.5 h and then polymerized as described above. The washed pellet was redissolved and dialyzed against the G-buffer containing 2 mM borate at pH 8.0.

Myosin was extracted from rabbit skeletal muscle according to the method of Tonomura et al. (1966). Myosin subfragment 1 (S-1) was prepared according to the method of Weeds and Pope (1977). Tropomyosin was extracted from the rabbit muscle following extraction of the myosin according to the method of Ebashi et al. (1968). Concentrations of actin, myosin S-1, and tropomyosin were determined spectrophotometrically. The following absorption coefficients were used for 0.1% solutions: actin, $A_{290} = 0.63$ cm^{-1}, 43 kDa (Lehrer & Kerwar, 1972); myosin S-1, $A_{280} = 0.77$ cm^{-1}, 120 kDa (Lehrer & Morris, 1982); and tropomyosin, $A_{277} = 0.24$ cm^{-1}, 66 kDa (Lehrer & Morris, 1982).

NMR Spectroscopy. ^{19}F NMR spectra of fluorinated actin were obtained in the Fourier transform mode with quadrature detection using a Varian XL-400 spectrometer operating at a ^{19}F frequency of 376.3 MHz while ^1H NMR spectra were obtained at 400 MHz. A 15% concentration of deuterium in the fluorinated samples acted as the lock signal. TFA was used as the external frequency reference (0.000 ppm). Upfield shifts from TFA are negative. A 16-kHz spectral width was used with a 90° pulse width and a data block of 32768 points. The repetition time between scans totaled 1.5 s of which 0.5 s was a preacquisition delay. The FITC–actin samples were exchanged on a 15 × 1 cm column of Sephadex G-25 equilibrated with D_2O (99.8%) buffered with 2 mM sodium borate, 0.1 mM $CaCl_2$, 0.1 mM ATP, and 1 mM NaN_3. The pH was adjusted to 8.0 using DCl and NaOD solutions. FITC–actin and AEDANS–DHT–actin samples were concentrated to 0.5 mM (20 mg/mL) on an Amicon filter assembly using a 10K molecular weight cutoff filter prewashed in D_2O. The concentrated protein samples were filtered through a 0.2-μm Acrodisc prior to transfer to the NMR tubes. The chemical shifts were referenced to (trimethylsilyl)propanesulfonic acid (TSS). A data block of 16384 points was used with a 0.5-s preacquisition delay, giving a repetition time of 2.5 s between scans. All samples were placed in 5-mm tubes, and spectra were recorded at 25 °C.

Results

PFPITC-Labeled Actin. The 376.3-MHz ^{19}F NMR spec-

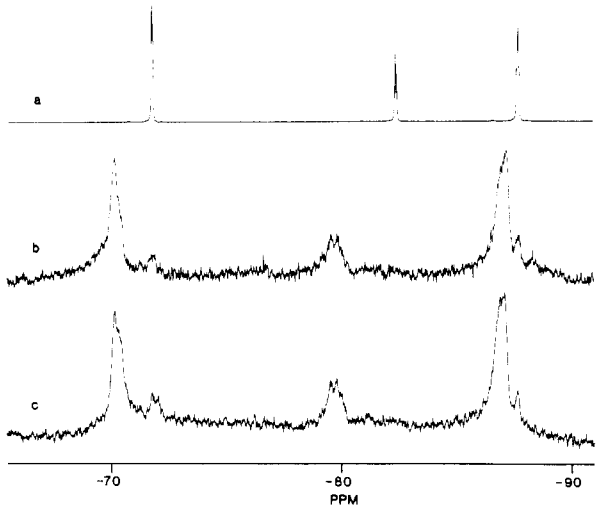

FIGURE 1: 376.3-MHz ^{19}F NMR spectrum of the free probe (a) pentafluorophenyl isothiocyanate (PFPITC). The ortho, para, and meta lines resonate at −71.85, −82.50, and −87.82 ppm upfield of trifluoroacetic acid (TFA) and exhibit intensities in the ratio 2:1:2, respectively. The spectrum in (b) was obtained from a 5 mg/mL solution of G-actin labeled with PFPITC at Lys-61. The ortho, para, and meta resonances are observed at −70.22, −79.87, and −87.05 ppm, only some 6-fold broader than in the free probe. Spectrum c was obtained after adding salt to polymerize the labeled G-actin solution used in (b). The resonances remain unaffected by actin polymerization. A small quantity of free label has been left in the actin samples as evidenced by the small resonances at −71.85 and −87.82 ppm in particular.

trum of the free PFPITC probe is shown in Figure 1a. Three resonance lines with multiplet structure are observed at −71.85, −82.50 and −87.82 ppm upfield from TFA. These correspond to the ortho, para, and meta atoms, respectively, with integrals in the ratio 2:1:2. Each of the lines shows fine structure due to the splitting caused by the various F–F couplings. The label was attached to G-actin at Lys-61, and the result in Figure 1b shows an NMR spectrum obtained after 16 000 scans of a 5 mg/mL solution of PFPITC–G-actin. The same ortho, para, and meta resonances are evident in the spectrum. They exhibit line widths which are 6-fold broader than the line widths recorded from the free probe. Each of the lines is shifted from the positions adopted by the free probe. The ortho resonance is found at −70.22 ppm, 1.63 ppm downfield of the free probe. The para resonance at −79.87 ppm is 2.63 ppm downfield of the free probe position while the meta resonance at −87.05 ppm is the least affected by binding to actin, being shifted only 0.77 ppm downfield of the position of the resonance in the free probe.

The addition of 50 mM KCl and 2 mM $MgCl_2$ to the G-actin sample resulted in the formation of filamentous or F-actin within 10 min. The ^{19}F NMR spectrum obtained after 16 000 scans (Figure 1c) remained unaffected, even though the solution was highly viscous. The probe did not interfere with the rate or extent of actin polymerization nor did polymerization affect either the chemical shifts of the PFPITC resonances or the line widths. A trace of free probe was left in the sample of actin as shown by the resonances slightly upfield of the ortho and meta resonances of the bound probe.

In order to verify that there were no additional labels attached to actin besides Lys-61, a limited tryptic digest of the labeled actin was performed (Jacobson & Rosenbusch, 1976; Hambly et al., 1986). The actin was separated into a proteolytic-resistant core consisting of residues 69–373 and a mixture of peptides consisting of residues 1–68 and 374–375 using an Amicon filtration apparatus operated with a YM-10

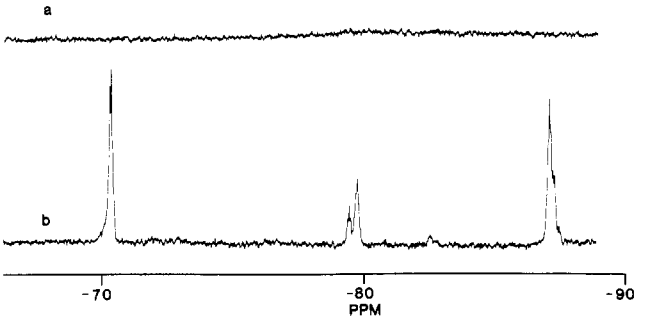

FIGURE 2: ^{19}F NMR spectrum (a) of a 0.4 mM solution of the actin core (residues 69–373) obtained from a limited tryptic digest. No label is present attached to the core. The spectrum in (b) contains a 0.4 mM sample of the N- and C-terminal peptide fragments including residues 1–68 and 374–375. The resonances from the label attached to Lys-61 have the same chemical shifts as in the G- and F-actin samples.

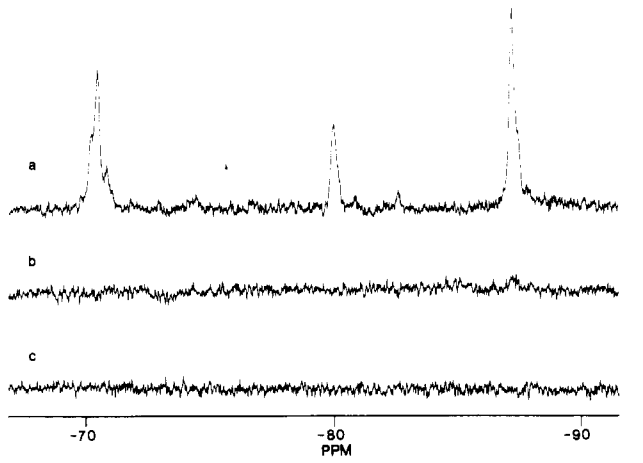

FIGURE 3: Effect of binding the relaxing protein Tm on the Lys-61 PFPITC label reveals that the resonances in the F-actin sample (a) are dramatically broadened in the presence of Tm (b). Spectrum c similarly reveals that the addition of myosin S-1 to the labeled F-actin results in the complete broadening of the resonances due to a large increase in the effective correlation time of the PFPITC probe.

membrane. Confirmation of the complete digestion of the actin into the core was obtained by using polyacrylamide gel electrophoresis. The core and the peptide mixture were individually lyophilized and redissolved at the same concentrations of 0.4 mM. ^{19}F NMR spectra were obtained from both samples to detect the presence of the PFPITC label. Figure 2 shows the results, with the core (Figure 2a) being devoid of any label. In case the label was inadvertently immobilized on the core, a spectrum also was obtained at pH 12 where the actin core is unfolded (Barden & dos Remedios, 1983). The results were unchanged (not shown). The sample containing the mixture of cleaved peptides (Figure 2b) clearly revealed the presence of label with the same chemical shifts as exhibited in the native actin samples. These spectra were obtained with 2000 scans.

The effect of tropomyosin (Tm) binding on the labeled Lys-61 resonance was examined by adding sufficient Tm to saturate all the actin binding sites in a sample of F-actin in 5 mM $MgCl_2$. There are seven actin monomers for every Tm. The spectrum in Figure 3a shows the resonances from a sample of newly labeled F-actin in the absence of Tm at a concentration of 6 mg/mL obtained after 4000 scans. The spectrum in Figure 3b was obtained with 12 mg/mL actin in the presence of Tm also after 4000 scans. A faint trace of the meta resonance was detected at −87 ppm, but essentially all the resonances were broadened beyond detection. Ultracentrifu-

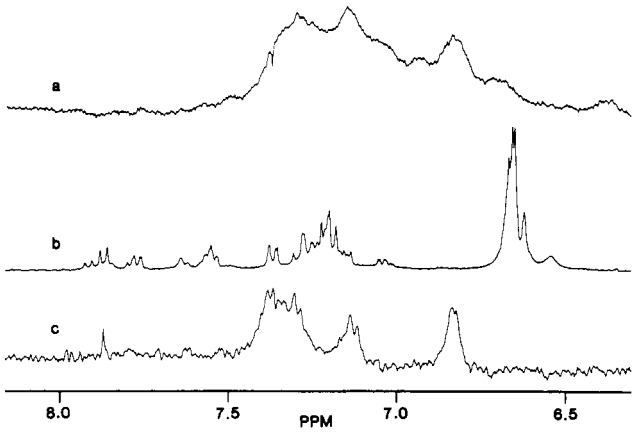

FIGURE 4: ^{1}H NMR spectrum of the aromatic region of a 0.5 mM solution of FITC–actin at pH 8.0 (a). Spectrum b is from a 2 mM solution of free FITC at pH 8.0, the resonances of which do not appear in spectrum a nor in spectrum c which was obtained by using labeled actin with a delay of 0.24 s in the $CPMGT_2$ pulse sequence to selectively broaden the most immobile resonances. While most of the actin resonances are broadened, no evidence was seen of the FITC resonances.

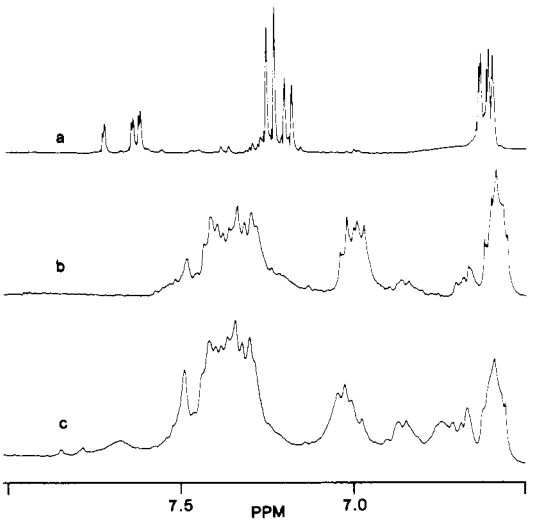

FIGURE 5: ^{1}H NMR spectrum of FITC (2 mM) at pH 12 is shown in (a). The aromatic region of the spectrum of unlabeled actin (0.35 mM) at pH 12 is shown in (b). Most of the aromatic proton resonances exhibit chemical shifts typical of an unordered structure. Spectrum c was obtained from a 0.4 mM sample of FITC–actin at pH 12 using the $CPMGT_2$ pulse sequence with a BT = 0.1 s. At this delay, more than half the actin resonances have been removed, leaving only the most mobile. The downfield cluster of resonances (7.66–7.71 ppm) from FITC are present at this pH due to partial mobilization although they are still broader than free FITC.

gation of the Tm–F-actin complex at 100000*g* for 1 h resulted in the sedimentation of the entire complex. The supernatant was clear of protein as revealed by UV absorption measurements at 280 nm.

A similar experiment was performed to determine the effect of binding myosin S-1 on the Lys-61 label resonances. An 8-mg sample of labeled F–actin was incubated with 25 mg of myosin S-1 for 1 h at pH 8.0 and the complex concentrated on an Amicon assembly to 50 mg/mL, the same concentration of label as used in the Tm–F-actin sample. The resulting spectrum is shown in Figure 3c. The label resonances were broadened completely by the binding of myosin. The presence of troponin (Tn) added in a Tm–Tn complex did not alter the results in Figure 3b.

FITC-Labeled Actin. PFPITC is not affected by actin–actin binding but is profoundly interfered with by both actin–myosin and actin–tropomyosin binding. The binding of the much larger FITC at the same site effectively blocks actin–actin binding. Thus, Lys-61 appears to be located close to all three binding sites, although somewhat more removed from the actin–actin site. Since the three sites cannot all overlap, an investigation was made of the mobility of FITC on Lys-61 with a view to establishing whether the bulk of FITC in a mobile environment is sufficient to sterically hinder actin–actin binding. Alternatively, FITC may fold back onto the actin surface some distance from Lys-61 and in so doing block the actin–actin site.

The aromatic region of a ^{1}H NMR spectrum (6–7.5 ppm) of a low ionic strength solution of 0.5 mM FITC–actin (20 mg/mL) at pH 8.0 and 25 °C is shown in Figure 4a. The aromatic proton resonances appear to be generally crowded and broad although no more so than unmodified G-actin spectra recorded at much lower concentrations (Barden et al., 1980; Barden & dos Remedios, 1985). The ^{1}H NMR spectrum of a 2 mM solution of FITC at pH 8.0 is shown in Figure 4b. A total of nine proton resonances are present in the spectrum with a major cluster at 6.6 ppm, a second cluster around 7.2 ppm, and the remainder from 7.5 to 7.9 ppm. No evidence can be seen of the FITC resonances in the spectrum of FITC–actin. In order to determine whether the FITC resonances are more mobile than the side chains of aromatic residues in actin, the resonances from the least mobile groups were progressively suppressed by using the $CPMGT_2$ pulse sequence and an evolution time ranging from 0.01 to 1.0 s. Most of the actin resonances were removed by using an evolution time of 0.24 s (Figure 4c). However, no evidence could be found of a cluster of FITC resonances remaining among the relatively few mobile actin resonances even though there was no overlap in the spectrum near the downfield FITC resonances, indicating that the FITC label is less mobile than most of the aromatic side chains on actin.

The viscosity of the FITC–actin sample in Figure 4a was measured by using an Ostwald viscometer at 20 °C with a water outflow time equal to 52 s. A value of $1.1 \times 10^{-3}\ \eta^{-1}$ was determined. Thus, the same viscosity was reproduced in a 2 mM sample of free FITC at pH 8.0 and 20 °C by adding 3.5% deuterated glycerol and then determining the T_2 relaxation times by using the $CPMGT_2$ pulse sequence. The spectrum remained essentially unaltered in the glycerol, with virtually the same mobility being recorded. A value of T_2 equal to 0.052 s was obtained.

The FITC–actin sample was partially unfolded by increasing the pH to 12. At this pH, the resonances of free FITC are slightly more equivalent (Figure 5a). The spectrum of unlabeled actin at pH 12 is more typical of unfolded protein (Figure 5b) although an unfolded core still exists (Mihashi & Ooi, 1965; Barden & dos Remedios, 1983). The spectrum of FITC–actin at pH 12 reveals the presence of the downfield FITC resonances at 7.66–7.71 ppm due to the three protons in the phenyl ring attached to the NCS side chain which is attached to Lys-61. The label is mobilized at this pH. A repeat of the $CPMGT_2$ sequence revealed that the FITC resonances were more mobile than many of the actin resonances. The downfield cluster of resonances of FITC at 7.66–7.71 ppm is more visible with a BT = 0.1 s (Figure 5c) although the resonances remain much broader than the resonances of free label, consistent with a much slower correlation time.

The absence of the resonances of bound FITC from the spectrum recorded from the native FITC–actin at pH 8.0 (Figure 4a) appeared to be due to a lack of mobility caused by the interaction of the fluorescein with the protein surface

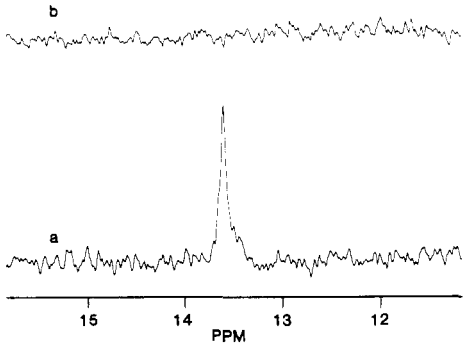

FIGURE 6: ^{19}F NMR spectrum of TFMPM–actin (10 mg/mL) with the fluorine label attached to Cys-10 exhibits a single resonance 13.61 ppm downfield from TFA (a). Spectrum b was obtained from a sample of the labeled actin containing equimolar bound myosin S-1 at a final protein concentration of 40 mg/mL. The myosin has caused the effective broadening of the CF_3 resonance.

resulting in a restriction in free rotation. The T_1 relaxation times of FITC were determined over the temperature range 15–65 °C. The variation was linear and varied between 2.4 and 3.9 s. Thus, the relaxation mechanism appears to be incoherent dipole–dipole. The fact that the T_2 is lower is not surprising since the protons are coupled and thus coherent dipole relaxation is also operating. From Doddrell et al. (1972), for pure ^1H–^1H dipolar relaxation, T_1 is expressed in terms of the correlation time τ_R, the interproton distance r, and K, which is equal to the product of $h/2\pi$ and the square of the proton gyromagnetic ratio γ_S, i.e.

$$1/T_1 = (1/10)K^2r^{-6}\chi$$

where χ is represented by

$$\chi = \frac{3\tau}{1 + \omega^2\tau^2} + \frac{6\tau}{1 + 4\omega^2\tau^2}$$

with ω the Larmor frequency.

The solution to the equation at 25 °C where T_1 is 2.7 s and the nearest-neighbor proton separation, r, on the fluorescein rings is 21.8 pm yields a value of the rotational correlation time of the free FITC equal to 86 ps. The correlation time of FITC bound to actin at pH 12 is much longer than 86 ps, but the line widths are not dramatically broadened at this pH. However, the FITC line widths are broadened beyond detection at pH 8 due to a severe reduction in the effective rotational correlation time, indicating that the value of τ_R for native actin-bound FITC is close to 50 ns, the value for actin as a whole (Mihashi & Wahl, 1975; Ikkai et al., 1979; Brauer & Sykes, 1981). The mobility of FITC on native actin appears to be as low as the actin-bound ATP (Barden et al., 1980). Various estimates of τ_R for the bound nucleotide range from 36 ns (Mihashi & Wahl, 1975) to 60 ns (Brauer & Sykes, 1981). Thus, FITC appears to fold back onto the actin surface, thereby sterically blocking the actin–actin binding site at some distance from the myosin and Tm binding site. From the size of the label, the actin site could be about 1 nm from the myosin/Tm site.

TFMPM-Labeled Actin. The relative immobility of FITC attached to Lys-61 was contrasted with the other myosin binding site close to Cys-10 (Barden et al., 1989). The very small CF_3Hg attached to Cys-10 was greatly affected by the binding of myosin but not by the binding of Tm. The CF_3 resonances were effectively broadened out in the F-actin spectrum when myosin S-1 was bound (Barden et al., 1989). In order to determine whether Cys-10, like Lys-61, was peripherally associated with an actin–actin site, a larger probe

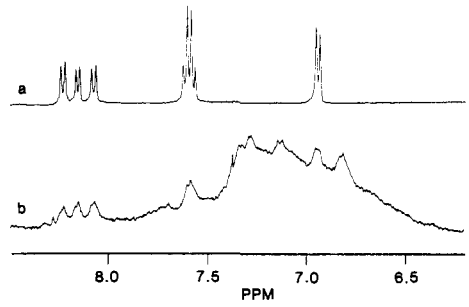

FIGURE 7: ^1H NMR spectrum in the aromatic region of 5 mM free IAEDANS at pH 8.0 (a). The spectrum of (0.5 mM) AEDANS–DHT–actin with the label attached to Cys-10 is shown in (b). The mobility of the AEDANS label is such that all the AEDANS resonances can be seen in the spectrum.

was used to label Cys-10. For this purpose (methylphenyl)-mercury (TFMPM) was synthesized and coupled to actin at Cys-10. The ^{19}F NMR spectrum (Figure 6a) recorded from a sample of F-actin at 10 mg/mL reveals a single resonance at 13.61 ppm downfield from TFA. A total of 4000 scans were collected. The spectrum of G-actin at 4 mg/mL was unchanged (not shown). Actin polymerization remained unaffected by the presence of the label on Cys-10, as revealed by the rate and extent of the increase in viscosity. Nor does the actin–actin interface affect the mobility of the label. The addition of Tm did not affect the spectrum while the addition of equimolar myosin S-1 (30 mg/mL) to the labeled F-actin (10 mg/mL) completely broadened out the ^{19}F resonance (Figure 6b, after 8000 scans) as expected from previous studies using the smaller CF_3Hg probe (Barden et al., 1989).

AEDANS-Labeled Actin. A still larger probe was attached to Cys-10 in order to compare the mobility of the two myosin binding regions on the G-actin monomer. For this purpose, AEDANS was bound to Cys-10 (Barden et al., 1986b) after the actin was made unpolymerizable by the initial modification of Tyr-53 with DHT (Bender et al., 1976; Barden et al., 1983; Miki et al., 1987b). Thus, the monomeric actin concentration was increased to 0.5 mM in order to greatly improve the ^1H NMR signal-to-noise ratio. Figure 7a shows the aromatic region of the spectrum of free IAEDANS at pH 8.0 in D_2O. The downfield-shifted resonances are located in a region of the actin spectrum well removed from any protein resonances. The spectrum of AEDANS–DHT–actin at pH 8.0 in Figure 7b clearly reveals the presence of mobile resonances from the Cys-10-bound AEDANS. The line widths of the AEDANS resonances are broadened only about 70% compared with the free label. The line width is approximately 6 Hz so that $1/T_2 = 18.8\ s^{-1}$. Doddrell et al. (1972) have shown that $1/T_2$ is represented by $(1/20)K^2r^{-6}[\chi + 4\tau_R + 6\tau_R(1 + \omega^2\tau_R^2)^{-1}]$ in this case where χ has been defined above and where $r = 21.8$ pm in AEDANS. Thus, $\tau_R \approx 17.6$ ns, indicating a considerable degree of mobility compared with the actin monomer as a whole which has a τ_R close to 50 ns as mentioned above. No evidence of any AEDANS resonances could be found when AEDANS–G-actin was polymerized and the spectrum recorded at an actin concentration of 20 mg/mL, indicating a large increase in the effective correlation time. This may indicate that the actin–actin site is close enough to the AEDANS at Cys-10 to sterically hinder free rotation and further reinforces the notion that the myosin site is adjacent to an actin–actin site as is the case near Lys-61.

Discussion

The trifluoroacetonyl-Cys-374 ^{19}F NMR resonance is present in G-actin but is broadened out when the actin po-

lymerizes (Brauer & Sykes, 1986). This result has been interpreted as revealing that the penultimate residue in actin (Cys-374) is associated with an actin–actin binding site. Indeed, Cys-374 on one actin monomer can be cross-linked to Lys-191 on an adjacent actin monomer in F-actin through a distance of 0.8–1.4 nm (Elzinga & Phelan, 1984; Sutoh, 1984). The effective correlation time of the probe attached to Cys-374 is dramatically increased following actin polymerization, indicating that the mobility of the probe is severely reduced in F-actin. While Cys-374 appears to be close to an actin–actin binding site, the labeling of Cys-374 with a wide variety of probes does not inhibit the formation of F-actin (Lin & Dowben, 1982). Thus, the label appears to be located at the periphery of the binding site rather than directly in the center.

A similar mechanism apparently applies in this work. The labeling of Lys-61 with FITC prevents actin polymerization, presumably via direct steric hindrance of an actin–actin binding site (Burtnick, 1984). However, this paper shows that labeling with the much smaller PFPITC does not interfere with actin polymerization. Moreover, unlike the situation with Cys-374 in which the label is broadened when actin polymerizes, the label on Lys-61 remains unaffected with line widths the same as in G-actin. It has therefore been possible to observe the influence of Tm and myosin S-1 binding on the ^{19}F label resonances. The addition of either of these proteins to the labeled F-actin results in the dramatic broadening of the resonances due to a large increase in the effective correlation time of the PFPITC label. This is consistent with a marked reduction in the mobility of the label in the actin–myosin and actin–Tm complexes. Just as Cys-374 is close to an actin–actin binding site (Brauer & Sykes, 1986), Lys-61 is close to binding sites for both myosin S-1 and Tm without directly inhibiting the binding of these proteins.

Tropomyosin is located in the two long-pitch grooves in F-actin and may compete for sites with myosin S-1 (Wakabayashi et al., 1975; O'Brien et al., 1983). Others have demonstrated that myosin S-1 binds to thin filaments containing Tm–Tn in the presence of ATP and Ca^{2+}, suggesting that the proteins do not compete for the same site (Chalovich et al., 1981; Chalovich & Eisenberg, 1982). However, the results in this paper provide evidence that both myosin S-1 and Tm profoundly influence a small localized region around Lys-61 on actin. Since the labeling of Lys-61 does not interfere with the binding of either protein, the results indicate that the binding sites for Tm and myosin S-1 on actin are at least immediatly adjacent to Lys-61 even if they do not overlap. These results support the view that myosin and Tm do compete for the same site on actin at one point at least.

The labeling of Lys-336 also is affected by the binding of both Tm and myosin S-1 (Lu & Szilagyi, 1981; Szilagyi & Lu, 1982). Moreover, Lys-336 was cross-linked to the bound ATP via C8 on the adenine (Hegyi et al., 1986). The distance between the FITC chromophore attached to Lys-61 and the nucleotide binding site was estimated to be 3.5 ± 0.3 nm (Miki et al., 1987a). Thus, the Tm and myosin S-1 binding sites may be coextensive over a substantial portion of the actin surface, given that the overall dimensions of the actin monomer are 6.7 × 4.0 × 3.7 nm (Kabsch et al., 1985).

The myosin binding site has been localized to residues 18–28 (Mejean et al., 1987). Miller et al. (1987) demonstrated that actin could simultaneously bind myosin S-1 and an antibody directed toward residues 1–7. This showed that the earlier results of Sutoh (1982a,b, 1983) linking actin residues 1–4 and 11 to the myosin site probably indicated that these residues were only adjacent to the site. Previous work has revealed that Cys-10 must be very close to the myosin binding site (Barden et al., 1989). Similarly, the current results reveal that Lys-61 is also adjacent to the site since the labeling of this residue does not interfere with myosin S-1 binding to actin.

The reactivity of Lys-61 is reduced when actin polymerizes (Lu & Szilagyi, 1981), indicating that polymerization reduces the accessibility of Lys-61 to the reagent within the actin–actin interface. However, labeling with PFPITC does not impede the polymerization of actin nor are the ^{19}F NMR resonances affected. This suggests that Lys-61 is even more closely associated with a Tm and a myosin S-1 binding site than it is with an actin binding site. The high degree of immobility of FITC on actin indicates that the moiety is folded back onto the actin surface, thus sterically blocking the actin–actin site to a large extent, perhaps 1 nm from the myosin/Tm site close to Lys-61. This finding that Lys-61 has a correlation time much longer than AEDANS on Cys-10 and probably the same as actin monomers may require a reinterpretation of FRET data which have relied on a mobile FITC group on Lys-61 (Miki, 1987).

In contrast, labels attached to another part of the myosin binding site on actin at Cys-10 (Barden et al., 1989) were shown to be significantly more mobile. Both (methylphenyl)mercury (TFMPM) and AEDANS exhibit a high degree of mobility compared with the actin residues as a whole. However, only the much larger AEDANS is affected by actin–actin binding so that the actin–actin and the actin–myosin sites are not overlapped but also appear to be adjacent, as they are at Lys-61. Cys-10 appears to be separated from the Tm site, but it may be peripherally associated with a site for Tn-I (Levine et al., 1988). Only Lys-61 has been shown to be involved in both Tm and myosin binding.

The localization of the myosin and Tm binding sites close to the actin–actin binding site supports the view that Tm and myosin bind to F-actin in the groove formed by the packing of actin monomers in the long-pitch helices. This structural relationship is entirely consistent with the reconstruction of F-actin–Tm from X-ray diffraction data (O'Brien et al., 1983).

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CORRECTION

Determination of the Roles of Active Sites in F_1-ATPase by Controlled Affinity Labeling, by Joe C. Wu, Johnson Lin, Hua Chuan, and Jui H. Wang*, Volume 28, Number 22, October 31, 1989, pages 8905–8911.

Page 8905. In the last sentence of the abstract, the phrase should read as follows: but is promoted by the binding of ATP at the other two active sites.

Page 8908. In column 1, line 1, the complete sentence should read as follows: The percentage of labeled Lys-301 should be higher than 64–72%, since there were peptides of different chain lengths which also contain Lys-β301.