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Registry No. ATPase, 9000-83-3; DNase, 9003-98-9; subtilisin, 9014-01-1.

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^{19}F NMR Studies of the Interaction of Selectively Labeled Actin and Myosin†

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ABSTRACT: Monomeric or G-actin contains five cysteine residues of which two (Cys-374 and Cys-10) can be labeled readily with 3-bromo-1,1,1-trifluoropropane and (trifluoromethyl)mercuric bromide. The ^{19}F NMR resonances were assigned to the particular cysteines. Polymerization of the actin resulted in the fluorinated Cys-374 resonances broadening out beyond detection while the addition of myosin subfragment 1 to the F-actin also resulted in the fluorinated Cys-10 resonance becoming dramatically broad. Thus, Cys-374 appears to be close to an actin–actin binding site, and Cys-10 appears to be close to the actin–myosin binding site. (Trifluoromethyl)mercuric bromide was used to label the two reactive sulfhydryls on myosin subfragment 1, SH1 (Cys-705) and SH2 (Cys-695), which were assigned on the basis of their reactivity with *N*-ethylmaleimide. Addition of polymerized or F-actin to the fluorinated myosin resulted in the complete broadening of the ^{19}F-labeled SH1 NMR resonance and the partial broadening of the nearby ^{19}F-labeled SH2 resonance, suggesting that the actin binding site on myosin subfragment 1 involves SH1.

Actin is present in all eukaryotic cells and is involved intimately with processes including cell division, the maintenance of cell shape, cytoplasmic streaming, and axonal transport (Korn, 1982). Actin and myosin, the two main muscle proteins, comprise the essential components of the contractile process. Actin in its monomeric form (G-actin) is a globular protein containing ATP which is converted to ADP when the actin polymerizes into its filamentous form (F-actin). This process occurs through the formation of strong bonds between monomers along the single-start left-handed "genetic" helix and weak bonds formed between actin monomers on opposite strands of the two-start long-pitch helix (Oosawa, 1983). The regions 40–69, 87–113, 168–226, and 283–291 have been

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implicated in actin–actin binding [see Hambly et al. (1986) for a review]. Moreover, Cys-374 on one monomer can be cross-linked to Lys-191 on an adjacent monomer through a distance of 0.8–1.4 nm (Elzinga & Phelan, 1984; Sutoh, 1984). The involvement of Cys-374 in an actin–actin binding site also was suggested in a ^{19}F nuclear magnetic resonance (NMR) study (Brauer & Sykes, 1986) on the basis of the broadening of a fluorinated Cys-374 resonance following actin polymerization.

Chemical cross-linking of actin to myosin using the "zero-length" cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) has shown that the acidic N-terminal residues on actin (1–4, 11) are very close to the 20- and 50-kDa fragments on myosin subfragment 1 (S-1) heavy chain while the actin residues 360–363 are close to the alkali light chain 1 of myosin (Sutoh, 1982a,b, 1983). However, Chen et al. (1985a,b) have questioned these findings after analysis of the actomyosin cross-linking reaction. A different approach was adopted by Mejean et al. (1986), who obtained antibodies to actin residues 1–7. They concluded that these residues do not bind to myosin heavy chain on the basis of results from enzyme-linked immunosorbent assay (ELISA) titrations of myosin S-1 binding to actin–antibody complexes. A similar conclusion was reached by Miller et al. (1987), who demonstrated the simultaneous binding to F-actin of myosin S-1 and an Fab fragment of an antibody to actin residues 1–7. The formation of such a ternary complex indicates that the acidic residues 1–7 do not form a part of the myosin heavy-chain binding site. Rather, the actin residues 18–28 appear to be more directly involved in the site (Mejean et al., 1986, 1988).

Myosin S-1 heavy chain contains two particularly reactive sulfhydryls, Cys-705 (SH1) and Cys-695 (SH2), which are close together in the sequence and in the tertiary structure (Cheung et al., 1985; dos Remedios et al., 1987). Fluorescence resonance energy transfer (FRET) spectroscopy has been used to estimate the distance between these reactive thiols. The distance was reported to decrease when F-actin was bound (Cheung et al., 1983).

A discrepancy exists between estimates of the distance between the reactive sulfhydryls and the actin surface. FRET spectroscopy has been used to estimate the distance between SH1 and several sites on actin such as the nucleotide binding site (Miki & Wahl, 1984; dos Remedios & Cooke, 1984), Cys-374 (Takashi, 1979; Trayer & Trayer, 1983; Miki & Wahl, 1984), and unidentified lysines (dos Remedios & Cooke, 1984). The distances all range between 5 and 7 nm. The overall dimensions of actin are 6.7 × 4.0 × 3.7 nm (Kabsch et al., 1985), thus placing SH1 at some distance from the actin surface. Moreover, SH1 was visualized by electron microscopy on the concave surface of S-1 using biotin–avidin. The binding of avidin was then estimated to be 13 nm from the head–rod junction or 6–7 nm from the tip of myosin S-1 (Sutoh et al., 1984). The same approach was adopted by Tokunaga et al. (1987) in visualizing both SH1 and the myosin ATPase sites. They concluded that SH1 is about 5 nm from the actin binding site.

These results contrast with the suggestion that SH1 and SH2 are very close to the actin binding site (Katoh et al., 1985; Suzuki & Morita, 1987). Recently, Suzuki et al. (1987) demonstrated that the heptapeptide Ile-Arg-Ile-Cys(SH1)-Arg-Lys-Gly coprecipitates with F-actin and inhibits the formation of an F-actin–myosin S-1 complex.

Skeletal muscle actin contains 5 cysteines in a sequence length of 375 residues. These occur at positions 10, 217, 257, 285, and 374 (Vandekerckhove & Weber, 1979). Actin can be alkylated at Cys-374, Cys-10, and, to a lesser extent, Cys-285 with small SH-directed reagents (Lusty & Fasold, 1969). Cys-374 is the most reactive sulfhydryl (Martonosi, 1968; Elzinga & Collins, 1975; Miki & Wahl, 1984), and Cys-10 is the next most reactive (Sleigh & Burley, 1973; Barden et al., 1986a). Removal of the divalent cation (Konno & Morales, 1985) or both cation and nucleotide (Katz, 1963, 1965; Faulstich et al., 1984) enables other cysteine residues to be alkylated due to the partial unfolding of the actin.

The use of the fluorine-19 nucleus in nuclear magnetic resonance (NMR) spectroscopy in the study of protein interactions has the advantage of a sensitivity almost as high as ^1H NMR coupled with a wide chemical shift range and 100% natural abundance. Moreover, the absence of ^{19}F background resonances generally means the spectrum is easily interpreted, consisting only of the resonances of the fluorine-containing reagent or substrate added to the sample. The exposed cysteine residues of the muscle proteins troponin C (Seamon et al., 1977) and actin (Brauer & Sykes, 1986) have been selectively alkylated using 3-bromo-1,1,1-trifluoropropanone (BTFP).

In order to help locate the actin–myosin interface, the reactive sulfhydryls SH1 and SH2 on myosin and Cys-10 on actin were labeled separately with BTFP and/or (trifluoromethyl)mercuric bromide (CF_3HgBr), and the effects of protein interactions on the ^{19}F NMR resonances were observed. Furthermore, the selectivity of actin sulfhydryl labeling is examined.

Materials and Methods

Actin was prepared with very high yield using the method of Barden et al. (1986b) from rabbit muscle acetone powder. The G-actin preparation in 0.2 mM $CaCl_2$, 0.2 mM ATP, 1 mM NaN_3, and 2 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris) at pH 8.0 was centrifuged at 100000*g* for 1.5 h. The supernatant was diluted to 3 mg/mL in the above buffer and the temperature increased from 4 to 25 °C. Urea was added as a solid to a final concentration of 1.5 M in order to increase the mobility of the N-terminal domain without causing an obvious alteration in the ^1H NMR spectrum (Barden & dos Remedios, 1983; Barden et al., 1986a). Thereafter, fluorination of the G-actin with BTFP (Brauer & Sykes, 1986) was accomplished by adding a 5 mM solution of BTFP in four aliquots over a 30-min period with continuous stirring. During this time, the pH was held constant with the dropwise addition of a dilute aqueous triethylamine solution. A further 1.5 h was allowed for the reaction. The labeled actin was dialyzed for 48 h against the G-actin buffer containing 0.5 mM dithiothreitol with frequent changes of dialysate. The actin was clarified at 100000*g* for 1.5 h and concentrated to 16 mg/mL with an Amicon PM10 membrane and apparatus.

The carbonyl leaving group on the BTFP made the probe unstable in basic media so that an alternative label was chosen. The (trifluoromethyl)mercuric label with a bromide leaving group, CF_3HgBr, was found to be suitable. This compound was prepared by the exchange reaction of the trifluoroacetate group on (trifluoromethyl)mercuric trifluoroacetate under reflux with excess aqueous sodium bromide (Rausch & Wazer, 1964; Bendall & Lowe, 1974). Synthesis of the trifluoroacetate precursor proceeded via the dry distillation of mercuric trifluoroacetate (Aldrich, 1962).

The G-actin was diluted to 1.7 mg/mL in a solution containing 0.2 mM $Ca(NO_3)_2$, 0.1 mM ATP, 1 mM NaN_3, and 2 mM sodium phosphate, pH 7.5. Chloride ions were omitted from all the actin solutions as a precaution against interference with the ^{19}F resonances in the NMR spectra (Bendall & Lowe,

1974). Actin was labeled in the required specific stoichiometric ratios (0.5, 1, 1.5, 2, and 3) by adding these molar equivalents of ^{19}F label and allowing the reactions to proceed for 3 h at 4 °C. The actin was dialyzed against the above solution for 48 h with frequent changes of dialysate, clarified at 100000*g* for 1.5 h, and then concentrated on an Amicon. The addition of more than 2 mol of label/mol of actin resulted in a reduction in the reduced viscosity of F-actin. At molar ratios of 3–4, the actin became unpolymerizable. The rate of increase of the extent of reduced viscosity of F-actin containing only 1–2 mol of label/mol of actin was indistinguishable from unlabeled actin. The labeling of sulfhydryls on actin was confirmed with a reactive sulfhydryl assay (Habeeb, 1972).

Actin was polymerized at 5 mg/mL in 0.1 M sodium acetate and 20 mM sodium phosphate at pH 7.0. A 10-fold molar ratio of *N*-ethylmaleimide was added to the F-actin for 3 h at 20 °C. The reaction was terminated by the addition of a 100-fold ratio of β-mercaptoethanol, and the F-actin was pelleted at 80000*g* for 1.5 h. F-Actin pellets were redissolved in 0.2 mM ATP, 0.2 mM calcium nitrate, and 2 mM sodium phosphate at pH 8.0 and dialyzed against the same buffer for 48 h with frequent changes of solution. The depolymerized actin was clarified at 100000*g* for 1.5 h. Labeling of Cys-10 was performed by adding the required ratio (e.g., 0.6 mol/mol of actin) of CF_3Hg to the G-actin for 3 h at 4 °C. The labeled G-actin was dialyzed for 24 h, clarified at 80000*g* for 1.5 h, and concentrated to 4 mg/mL (G-actin) or up to 20 mg/mL (F-actin) on an Amicon assembly.

Tropomyosin (Tm) was extracted from rabbit skeletal muscle according to the method of Ebashi et al. (1968). Tropomyosin was bound to F-actin at a ratio of 1 Tm/7 actin monomers.

Myosin was extracted from rabbit skeletal muscle by the method of Tonomura et al. (1966). Myosin S-1 was prepared according to the method of Weeds and Taylor (1975). The most reactive thiol on myosin S-1, SH1, was reacted with CF_3HgBr essentially according to the method of Ando et al. (1980). Myosin S-1 (8.6 mg/mL) was dissolved in 0.5 M sodium acetate, 1 mM NaN_3, and 20 mM sodium phosphate at pH 6.0. An equimolar ratio of label was added and the reaction stirred for 4 h at 4 °C, and then the protein was dialyzed for 12 h against the same buffer. The labeled S-1 was then dialyzed for 12 h against 50 mM sodium acetate, 2 mM magnesium acetate, 1 mM NaN_3 and 20 mM sodium phosphate, pH 7.0, centrifuged at 80000*g* for 1 h, and concentrated on an Amicon assembly to 43 mg/mL.

The second most reactive thiol, SH2, was labeled according to the method of Sekine and Kielley (1964). Myosin S-1 (3.5 mg/mL) was dissolved in 0.5 M sodium acetate and 50 mM Tris–histidine, pH 7.0. A 4-fold molar excess of *N*-ethylmaleimide was added to the myosin S-1 to label the SH1 for 40 min at 4 °C. The reaction was terminated by adding a 50-fold excess of β-mercaptoethanol and the S-1 dialyzed against 50 mM sodium acetate, 1 mM ADP, and 20 mM Tris–maleate at pH 7.0 for 12 h. The SH2 was labeled by adding 1 mol of CF_3HgBr/mol of S-1 at 4 °C for 3 h with constant stirring. Thereafter, the S-1 was dialyzed for 12 h against the same buffer, centrifuged at 80000*g* for 30 min, and concentrated on an Amicon assembly to 65 mg/mL.

^{19}F NMR spectra of fluorinated actin and myosin 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. A 15% concentration of deuterium in the samples acted as the lock signal. Trifluoroacetic acid (TFA) was used as the external frequency reference

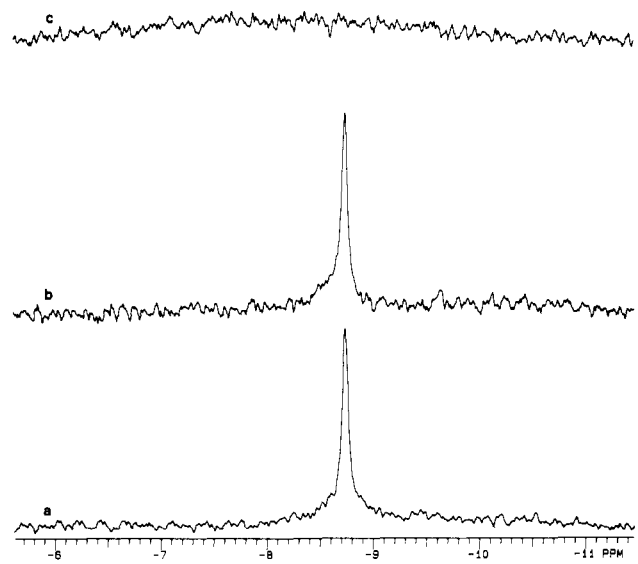

FIGURE 1: ^{19}F NMR spectra of BTFP–F-actin showing the resonance due to the Cys-10 label (a), F-actin with bound Tm (b), and F-actin with bound myosin S-1 (c). The F-actin concentration was 16 mg/mL in 85% H_2O/15% D_2O, 50 mM KCl, and 10 mM Tris (pH 8.0). Equimolar myosin S-1 was added from a 65 mg/mL solution. Chemical shifts are referenced to TFA at 0.000 ppm.

(0.000 ppm). Upfield shifts from TFA are negative (BTFP samples), and downfield shifts are positive (CF_3HgBr samples). A 5000-Hz spectral width was used with a 90° pulse width and a data block of 8192 points. The repetition time between scans totaled 1.6 s of which 0.8 s was a preacquisition delay. Samples were placed in 5-mm tubes, and spectra were run at 25 °C. T_1 values were determined by the progressive saturation method (Freeman & Hill, 1971), and the T_2 values were determined by using the Carr–Purcell–Meiboom–Gill sequence available in the Varian software.

Results

BTFP-Labeled Actin. The ^{19}F NMR spectrum of F-actin (16 mg/mL) in 85% H_2O/15% D_2O, 50 mM KCl, and 10 mM Tris at pH 8.0 is shown in Figure 1a, after being labeled with 2 mol of BTFP. The resonance (−8.47 ppm) of the label attached to Cys-374, the most reactive sulfhydryl, is broadened out when G-actin polymerizes as observed by Brauer and Sykes (1986). The resonance in Figure 1a is due solely to BTFP attached to Cys-10, the second most reactive sulfhydryl (Barden et al., 1986a). Free label, which resonates at −7.877 ppm at pH 8.0, was removed completely by dialysis. No evidence was found of a third reactive sulfhydryl being labeled to any detectable extent, in contrast to the results of Brauer and Sykes (1986). The labeling conditions employed were designed to be more selective in only labeling two Cys residues (Barden et al., 1986a). The chemical shift of the trifluoroacetonyl-Cys-10 resonance was −8.75 ppm upfield of TFA.

The effect of tropomyosin (Tm) binding on the labeled Cys-10 resonance was examined by adding Tm to the labeled F-actin at a ratio of 1 Tm for every 7 actin monomers in 5 mM $MgCl_2$, sufficient to saturate all the Tm binding sites on F-actin. The spectrum in Figure 1b reveals that the Cys-10 label remains unaffected. Ultracentrifugation of the Tm–F-actin complex at 100000*g* for 1 h resulted in the sedimentation of the F-actin pellet together with the bound Tm. Essentially none of the Tm remained unbound in the supernatant as revealed by SDS gel electrophoresis and UV absorption measurements at 280 nm.

A similar experiment was performed to determine the effect of myosin S-1 binding on the Cys-10 label. An 8-mg sample

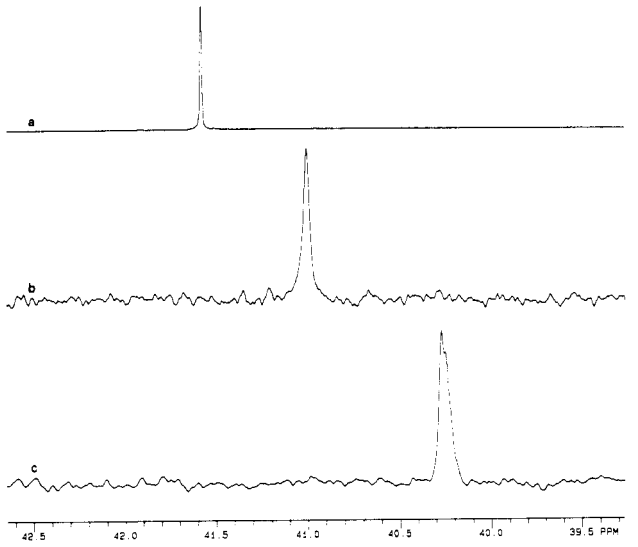

FIGURE 2: ^{19}F NMR spectra of the free probe CF_3HgBr (a), G-actin at 3.5 mg/mL containing 0.6 mole of CF_3Hg/mol of actin at Cys-374 (b), and G-actin containing Cys-374 blocked with NEM and 0.6 mol of CF_3Hg/mol of actin at Cys-10 (c).

of the labeled F-actin (16 mg/mL) was incubated with 25 mg of myosin S-1 (65 mg/mL) for 1 h at pH 8.0 Twice the number of scans (4096) were accumulated in the spectrum of Figure 1c compared to the spectra in Figure 1a and Figure 1b to improve the signal-to-noise ratio as partial compensation for the dilution resulting from the addition of myosin S-1. The BTFP label on Cys-10 is fully broadened by the myosin binding just as the label on Cys-374 is broadened upon polymerization of the actin.

CF_3HgBr-Labeled Actin and Myosin. The carbonyl leaving group on the BTFP made the label somewhat unstable. The $-COCF_3$ group presumably dissociates to yield CHF_3 particularly in the presence of a nucleophilic base. A similar mechanism applies with the $-COCI_3$ group used in the anlytical iodoform test to yield CHI_3 (Shriner et al., 1964). Thus, the much more stable compound CF_3HgBr was used to repeat the above experiments and to extend them to new label sites on myosin S-1, namely, the two most reactive sulfhydryls SH1 and SH2.

The ^{19}F NMR spectrum of the free probe is shown in Figure 2a. The T_1 value for the free probe was determined to be 2.72 s while the T_2 value for the free probe was 1.59 s. The label was added to G-actin (3.5 mg/mL) at a molar ratio of 0.6 to ensure that only the most reactive site was occupied. The result is shown in the spectrum in Figure 2b. A single resonance is observed at 41.00 ppm downfield of TFA, due to the labeling of Cys-374. Preincubation of actin with *N*-ethylmaleimide (NEM) was used to block the Cys-374 and unreacted NEM removed. Thereafter, the next most reactive sulfhydryl, Cys-10, was labeled by the addition of CF_3HgBr at a molar ratio of 0.6. The label attached to Cys-10 was found to resonate at 40.27 ppm (Figure 2c), well removed from the resonance due to the labeled Cys-374. The T_1 values for the resonances of the two protein-bound labels were virtually identical, 0.82 s, about half the repetition time between scans.

Actin was cleaved by trypsin in order to separate labels attached to Cys-10 and Cys-374. Actin labeled with 1.8 mol of CF_3Hg/mol of actin was digested according to the method of Jacobson and Rosenbusch (1976). This resulted in a proteolytic resistant core consisting of residues 69–372 and a mixture of peptides consisting of residues 1–68 (mostly cleaved into smaller fragments) and the C-terminal tripeptide 373–375. SDS gels were used to confirm that complete digestion to a

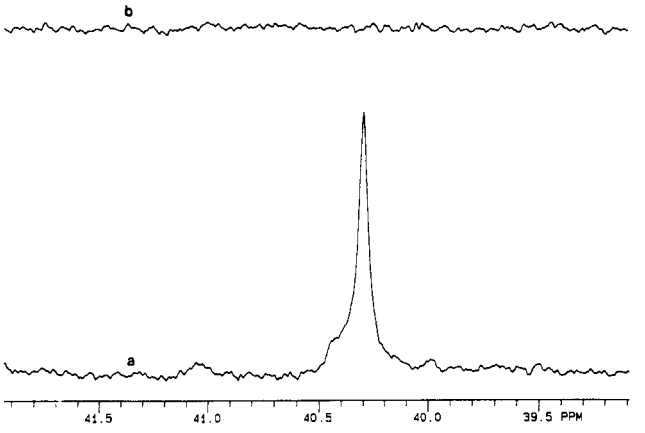

FIGURE 3: ^{19}F NMR spectra of actin reacted with 1.8 mol of CF_3HgBr before digestion with trypsin. Spectrum a shows that the N-terminal 68 and the C-terminal 3 residues contain labeled Cys residues, 10 and 374. Spectrum b shows that the tryptic core of actin, 69–372, does not contain any resonances due to the presence of labeled Cys residues.

33-kDa core occurred. The only Cys residues contained within the cleaved peptides were Cys-10 and -374. The core, containing Cys-217, -257, and -285, was separated from the N- and C-terminal peptides by passing the digest products through an Amicon PM-10 membrane which retained only the high molecular weight actin core. The core was concentrated to 16 mg/mL, and the peptides were collected, freeze-dried, and redissolved at 3.75 mg/mL, the same molarity as the core. Samples of each at pH 8.0 were examined by using NMR. The spectrum of the peptide sample in Figure 3a reveals a ^{19}F NMR resonance in close proximity to the chemical shift of the Cys-10 label resonance in F-actin and minor resonances near 41.0 and 40.4 ppm. The spectrum of the actin core (Figure 3b) reveals no resonances. The core therefore appears to be devoid of any labels, in full agreement with the results of Barden et al. (1986a).

In the unlikely event that labeling of a third Cys residue in actin was masked by the subsequent broadening of the resonance due to a conformational change occurring in the core after tryptic digestion, the level of Hg in the core was detected. A Varian Techtron Model 1200 atomic absorption spectrometer was used to measure Hg with the cold vapor technique (Omang, 1971). Although as little as 0.05 Hg/actin could be detected, no difference was detected between the level of Hg in the buffer and the actin core. All the Hg was present in the peptide sample.

Polymerization of the actin resulted in the complete broadening of the Cys-374 resonance as occurred with the BTFP label. Consequently, NEM was not used to block the Cys-374. Rather, 1.8 mol of label was added to each mole of G-actin in order to label both Cys-374 and Cys-10. The spectrum of the labeled F-actin (16 mg/mL) is shown in Figure 4a. Only the Cys-10 resonance remains at 40.27 ppm.

The addition of myosin S-1 to the F-actin resulted in the complete broadening of the Cys-10 resonance (Figure 4b), in full agreement with the results in Figure 1c using BTFP. The conditions were the same as those described in the previous section with the BTFP label. A 3-fold increase in the number of scans was used (3072) to compensate for the dilution effect resulting from the addition of myosin S-1.

Myosin S-1 contains two sulfhydryls which are readily labeled and which have been well characterized. These are commonly referred to as SH1 and SH2 and are the residues Cys-705 and Cys-695 (Strehler et al., 1986). The labeling of SH1 with CF_3HgBr resulted in the spectrum in Figure 5a.

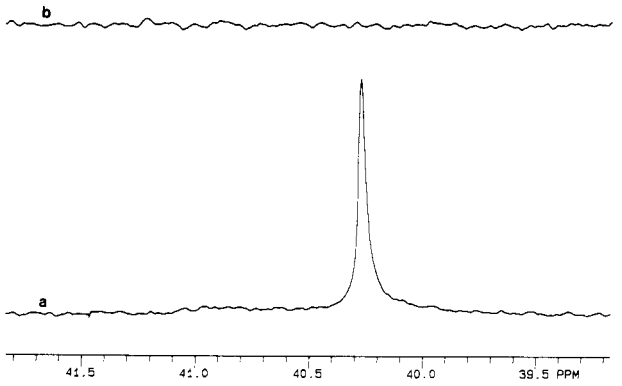

FIGURE 4: ^{19}F NMR spectra of F-actin (16 mg/mL) labeled with 1.8 mol of CF_3Hg/mol of actin. Only the resonance from the label attached to Cys-10 at 40.27 ppm remains unbroadened. The addition of myosin S-1 (b) results in the complete broadening of the Cys-10 label resonance.

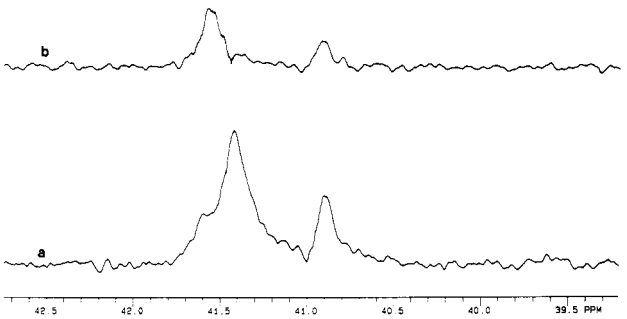

FIGURE 5: ^{19}F NMR spectrum of myosin S-1 (60 mg/mL) containing 1 mol of CF_3Hg/mol of S-1. The ^{19}F label attached to SH1 resonates at 41.4 ppm while the minor resonances at 41.55 and 40.95 ppm are due to labels attached to other sulfhydryls (a). Spectrum b resulted from the saturation of all the S-1 binding sites by the addition of F-actin. The major resonance due to the label on SH1 is broadened out while the intensity of the minor resonance at 40.95 ppm is reduced by half.

Three resonances were detected. A major resonance at 41.1 ppm was bounded by two minor resonances at 40.95 and 41.55 ppm. More selective labeling conditions in which the molar ratio of label to myosin S-1 was lowered to 0.5 resulted in the virtual elimination of the minor resonances. Thus, the SH1 resonance is the major peak at 41.4 ppm. The presence of the minor resonances in the spectrum proved to be useful as internal standards following the addition of F-actin. The spectrum in Figure 5a was obtained after 2048 scans using a sample of labeled myosin S-1 at 60 mg/mL. An aliquot of F-actin (at 20 mg/mL) was added to saturate all the actin binding sites on the myosin. This resulted in the concentration of the label falling to half the level in Figure 5a. A total of 8192 scans were collected in the spectrum of the actomyosin complex shown in Figure 5b as compensation for the reduced concentration. The labeled SH1 resonance at 41.4 ppm was completely broadened out while the minor resonance at 41.55 ppm remained unaffected. The second minor resonance at 40.95 ppm was partially broadened, with approximately half the intensity remaining in the spectrum.

The identity of the partially broadened resonance at 40.95 ppm was revealed by blocking SH1 with NEM. The second most reactive sulfhydryl was labeled, i.e., SH2, together with a proportion of the third reactive sulfhydryl as shown in the spectra in Figure 5. The same two resonances present in Figure 5 also were seen in Figure 6a, at 41.55 and 40.95 ppm. However, the SH1 resonance at 41.4 ppm was absent, effectively having been blocked with the NEM. The larger resonance at 40.95 ppm was presumably due to SH2. F-Actin was

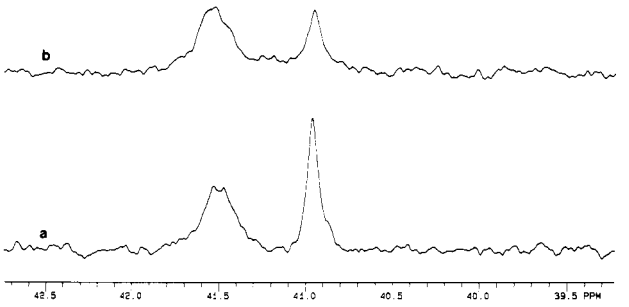

FIGURE 6: Spectrum in (a) resulting from the blocking of SH1 with NEM and the labeling of SH2 and an unknown third sulfhydryl. The sharp resonance at 40.95 ppm is due to the label on SH2. Addition of F-actin results in the partial broadening of the SH2 resonance while the third sulfhydryl remains unaffected (b).

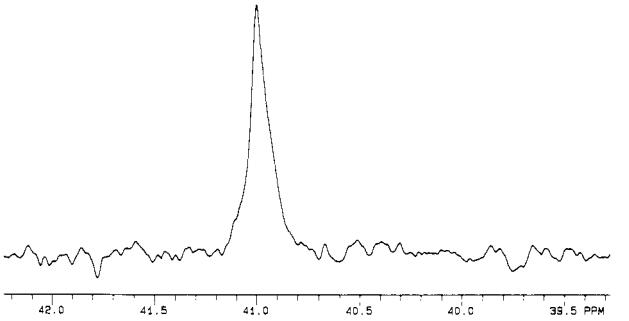

FIGURE 7: NMR spectrum obtained by more selectively labeling SH2 with the addition of only 0.5 mol of CF_3HgBr/mol of S-1. A single resonance at 40.95 ppm is observed, the same as revealed in Figures 5 and 6.

added to the myosin S-1 sample at the same concentration used in Figure 5b and the effect on the SH2 resonance observed. The spectrum in Figure 6b reveals that the resonance at 41.55 ppm due to the unknown label again remains unaffected by F-actin binding but the SH2 resonance at 40.95 ppm is broadened by about 60%, indicating that while SH1 is very closely associated with the actin binding site SH2 is less involved while remaining close to the site. The third sulfhydryl is not involved in the actin binding site.

Selective labeling of SH2 was accomplished by using lower molar ratios of added label to abolish the labeling of the third sulfhydryl. The spectrum in Figure 7 shows the result obtained from a sample of myosin S-1 labeled with 0.5 mol of CF_3HgBr after SH_1 was blocked with NEM. A single resonance is present at 40.95 ppm. No changes to the spectrum of the labeled myosin S-1 were observed when the substrate ATP was added at a molar ratio of 20.

Discussion

Actin was specifically labeled with BTFP and CF_3HgBr at Cys-374 and Cys-10. No NMR resonances were observed from a sample of the tryptic actin core (residues 69–372) which indicated that the core remained unlabeled under the stated conditions. In the unlikely event that the core contained an undetectable broadened resonance due to a conformational change in the core resulting from the proteolytic digestion process, the Hg content was measured. AAS measurements of Hg content established beyond doubt that the core remained free of label. These measurements clearly revealed that the three cysteines Cys-217, -257, and -285, in the core were devoid of bound Hg. Thus, the results are in full agreement with an earlier paper (Barden et al., 1986a) in which the conditions were established for the selective labeling of Cys-10 with larger fluorescent labels.

The ^{19}F NMR resonances of both label types (BTFP and CF_3HgBr) attached to the most reactive cysteine (Cys-374)

were downfield-shifted with respect to the positions of the resonances of the labels attached to Cys-10. Therefore, the label on Cys-374 is probably in a more hydrophobic environment than is the label on Cys-10 (Wuthrich, 1986). This may be due to the close proximity of the Phe-375 side chain.

Actin polymerization resulted in the dramatic broadening of the ^{19}F resonances of both BTFP [in full agreement with the results of Brauer and Sykes (1986)] and CF_3Hg. The resonances of the label attached to Cys-10 remained unaffected. Thus, the effective correlation times for the labeled Cys-10 remained unaffected by the polymerization process whereas the label on Cys-374 experienced a large increase in the effective correlation time due to a severe reduction in its mobility in F-actin. The use of bifunctional cross-linking reagents has indicated that Cys-374 on one actin monomer can be cross-linked to Lys-191 on an adjacent monomer in F-actin through a distance of 0.8–1.4 nm (Elzinga & Phelan, 1984; Sutoh, 1984). Thus, Cys-374 appears to be close to an actin–actin binding site. However, the labeling of Cys-374 with a wide variety of probes does not inhibit F-actin formation (Lin & Dowben, 1982). Consequently, the label is not centered at the actin–actin binding site but rather appears to be located more peripherally.

Similarly, the ^{19}F resonance of the label on Cys-10 in F-actin is broadened dramatically upon the binding of myosin S-1. Just as Cys-374 is close to an actin–actin binding site, Cys-10 must be close to the actin–myosin binding site. The small size of the CF_3Hg group does not inhibit myosin binding. The large increase in the effective correlation time of the Cys-10 label must result from a significant reduction in the mobility of the label imposed by the very close approach of the myosin heavy chain. This result is in close agreement with the results of Mejean et al. (1988), who localized the myosin binding site on actin to residues 18–28. The earlier results of Sutoh (1982a,b, 1983) which suggested the site involved the acidic residues 1–4 and 11 also are consistent with the results in this paper. While these residues are not directly involved in the myosin site (Miller et al., 1987), they clearly appear to be immediately adjacent to the site.

Tropomyosin lies in the two long-pitch grooves in F-actin and may compete for binding sites with myosin S-1 (Wakabayashi et al., 1975; O'Brien et al., 1983). Others have shown that the binding of S-1 to thin filaments in the presence of ATP remains unaffected by Ca^{2+} (Chalovich et al., 1981; Chalovich & Eisenberg, 1982). The results in this paper show that while the label on Cys-10 is affected by S-1 binding, the binding of Tm has no effect on the line width. If Tm does interfere with S-1 binding to F-actin, it must do so at a site well removed from Cys-10.

The chemical shift of the resonance of the CF_3Hg label attached to SH1 (Cys-705) on myosin S-1 is downfield of the position of the labeled SH2 (Cys-695) resonance and thus appears to be situated in a more hydrophobic environment (Wuthrich, 1986). Both of the residues are probably surface accessible as are the residues Ile^{702}-Arg^{703}-Ile^{704} (Suzuki et al., 1987) adjacent to SH1. These Ile residues may be the source of the hydrophobic shift.

The label attached to SH1 was completely broadened by the binding of F-actin while the label on SH2 was partially broadened. A large reduction in the effective correlation time of the SH1 label in particular is consistent with a marked reduction in its mobility in the presence of actin. This result is in full accord with other investigations (Katoh et al., 1984, 1985; Kato & Morita, 1984; Suzuki & Morita, 1987; Suzuki et al., 1987) which have placed an actin binding site on the myosin chain between SH1 and SH2. The myosin peptide 702–708 has been shown to cosediment with F-actin after ultracentrifugation and to inhibit formation of the acto–S-1 complex by direct competition with S-1 for actin (Suzuki et al., 1987). Thus, the complete broadening of the trifluoromethyl resonance on SH1 resulting from actin binding does not appear to result simply from a conformational change in myosin around the SH1, well removed from the actin binding site. Rather, SH1 must be in contact with the actin surface.

This conclusion is at variance with the report by Tokunaga et al. (1987), who placed SH1 5 nm from the actin binding site on the basis of three-dimensional electron microscopy reconstructions. Moreover, a fluorescence resonance energy transfer spectroscopy study of the distance between SH1 and several unidentified reactive Lys residues on actin concluded that SH1 was 6.6 nm from actin (dos Remedios & Cooke, 1984). Perhaps the presence of the label *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS) interfered with actin binding in such a way as to severely affect the result, or perhaps actin binding to the myosin affected the value of the orientation factor K^2.

The existence of a third reactive sulfhydryl group on myosin S-1 which can be specifically labeled was revealed in this paper. The selective labeling of this residue may prove to be a valuable aid in determining distances between various identified residues on actin and myosin and in orienting the monomers within the actin–myosin complex, particularly if the sulfhydryl is located within the heavy chain rather than one of the myosin light chains.

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Registry No. Cys, 52-90-4.

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