Spin-Echo ^1H NMR Studies of Differential Mobility in Gizzard Myosin and Its Subfragments†

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ABSTRACT: The unexpectedly narrow resonances in the ^1H NMR spectra of gizzard myosin, heavy meromyosin, and subfragment 1 were examined by spin-echo NMR spectroscopy. These resonances originated predominantly in the myosin heads, or subfragment 1 units. Smooth muscle myosin undergoes a dramatic change in hydrodynamic properties and can exist either as a folded (10S) or as an extended (6S) species. Factors that influence this transition, namely, ionic strength and phosphorylation (or thiophosphorylation), were varied in the NMR experiments. T_2 relaxation experiments on dephosphorylated myosin indicated several components of different relaxation times that were not influenced by changes in ionic strength. Our experiments focused on the components with longer relaxation times, i.e., corresponding to nuclei with more mobility, and these were observed selectively in a spin-echo experiment. With dephosphorylated myosin and HMM, increases in ionic strength caused an increased intensity in several of the narrower resonances. The ionic strength dependence of these changes paralleled that for the 10S to 6S transition. With thiophosphorylated myosin and HMM, changes in ionic strength also influenced the intensities of the narrower resonances, and in addition changes in the ^1H NMR spectrum due to thiophosphorylation were observed. The narrow resonances seen with myosin and HMM were observed with S1, but the spin-echo spectra of S1 were not influenced either by changes in ionic strength or by phosphorylation. These results suggest that a fraction of the ^1H resonances in smooth muscle myosin and its fragments originates from both aliphatic and aromatic residues of increased mobility compared to the mobility expected from hydrodynamic properties of these proteins. In general, the intensities of these residues increase with increasing ionic strength, and this is consistent with an increase in the percentage of mobile residues during the 10S to 6S transition. Segmental flexibility appeared also to be influenced by phosphorylation within the 6S conformation. These changes were not detected in the isolated myosin heads and thus required a higher order of structure, either the subfragment 2 region or the interaction of myosin heads.

Regulation of contractile activity in smooth muscle involves phosphorylation and dephosphorylation of myosin (Hartshorne, 1987). It is generally accepted that phosphorylation of the serine-19 residues on each of the two 20000-dalton light chains converts an inactive to an active state, and is essential for the initiation of a contractile response. From experiments carried out with isolated proteins, it is known that phosphorylation increases the actin-activated ATPase of myosin, and it is assumed that this event leads to increased cross-bridge cycling in the intact muscle. Myosin is phosphorylated by myosin light chain kinase, and the link between contraction and intracellular Ca^{2+} transients is provided by the calmodulin dependence of this enzyme. The identity of the phosphatase is not established, and it is assumed that the dephosphorylation reaction is not regulated.

Smooth muscle myosin, unlike striated muscle myosin, undergoes a remarkable transition. At low ionic strength, in the presence of ATP, smooth muscle myosin exists in a folded conformation with a sedimentation coefficient of 10 S. Electron micrographs of the folded state have shown that the myosin tail loops back upon itself to interact near the head–neck junction. The heads themselves also appear to be directed back toward the tail. At higher ionic strengths, the familiar extended 6S conformation is found (Suzuki et al., 1982; Onishi et al., 1983). In general, phosphorylation shifts the equilibrium toward the 6S state (Ikebe et al., 1983; Craig et al., 1983; Onishi et al., 1983; Trybus & Lowey, 1984). Although lacking most of the myosin tail, heavy meromyosin (HMM)[1] appears to undergo a comparable transition (Suzuki et al., 1985). Of particular interest is the observation that during the 10S–6S transition, enzymatic activity is altered, and it was suggested that 6S and 10S myosins are characterized by distinct enzymatic properties (Ikebe et al., 1983). From these data, a simple hypothesis was developed, namely, that the conformation of myosin (either 6S- or 10S-like) determined enzymatic activity (Ikebe et al., 1983). In this scheme, the function of phosphorylation would be to drive a conformational change. It is unlikely that the entire folding transition is allowed with filamentous myosin, and an important assumption is that some of the changes occurring as part of the 6S–10S transition may also occur in the myosin molecules in the thick filaments. The problem is to identify those changes that are linked to biological properties.

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[1] Abbreviations: S1, heavy meromyosin subfragment 1; S2, heavy meromyosin subfragment 2; HMM, heavy meromyosin; LMM, light meromyosin; AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; T_2, NMR spin–spin relaxation time; DSS, 4,4′-dimethyl-4-silapentane-1-sulfonate; CPMG T_2, Carr–Purcell–Meiboom–Gill spin-echo NMR pulse sequence.

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One technique that has been useful is the application of limited proteolysis as a conformational probe. Using various proteases, it was found that two sites are altered during the 10S–6S transition, sites A and B. Site A is located about 68 kDa from the N-terminus of the heavy chain, and site B is at the S1–S2 junction. Both of these sites are protected (from proteolysis) in the 10S state, and in addition, site A is blocked by the binding of actin (Ikebe & Hartshorne, 1986). As a working hypothesis, it was suggested (Ikebe et al., 1988) that the effects at site B reflected the flexibility at the head–neck junction. In the dephosphorylated state, the S1 units are thought to be in a constrained configuration. Phosphorylation induces an increased flexibility in the S2 region that allows interaction of the cross bridges with actin. Suzuki et al. (1985) also suggested that the orientation of the myosin heads is important in determining enzymatic activity.

In order to examine dynamic events on a molecular level, the folding transition in smooth muscle myosin was followed by ^{1}H NMR spectroscopy. The motional properties of a given nucleus are most clearly revealed in the NMR spectrum by the resonance line width; small molecules tumble rapidly in solution and give rise to narrow lines, whereas large proteins such as myosin (molecular weight 470 000) would normally be expected to contribute extremely broad, overlapping resonances. In solution-state NMR experiments, the line width is sensitive to dynamic events which occur with a time period shorter than the overall rotational correlation time, which for myosin is on the order of 0.1–1.0 μs. Segmental internal motion, or flexibility, of a section of the polypeptide chain results in a shorter effective correlation time for protons in that region and concomitant narrower resonance line widths.

The line width of the NMR resonance corresponding to a given proton is related to its spin–spin relaxation time T_2 by the equation $\Delta\nu = 1/\pi T_2$. This forms the basis of experiments designed to measure line widths in spectra with many overlapping resonances. The T_2 relaxation time is typically measured in a spin-echo experiment in which xy magnetization, produced by an initial 90° pulse, is allowed to decay over a variable time period τ. In the simplest experiment, the natural defocusing of spins, caused by precession at their individual frequencies, is reversed by the application of a refocusing pulse at $\tau/2$. This produces the so-called spin-echo at time τ, immediately prior to data acquisition. T_2 is obtained from an exponential fit to the resonance intensity plotted as a function of τ. Molecules such as myosin which tumble slowly in solution have short T_2's and narrower line widths. The spin echo experiment thus allows one to distinguish mobile regions of myosin from those tumbling more slowly with the molecule as a whole.

Skeletal muscle myosin and its subfragments have previously been the subject of several NMR investigations. Highsmith et al. (1979) noted a number of very narrow resonances superimposed upon the expected broad resonance envelope. These are of particular interest, as they show the myosin molecule to contain one or more regions of independent local motion [see also Highsmith and Jardetzky (1981)]. Since the narrow resonances are conserved in the spectra of both heavy meromyosin and S1, the local motions observed by NMR must be confined almost exclusively to the myosin head region. The possibility of a small contribution from the S1–S2 junction, however, cannot be discounted (Highsmith et al., 1979; Prince et al., 1981).

The ^{1}H NMR spectra of smooth and skeletal muscle myosins were found to be very similar inasmuch as both were clearly divided into broad and narrow components. The broad component, which arises from immobile regions of the molecule, was difficult to quantify. It may be eliminated conveniently, however, in a spin-echo experiment which takes advantage of the vastly different transverse relaxation rates ($1/T_2$) of the two components [see Bloom et al. (1986)]. The intensity changes which occur in the narrow component of the spectrum were used to monitor the conformational transition between the folded 10S and the extended 6S states. Myosin spectra, obtained as a function of ionic strength, were compared with those of heavy meromyosin and S1. The effects of phosphorylation and nucleotide binding on the conformational change were also examined.

Materials and Methods

Myosin (Ikebe & Hartshorne, 1985a) and myosin light chain kinase (Ikebe et al., 1987) were isolated from frozen turkey gizzard. Calmodulin was isolated from bull testes (Walsh et al., 1983). HMM and S1 were prepared by digestion of gizzard myosin with *Staphylococcus aureus* protease at 25 °C (Ikebe & Hartshorne, 1985b). S1 prepared by this procedure retains the intact 20 000-dalton light chain. Myosin, HMM, and S1 were freeze-dried in 5% sucrose, 1 mM $NaHCO_3$ (pH 7.6), 5 mM dithiothreitol, and 0.5 M KCl. These proteins were prepared for NMR experiments by dialyzing for 24 h against 20 mM potassium phosphate (pH 7.5), 5 mM $MgCl_2$, 0.44 M KCl, and 1 mM dithiothreitol in H_2O at 4 °C. (This procedure removed most of the sucrose.) Insoluble protein was removed by centrifugation in an Eppendorf centrifuge for 10 min. The protein was then dialyzed for 12–18 h against 50 volumes of the required D_2O solution (three to five changes of dialysis solution). Protein concentrations were measured by using $E^{1\%}_{280nm}$ values of 7.0, 7.0, and 4.7 for HMM, S1, and myosin, respectively.

Myosin, HMM, and S1 were examined in the dephosphorylated and thiophosphorylated states. Thiophosphorylation was carried out at 25 °C prior to freeze-drying in 0.5 M KCl, 20 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 0.2 mM adenosine 5′-*O*-(3-thiotriphosphate) (Boehringer Mannheim), 0.5 mM dithiothreitol, 0.05 mM $CaCl_2$, 2–4 mg/mL protein, 20 μg/mL myosin light chain kinase, and 10 μg/mL calmodulin. After approximately 15 min, the reaction mixture was dialyzed against the solvent used for freeze-drying. The extent of thiophosphorylation was greater than 90% (i.e., for serine-19) as monitored by urea gel electrophoresis (Perrie & Perry, 1970). Electrophoresis in 0.1% sodium dodecyl sulfate was carried out on 7.5–20% polyacrylamide gradient slab gels using the discontinuous buffer system of Laemmli (1970). Adenosine 5′-(β,γ-imidotriphosphate) (AMPPNP) was from Sigma Chemical Co.

Viscosity was measured by using the low-shear falling ball assay as described by MacLean-Fletcher and Pollard (1980). This procedure was validated for use with monomeric proteins using a Cannon-Ubbelohde viscometer (Ikebe et al., 1983).

^{1}H NMR spectra were recorded at 500 MHz on a Varian VXR 500 NMR spectrometer. A wide sweep width (±29 900 Hz or 120 ppm) was used to allow the broad component of the spectrum to be observed quantitatively; however, the full spectral width is generally not included in the figures in order that the narrow resonances may be seen in more detail. A relaxation delay of 5 s was left between pulses. No attempt was made to eliminate the residual HDO resonance as irradiation results in intensity changes due to the effects of spin diffusion (Akasaka et al., 1978). T_2 measurements were made by using a Carr–Purcell–Meiboom–Gill pulse train [see, for example, Harris (1983)], and the data were fitted to two or three exponential decays (as appropriate) by using a nonlinear

least-squares fitting routine. Intensity changes in the narrow component of the spectra were observed by using a spin-echo experiment identical with that used for the T_2 measurement, but with a single total refocusing time long enough to allow the broad component to decay. This value varied according to the size of the molecule; 1.6–2.4 ms was used for myosin, 8 ms for heavy meromyosin, and 12 ms for S1. The 10S to 6S transition was induced by increasing the ionic strength of the solution, which in turn affects the performance of the probe. 90° pulse lengths were determined at each salt concentration and were found to vary linearly between 15 μs (I = 0.2) and 20 μs (I = 0.5). Peak intensities were thus corrected by multiplying the absolute intensity at each salt increment by the fractional increase in pulse length; this is based on the principle of reciprocity, stated by Hoult (1976), that the performance of the NMR coil as both the receiver and the transmitter degrades equally as the ionic strength of the sample increases. The validity of this procedure was confirmed by the unchanging height of the methyl proton resonance of DSS in the corrected spectra. The fractional contribution of the broad and narrow components was determined from a comparison of the total integrated intensity of a normal spectrum (wide sweep width) with that of a spin-echo spectrum obtained after the broad component had been allowed to die away (Bloom et al., 1986). Correction for the T_2 values of the narrow peaks was made if necessary. Chemical shifts are reported with respect to the methyl resonance of DSS at 0 ppm (internal standard), and the sample temperature was 25 °C. All other details are reported in the figure legends.

Results

For these studies, the NMR spectra were recorded in D_2O and at relatively high protein concentrations (≥6 mg/mL). Previously, the 6S–10S transition was investigated in H_2O-based solvents and at 1–2 mg/mL [e.g., see Ikebe et al. (1983)]. To determine the influence of protein concentration and D_2O on the 6S–10S transition, the experiments shown in Figure 1 were carried out. The reduced viscosity (η_{sp}/c) was determined at different myosin concentrations (Figure 1A) for 6S myosin in 0.5 M KCl and for 10S myosin in 0.2 M KCl (other conditions given in the figure legend). Over the range of myosin concentrations used in the NMR experiments (3–16.6 mg/mL), the concentration dependence of viscosity was linear for both 6S and 10S myosin (Figure 1A). At low concentrations of myosin (1 mg/mL and less), there was a sharp increase in viscosity for 6S and 10S myosin. This effect is unusual and is not understood.

The effect of D_2O on the 6S–10S transition was determined by measuring relative viscosity over a range of ionic strengths in H_2O and D_2O solvents (Figure 1B). At the higher ionic strengths (>0.4 M KCl), myosin exists as 6 S and at the lower ionic strengths as 10 S. Clearly, the 6S–10S transition occurs in both solvents, although the midpoints of the transitions differ slightly (approximately 0.28 M KCl in H_2O and 0.34 M KCl in D_2O). The extent of this shift was independent of myosin concentration (over a concentration range suitable for NMR experiments).

The ^{1}H NMR spectrum of dephosphorylated smooth muscle myosin in the 6S state is shown in Figure 2B. The large size of the myosin molecule results in a very broad spectrum with intensity extending over a wide frequency range. Collection of such a spectrum requires the use of a much wider spectral width (120 ppm) than is normally used for ^{1}H spectra. The most noteworthy feature of the spectrum is the number of narrow lines that are seen to superimpose the broad resonance envelope. This means that a significant amount of internal

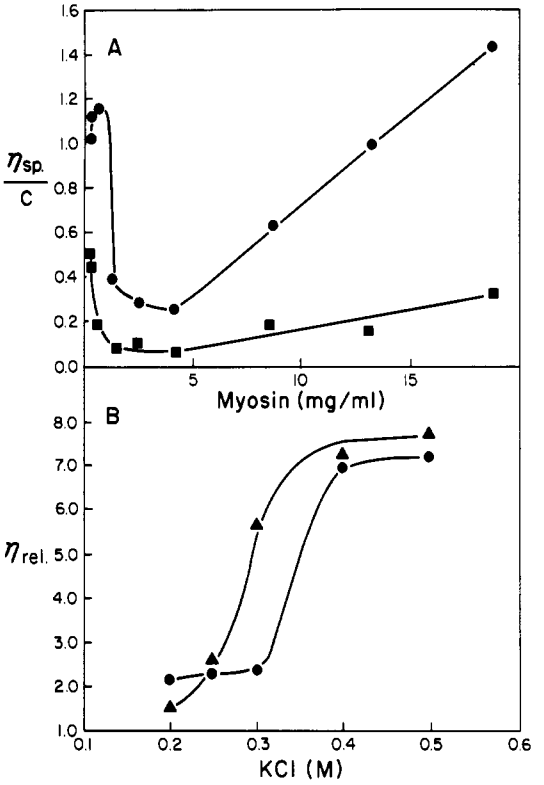

FIGURE 1: Viscosity measurements with gizzard myosin. (A) Reduced viscosity (η_{sp}/c; mL/g × 10^{-3}) versus myosin concentration. Conditions: 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM ATP, and 0.2 M KCl (■) or 0.5 M KCl (●). (B) Relative viscosity versus [KCl] in H_2O and D_2O solvents. Conditions: 30 mM potassium phosphate (pH 7.2), 10 mM $MgCl_2$, 0.2 mM dithiothreitol, 1 mM ATP in either H_2O (▲) or D_2O (●), and 9.8 (in H_2O) and 10.8 mg/mL myosin (in D_2O).

mobility exists within the smooth muscle myosin molecule and in this respect it appears very similar to skeletal muscle myosin (Highsmith et al., 1979). A version of the same spectrum, horizontally expanded along the frequency axis, shows the narrow lines in more detail (Figure 2A, upper trace). In order to follow the 6S–10S transition using NMR, we might look for changes in intensity in some or all of these resonance lines. A useful NMR technique, which allows the narrow resonances to be observed in isolation, is the spin-echo (described under Materials and Methods).

Selected time points from a T_2 experiment performed on 6S myosin are given in Figure 3. The data can be fitted to three exponential decays (several peaks are plotted in Figure 4A): most of the broad component is gone in less than 0.8 ms (the first time point), a second fast component decays with a T_2 of about 2 ms, and the narrow lines have T_2 values between 35 and 75 ms. These last values are remarkably long for a protein as large as myosin. By judicious selection of the delay time, τ, the spin-echo experiment may be used to observe the narrow resonances of the spectrum of myosin and its subfragments, selectively.

The spin-echo spectrum of dephosphorylated 6S myosin is also presented in Figure 2A (lower trace). A delay of only 2 ms was sufficient to remove the entire broad component; during this time, the decay of the narrow component was considered to be insignificant. Comparison of the spin-echo spectrum with that of Figure 2B shows the mobile residues to account for 4–5% of the total intensity. This corresponds to about 200 amino acid residues; therefore, each peak must be composed of many individual resonances. Most of these cannot be assigned reliably to residue types; however, the peak

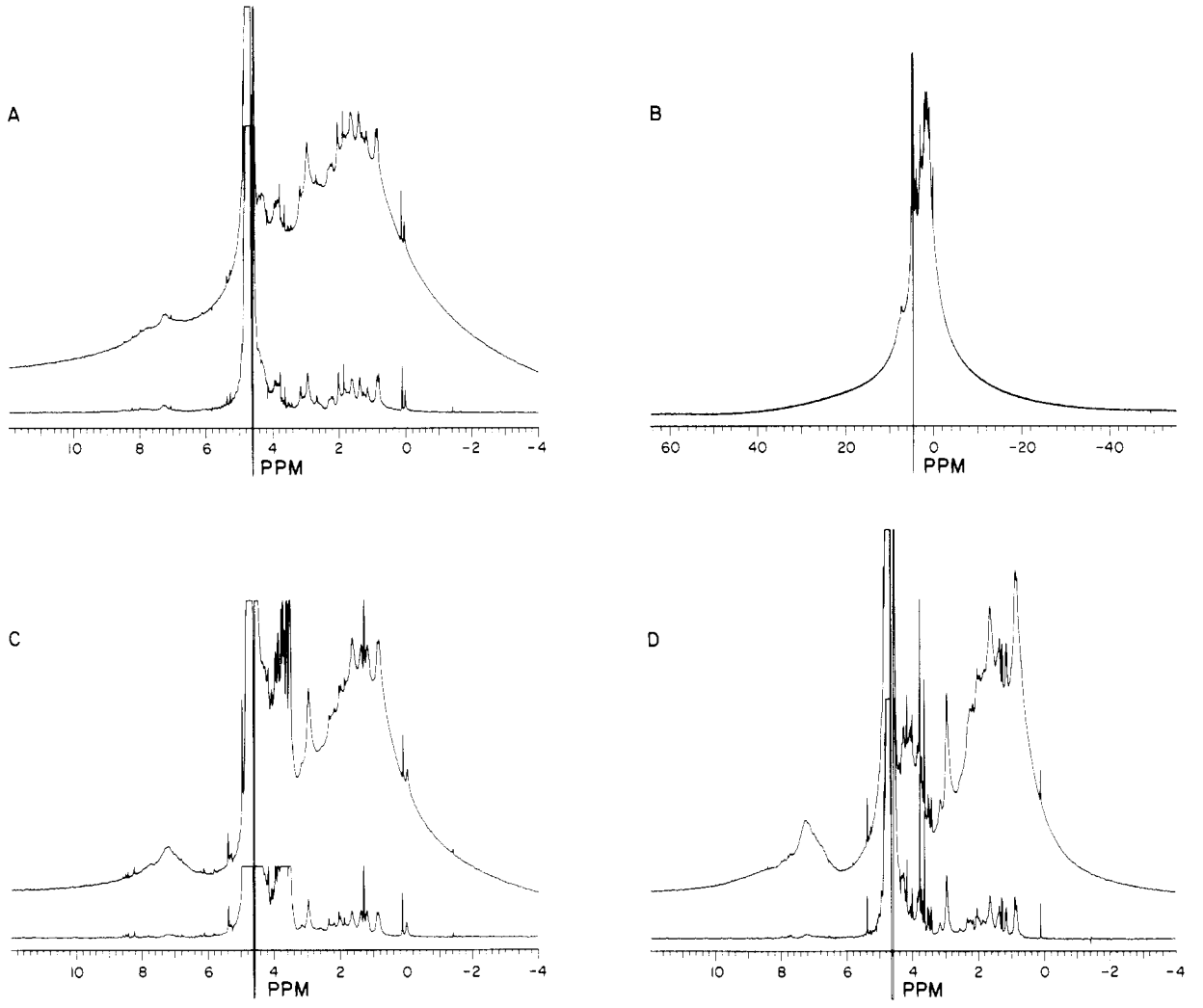

FIGURE 2: ^{1}H NMR spectra of gizzard myosin (A and B), gizzard HMM (C), and gizzard S1 (D). The lower spectra in panels A, C, and D are the CPMG T_2 spin-echo ^{1}H NMR signal for each protein; upper spectra in panels A, C, and D are expansions of wide sweep-width spectra. Vertical scales are constant. Panel B shows a broad sweep-width spectrum. Conditions for panels A and B: 16.6 mg/mL (35 μM) dephosphorylated myosin, 20 mM potassium phosphate (pH 7.5), 0.4 M KCl, and 5 mM $MgCl_2$; 256 transients. Conditions for panel C: 4.8 mg/mL (15.5 μM) dephosphorylated HMM, 31 μM AMPPNP; other conditions same as panels A and B. Conditions for panel D: dephosphorylated S1, 10.3 mg/mL (904 μM); 128 transients; other conditions same as panels A and B. Spectra were recorded at 25 °C, and a line broadening of 2 Hz was applied to all spectra. Residual HDO (not irradiated) appears in all spectra at 4.7 ppm.

at 0.9 ppm probably consists largely of aliphatic methyl group resonances (leucine, isoleucine, and valine), alanine methyls should contribute at 1.4 ppm, and the peak at 2.9 ppm would be expected to contain significant intensity from the lysine ϵ-CH_2. Intensity observed above 7 ppm comes from aromatic side chains. A statistical analysis of the ^{1}H chemical shifts of amino acid side chains in proteins was presented by Gross and Kalbitzer (1988).

While some of the line narrowing of these resonances can result from internal motions of the side chains, the very narrow line widths observed for these residues in the large myosin molecule imply backbone mobility as well. The effect of any given motion is to narrow the line width by a factor $[(3\cos^2\theta - 1)/2]^2$ (Marshall et al., 1972) where θ is the angle between the axis of internal motion and the internuclear vector between the spins. Thus, for a short-chain amino acid such as alanine, methyl group rotation will reduce the line width by a factor of $^1/_4$. This is not sufficient to account for the reduction in line widths observed in these studies. Further, side chain mobility is present in all proteins and not unique to myosin.

Spectra of the myosin subfragments HMM and S1, obtained under identical conditions, are presented in Figure 2C,D; however, since HMM is more difficult to prepare, the concentration is significantly lower, and the data are not of the quality achieved with myosin and S1. The narrow resonances are retained in both these sets of spectra. T_2 experiments were performed on both S1 and HMM (spectra not shown) to establish appropriate delays for the spin-echo spectra of Figure 2 (lower traces). Data obtained for various individual peaks yielded biphasic decays comprising a fast ($T_2 \approx 2$ ms for HMM) and a slow component (T_2 values of 35–75 ms). Longer refocusing delays (8 ms for HMM, 12 ms for myosin) were required to remove the broad component in the spin-echo spectra of Figure 2C,D. This is to be expected as the molecules are smaller. Two points are worthy of note. First, although small differences are apparent in comparison of the spin-echo spectra of myosin, HMM, and S1, it is clear that the great majority of the mobile residues reside in the S1 subunit or myosin head, as is the case for skeletal muscle myosin (Highsmith et al., 1979). Second, the T_2 of the narrow lines is thus independent of the overall size of the molecule, suggesting rapid local motion, independent of the overall tumbling of the molecule as a whole.

T_2 measurements at high and low salt concentrations were compared (Figure 4). The observed independence of T_2 values of the narrow resonances upon salt concentration strongly

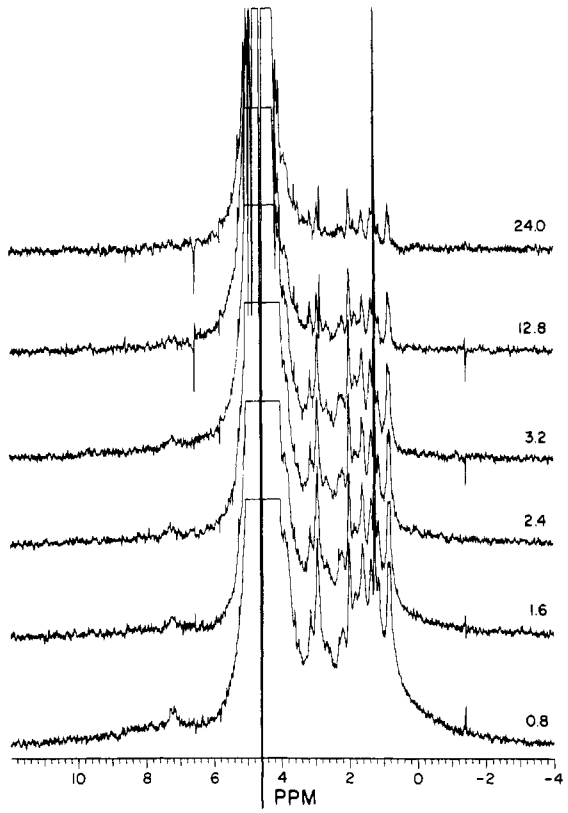

FIGURE 3: T_2 relaxation of the ^{1}H NMR signal of gizzard myosin in a CPMG T_2 experiment. Conditions: dephosphorylated myosin, 15.6 mg/mL (33 μM); absence of AMPPNP; other conditions as in Figure 2A,B. Spectra acquired with total relaxation delay times ranging from 0.8 to 24.0 ms (as indicated). Resonance at 1.3 ppm is an impurity.

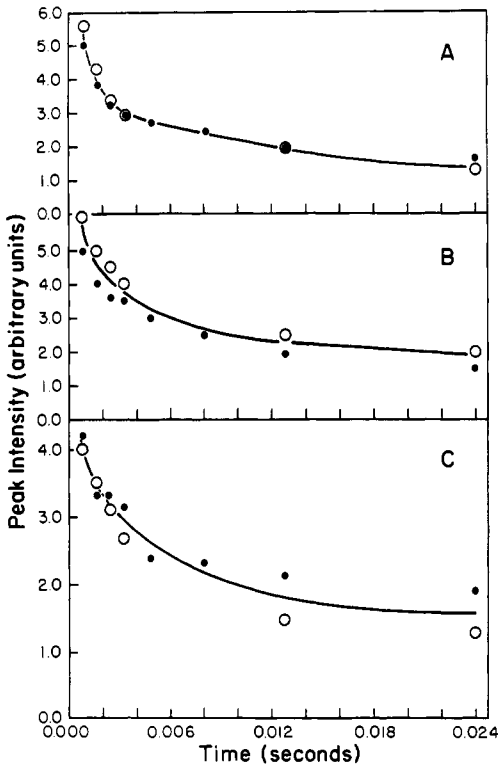

FIGURE 4: Comparison of T_2 relaxations for selected resonances of dephosphorylated myosin in 0.2 M (O) and 0.4 M KCl (●). Intensities of selected resonances at 0.9 (A), 2.0 (B), and 3.2 ppm (C) were measured and plotted versus the total delay time, τ. T_2 values were estimated by best fit to a double-exponential function of the type intensity = $Ae^{-k_1 t} + Be^{-k_2 t}$, where $k = 1/T_2$, and were (A) 1.04 and 35 ms, (B) 2.4 and 52 ms, and (C) 2.7 and 75 ms.

suggests that an increased number of resonances contribute to the narrow component at higher ionic strengths (as opposed to existing lines becoming narrower).

The effects of the 6S–10S transition on the mobile component of myosin and its subfragments were examined by recording spin-echo spectra at a fixed delay time, τ, at different ionic strengths. The results obtained with dephosphorylated myosin in the absence of nucleotide are shown in Figure 5A. As the KCl concentration was increased from 0.2 M (lower trace) to 0.5 M (upper trace), the intensities of many peaks increased. As suggested earlier (Ikebe et al., 1983), gizzard myosin in the absence of nucleotide still undergoes a salt-dependent conformational change. The spectra shown in Figure 5A reflect this change and suggest an increase in the number of mobile residues as ionic strength is increased. In the presence of AMPPNP, the dephosphorylated (Figure 5B) and thiophosphorylated (Figure 5C) myosins also showed an increase in several intensities as the KCl concentration was increased. With dephosphorylated myosin, the two spectra represent the 10S (at 0.15 M KCl) and 6S (at 0.5 M KCl) states, and thus the increased intensity of several resonances reflects this conformational transition. At the two ionic strengths used in Figure 5C (i.e., 0.15 and 0.5 M KCl), thiophosphorylated myosin exists as 6 S as defined by viscosity measurements (Ikebe et al. 1983). Differences in the spectra of these 6S myosin samples indicate that different conformations may be allowed within the hydrodynamic definition of the 6S conformation. In addition, comparison of the spectra shown in Figure 5A,B with those of Figure 5C suggested that thiophosphorylation of 6S myosin induces an alteration in conformation. For example, the resonances at 2.0 and 3.2 ppm are less prominent in thiophosphorylated 6S myosin compared to dephosphorylated 6S myosin.

The addition of nucleotide, as AMPPNP, did not affect the smooth muscle myosin spectra directly. This was also shown for skeletal muscle myosin (Highsmith et al., 1979); nucleotides have been shown to bind to a rigid site in the molecule (Shriver & Sykes, 1981).

A similar sequence of spectra is presented in Figure 6 for HMM. Figure 6A shows spin-echo spectra of dephosphorylated HMM in 0.15 M KCl (lower spectrum) to 0.45 M KCl (upper spectrum). Increasing ionic strength increased the intensity of several resonances although this was not as marked for HMM as for myosin. The aromatic region (around 7.2 ppm) did not show a clear ionic strength dependent increase; the signal to noise ratio for the aromatic resonances is much less than for the aliphatic resonances because they are fewer in number. Spin-echo spectra of dephosphorylated and thiophosphorylated HMM in the presence of AMPPNP are shown in panels B and C, respectively, of Figure 6. In general, the increase in segmental flexibility (on raising the KCl concentration) was greater for the thiophosphorylated HMM. Differences also were observed between dephosphorylated and thiophosphorylated HMM (i.e., at constant ionic strength). For example, the resonances at 0.9, 1.9, and 2.0 ppm were more prominent with the thiophosphorylated sample. The resonance at 3.2 ppm was small with both sets of spectra and did not increase significantly on raising ionic strength.

Spin-echo spectra of S1 are shown in Figure 7, using a similar experimental format as in the two previous figures (see figure legends for conditions). The important point from these data is that the S1 resonances do not show any obvious change with these experimental variables. Neither ionic strength alterations nor thiophosphorylation induced conformational changes that were detectable with this technique.

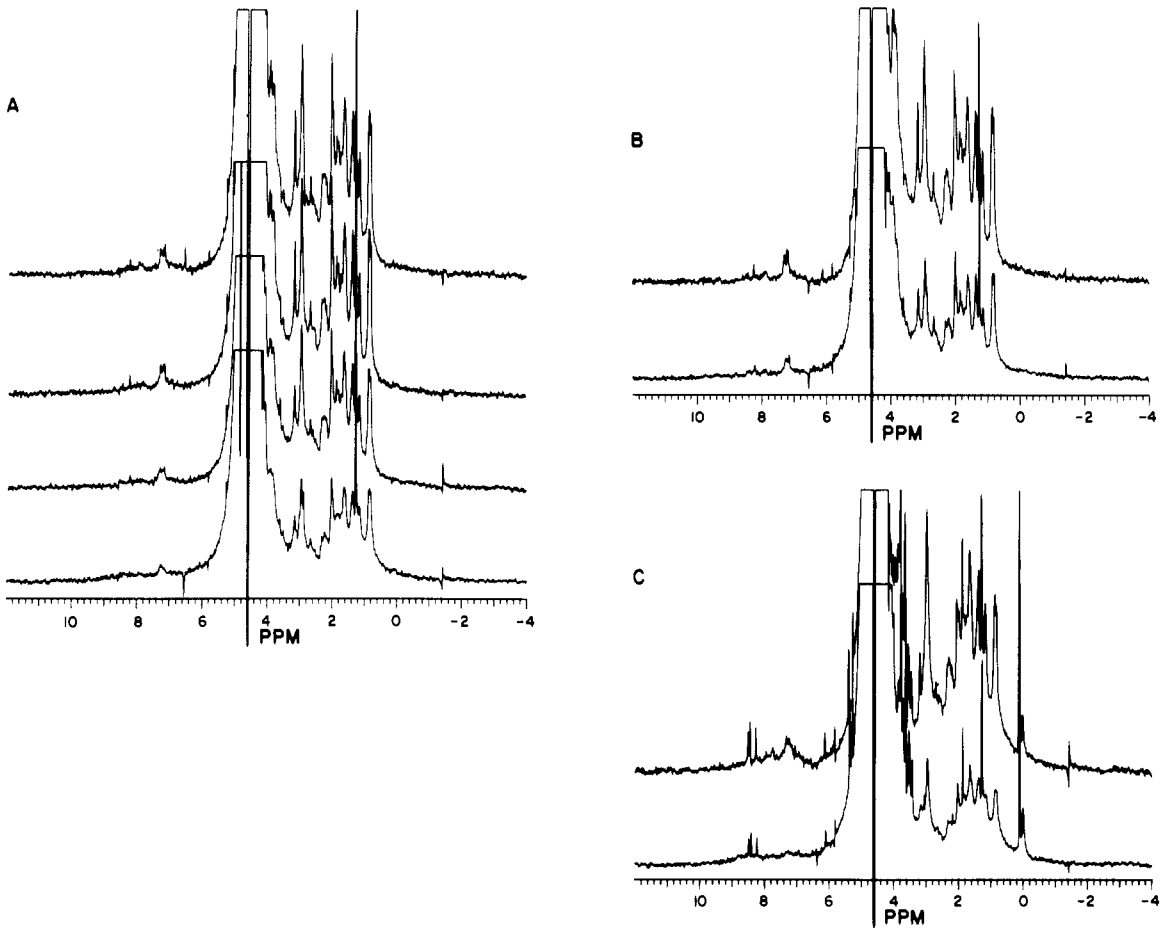

FIGURE 5: Spin-echo spectra of gizzard myosin. (A) Dephosphorylated myosin minus AMPPNP in different KCl concentrations: 0.2 M (lower); 0.3, 0.4, and 0.5 M (upper). (B) Dephosphorylated myosin plus 70 μM AMPPNP in 0.15 M KCl (lower) and 0.5 M KCl (upper). (C) Thiophosphorylated myosin plus 30 μM AMPPNP in 0.15 M KCl (lower) and 0.5 M KCl (upper). [Myosin], in panels A and B, 15.6 mg/mL (33 μM); panel C, 6.7 mg/mL (14 μM); 1024 transients. The line broadening in all spectra was 5 Hz. The vertical scale was corrected for the effect of salt on the transmitter/receiver coil (Materials and Methods), leading to an increase in the height of the noise as the titration progressed. Two of the narrow resonances around 8.4 ppm correspond to excess AMPPNP. In these spectra, and those of Figures 6 and 7, the heights of the AMPPNP resonances appear to vary. This is due to the very short acquisition times, and concomitant poor digital resolution, for these wide sweep-width spectra so that very narrow lines such as those for free AMPPNP vary in apparent width. The resonance intensities (i.e., areas) are correct. In addition, there is sometimes a contaminant resonance from formate ion near 8.45 ppm. Other conditions as in Figure 2.

Certain resonances from the spin-echo spectra were collected and their heights plotted as a function of KCl concentration. As shown in Figure 8A, the ionic strength dependent increase was most marked for thiophosphorylated myosin (using resonances at 0.9, 2.0, 3.0, and 3.2 ppm). These data suggest that as the ionic strength is increased a region of the molecule becomes more mobile. The midpoint of this transition is about 0.25 M KCl. Thiophosphorylated myosin exists over this range of ionic strengths as the 6S conformation (Ikebe et al., 1983). Thus, it is apparent that within the operational definition of the 6S state different conformations are found. With thiophosphorylated HMM (figure 8B), the changes (illustrated for three peaks) were not as abrupt as with myosin, but a similar trend was indicated, namely, an increased mobility at higher ionic strengths. Since the end points of these transitions were not established, the midpoints cannot be determined, but it is likely that the ionic strength range over which the HMM transitions occurred is similar to that for myosin.

With thiophosphorylated S1, there was no effect of ionic strength on any peaks; four peaks are selected to illustrate this (Figure 8C). The same resonances are detected in S1 as in HMM and myosin. It is likely, therefore, that the resonances monitored in myosin and HMM originate in the S1 parts of the molecule (including the light chains). It is important to emphasize that the changes observed for myosin and HMM reflect changes in the S1 unit only when these are incorporated into the more complex structures.

For dephosphorylated myosin, the peak heights of selected resonances from spin-echo spectra in the presence (Figure 9A) and absence of AMPPNP (Figure 9B) are plotted against KCl concentration. (This allows comparison with the 6S–10S transition shown in Figure 1.) In both cases, the peak heights increase as the ionic strength is raised, although the data obtained in the absence of AMPPNP show a more marked transition. The midpoint of these transitions is about 0.32 M KCl (i.e., similar to the midpoint obtained from the viscosity measurements of dephosphorylated myosin in Figure 1). Thus, it appears likely that the changes of these resonances occur during the transition from 10S to 6S myosin. These data are consistent with the suggestion that segmental flexibility is greater in the 6S state obtained at high ionic strength. The change of the resonances in the presence of AMPPNP (Figure 9A) suggested that the underlying conformational transition is not as extensive as in the absence of AMPPNP.

Discussion

The definition of 6S or 10S is based on sedimentation velocity, and the 6S–10S transition is usually monitored via changes in hydrodynamic properties. This conformational change is dependent on ionic strength (as shown in Figure 1),

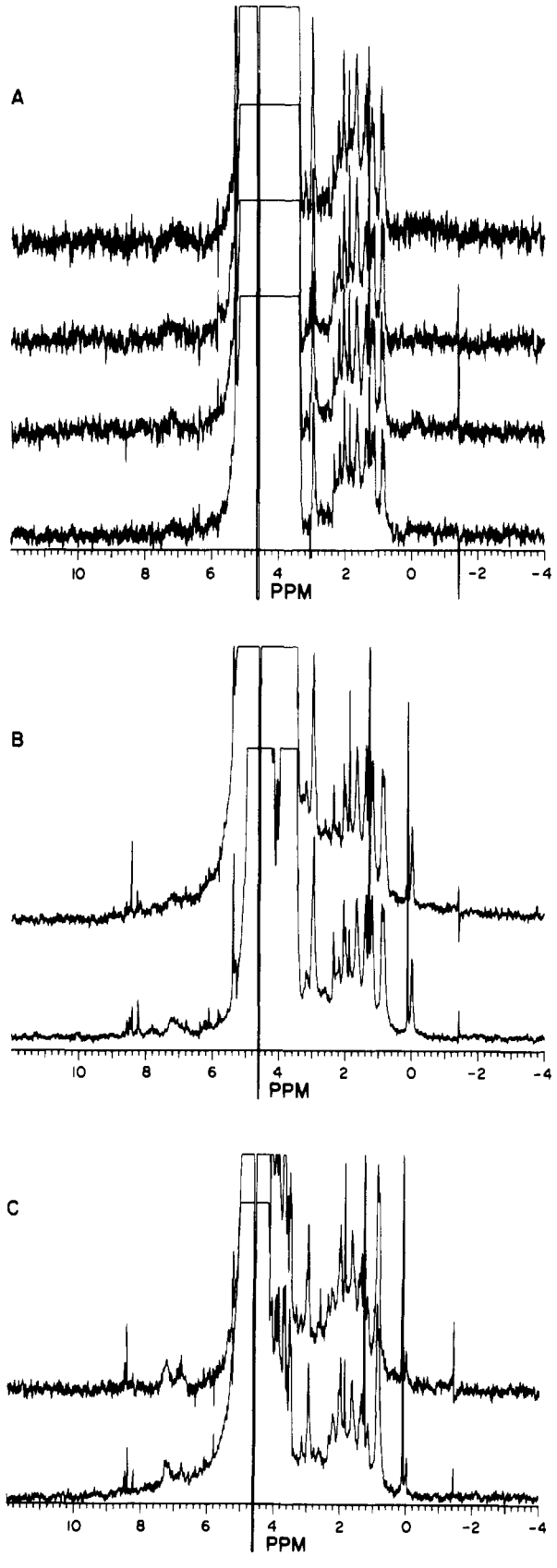

FIGURE 6: Spin-echo spectra of gizzard HMM. (A) Dephosphorylated HMM minus AMPPNP in different KCl concentrations: 0.15 M (lower); 0.25, 0.35, and 0.45 M (upper). (B) Dephosphorylated HMM plus 26 μM AMPPNP in 0.15 M KCl (lower) and 0.45 M KCl (upper). (C) Thiophosphorylated HMM plus 20 μM AMPPNP in 0.15 M KCl (lower) and 0.5 M KCl (upper). [HMM], in (A) 2.5 mg/mL (8.1 μM); in (B), 4.0 mg/mL (12.9 μM); in (C), 3.1 mg/mL (10 μM). The line broadening in all spectra was 5 Hz. Other conditions as in Figure 2.

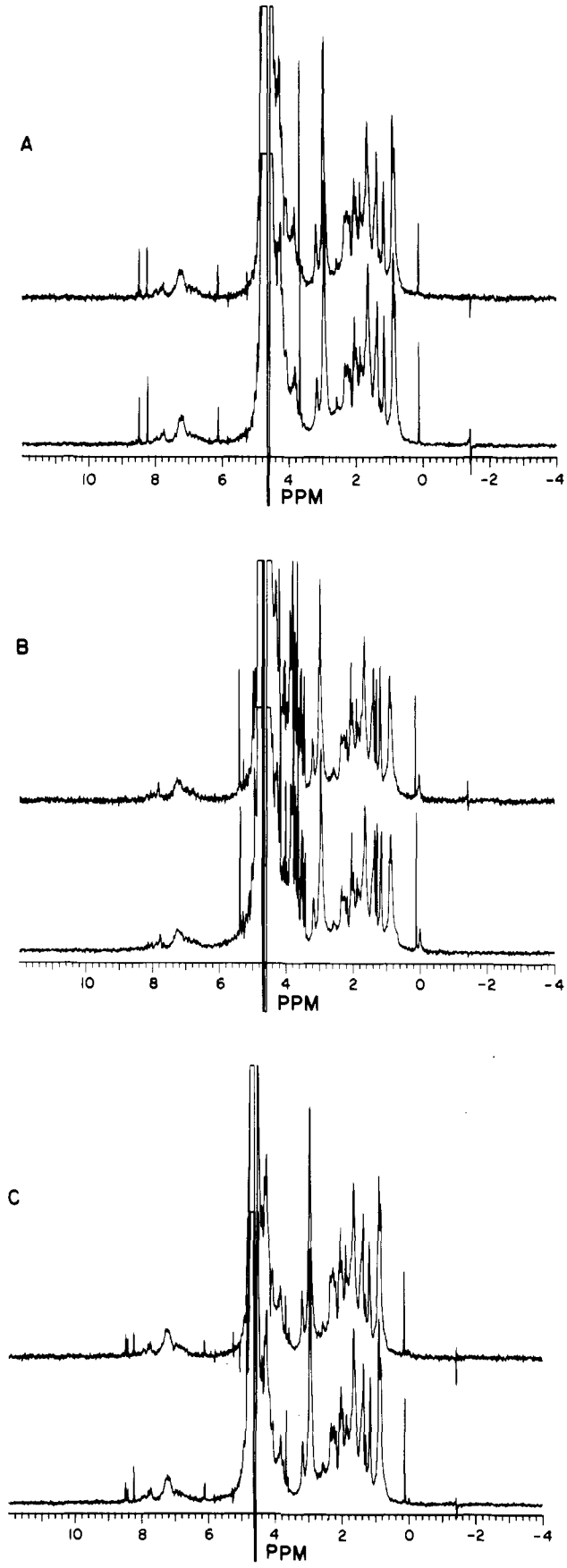

FIGURE 7: Spin-echo spectra of gizzard S1. (A) Dephosphorylated S1 plus 100 μM AMPPNP in 0.15 M KCl (lower) and 0.45 M KCl (upper). (B) Dephosphorylated S1 minus AMPPNP in 0.2 M KCl (lower) and 0.5 M KCl (upper). (C) Thiophosphorylated S1 plus 100 μM AMPPNP in 0.15 M KCl (lower) and 0.45 M KCl (upper). The line broadening in all spectra was 2 Hz; 128 transients were averaged. Other conditions as in Figure 2.

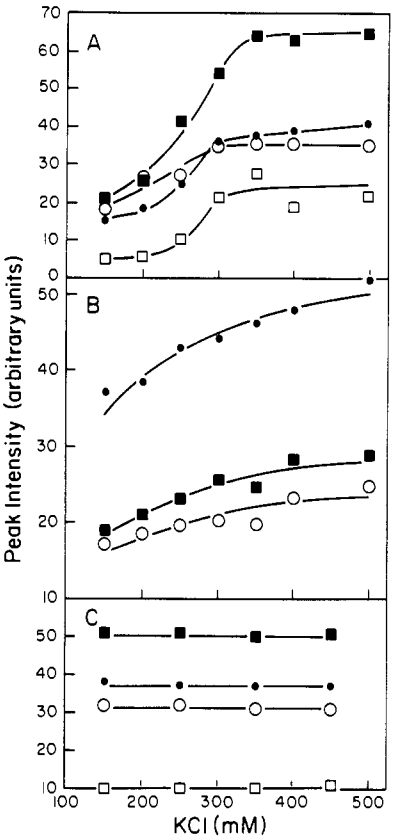

FIGURE 8: [KCl] dependence of various resonances for thiophosphorylated myosin (A), thiophosphorylated HMM (B), and thiophosphorylated S1 (C). The selected resonances were centered around 0.9 (●), 2.0 (○), 3.0 (■), and 3.2 ppm (□). The height of each resonance was measured from corrected spin-echo spectra determined at different KCl concentrations (for example, as in Figures 5C, 6C, and 7C).

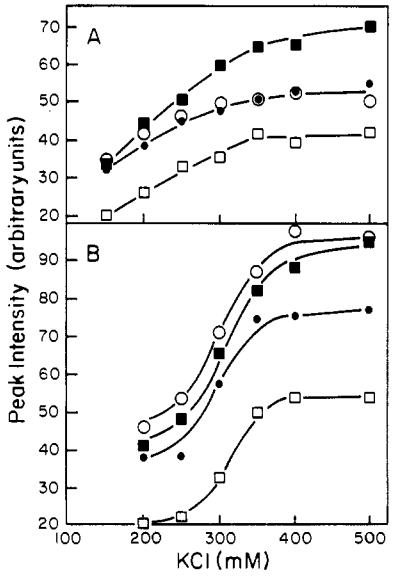

FIGURE 9: [KCl] dependence of various resonances of dephosphorylated myosin in the presence (A) and absence of AMPPNP (B). Resonances as indicated in Figure 8. [AMPPNP], 70 μM.

the presence or absence of ATP, and the phosphorylation state of the 20-kDa light chains. In this study, spin-echo ^{1}H NMR showed that the number of residues contributing to the mobile region(s) of turkey gizzard myosin increased with ionic strength. Changes were also observed with HMM but not with S1. The narrow resonances originate in the S1 units, as shown also for skeletal muscle myosin (Highsmith et al., 1979; Ribeiro et al., 1984), and account for about 5% of the total ^{1}H resonances in smooth muscle 6S myosin. Thus, localized changes in the myosin heads can be detected as part of the 6S–10S transition in addition to the more obvious changes in hydrodynamic parameters. Since these changes are not observed in the isolated S1 units, additional structure is required to allow the conformational transitions.

At the concentrations of myosin necessary for the NMR experiments, there is clearly the possibility of aggregation, and this could influence the spin-echo ^{1}H NMR signals. While this cannot be entirely eliminated, it is considered that myosin aggregation, or polymerization, is unlikely to be a dominant factor in the observed ionic strength dependence of the spin-echo results for the following reasons: (1) Aggregation is not detected by viscosity measurements, and the profile of the 6S–10S transition is independent of myosin concentration. (2) If aggregation did dominate the spin-echo ^{1}H NMR spectra of myosin, one would expect to detect a concentration dependence (over the range of 3–16 mg/mL) of the observed signals. This was not the case. (3) HMM, a soluble subfragment of myosin, also exhibited increased intensity of several resonances with increasing ionic strength (see Figures 6 and 7B). (4) For dephosphorylated gizzard myosin, Kendrick-Jones et al. (1987) showed that at 0.2 M NaCl myosin was almost completely soluble, both in the absence and in the presence of ATP. The change in peak intensities occurs above an ionic strength of 0.2 (see Figure 9), i.e., under conditions where myosin is predominantly monomeric.

One explanation for the observed changes is that they are due, in part, to the 6S–10S transition. For example, the intensity changes found with dephosphorylated myosin as a function of KCl concentration (Figure 9B) closely follow the 6S–10S conformational transition. But changes can also be observed for a given conformation as a result of thiophosphorylation. As shown in Figure 5, the spectra for thiophosphorylated myosin at 0.2 and 0.4 M KCl (both operationally defined as 6S myosin) are different from the spectrum of dephosphorylated myosin at 0.4 M KCl, also in the 6S state. Thus, some additional descriptor is required, particularly for the thiophosphorylated 6S myosin at the lower ionic strengths. This species, 6S*, showed less segmental flexibility than either conventional 6S or 10S myosins. In this regard, it is interesting that the spin-echo ^{1}H NMR spectrum of 6S thiophosphorylated myosin showed less intensity at three resonance positions, compared to 6S dephosphorylated myosin.

Several different types of motion are thought to occur within the myosin molecule. A "hinge" has been proposed to exist at the LMM–HMM junction, and the myosin heads themselves are believed to be flexibly attached [reviewed by Harvey and Cheung (1981)]. The NMR experiment is sensitive to certain frequencies of motion, i.e., those that occur with a time period shorter than the overall rotational correlation time. It is clear that the majority of the flexible segments observed in myosin by ^{1}H NMR occur within the head region (Highsmith et al., 1979; Stewart & Roberts, 1982) and, in this respect, smooth muscle myosin appears to be similar to the myosin of skeletal muscle. However, some differences in the relative peak intensities are observed when the spectra of myosin and its subfragments are compared, and it is possible that some of the mobile component of smooth muscle originates elsewhere in the molecule. The location of the mobile component is obviously of interest; apart from their probable involvement in the 6S–10S transition, the mobile residues in skeletal muscle myosin are known to be largely immobilized upon binding actin (Highsmith et al., 1979; Prince et al., 1981). In our experi-

ments, the mobile component contributes about 5% of the total intensity, corresponding to about 100 residues in each myosin head. This is about half the number estimated by Highsmith et al. (1979). However, it was shown for skeletal myosin that the essential Al light chain possesses a 40-residue N-terminal extension which is highly mobile and contributes significantly to the narrow component of the ^{1}H NMR spectrum (Prince et al., 1981; Henry et al., 1985). No such extension exists in the smooth muscle essential light chain (Matsuda et al., 1981), and this might account for some of the discrepancy. A mobile region (about 20 residues) has been demonstrated at the N-terminus of isolated gizzard regulatory light chain (with or without phosphorylation), and ^{31}P NMR suggests the phosphorylated serine residue to be mobile in myosin itself (Levine et al., 1988). It is interesting to note that another large actin binding protein, spectrin, has been shown to possess an unexpectedly large number of narrow resonances, amounting to almost 15% of the total intensity (Fung et al., 1989).

Movement of domains within the myosin head has also been suggested [see Vibert and Cohen (1988)], and it is tempting to speculate that mobile residues may be in some way involved with the "domain" structure. Independent motion can sometimes be associated with an entire domain in a molecule, for example, the kringle domain of urokinase (Oswald et al., 1989), but the number of residues available in myosin appears to be too small for this. Furthermore, line widths of the isolated 20-kDa domain–essential light chain complex of skeletal muscle are not as narrow as those of intact myosin (Chaussepied et al., 1986). It is possible that a flexible linker, or linkers, exists which connects the domains of the myosin head and results in narrow lines. (S1 is composed of three domains: the N-terminal 25 kDa, a central 50 kDa, and the C-terminal 20 kDa. The 20-kDa domain is attached to S2.) The primary structures of the 25–50-kDa, the 20–50-kDa, and the 20-kDa to S2 linker regions are rich in amino acids that could contribute to these mobile residues (Yanagisawa et al., 1987). On the basis of the sequence of the gizzard myosin heavy chain (Yanagisawa et al., 1989), there are several other areas of interest, including the lysine-rich putative actin binding site (lysine-568 to lysine-578 in the 50-kDa domain and lysine-639 to lysine-652 near the 20–50-kDa linker region), the glycine-rich region in the 25-kDa domain (glycine-177 to glycine-182), the trimethyllysine at position 128 (possibly involved in binding the pyrophosphate group of ATP), and the aromatic amino acid rich putative S1 to S2 hinge region (residues 838–849).

From the above data, it is evident that smooth muscle myosin, like its skeletal muscle counterpart, contains regions of independent local motion, probably associated with the two S1 units, or myosin heads. However, the myosins from the two tissues differ in that smooth muscle myosin can undergo the 10S–6S transition and this in turn influences resonance intensities. In the 10S folded state, the myosin heads appear to be oriented back toward the S2–LMM region (Suzuki et al., 1982; Craig et al., 1983). The NMR data suggest that part of the myosin head is restricted in motional freedom in the folded state and assumes a more mobile character upon formation of the extended 6S conformation. The decrease in mobility in the 10S state may be due to direct immobilization of a site, or sites, in the head region via its interaction with the myosin tail. Alternatively, the heads themselves may assume a restricted configuration and indirectly form a binding site for the tail. The latter possibility is supported by experiments with HMM, which although lacking LMM still undergoes a conformational transition analogous to 6S–10S (Suzuki et al., 1985). The increase in the number of narrow resonances in the ^{1}H NMR spectra of HMM with increasing ionic strength also mimics the changes observed with myosin. In addition to the changes observed during the 10S–6S transition, certain of the heavy-chain resonances also are altered by phosphorylation of the myosin light chains. It is important to emphasize that although the same resonances are detected in the isolated S1 units, these resonances are not modified by changes in either ionic strength or phosphorylation. Thus, although the S1 units display independent motion, the ionic strength dependent or phosphorylation-dependent alteration of such requires an interaction between the two heads or an S1–S2 interaction. Regulation of myosin ATPase activity via phosphorylation also requires the same interactions, and thus it is possible that the structural changes detected by NMR form part of the contractile process in smooth muscle.

References

Akasaka, K., Konrad, M., & Goody, R. S. (1978) *FEBS Lett. 96*, 287–290.

Bloom, M., Holmes, K. T., Mountford, C. E., & Williams, P. G. (1986) *J. Magn. Reson. 68*, 73–91.

Craig, R., Smith, R., & Kendrick-Jones, J. (1983) *Nature 302*, 436–439.

Fung, L. W.-M., Lu, H.-Z., Hjelm, R. P., & Johnson, M. E. (1989) *Life Sci. 44*, 735–740.

Gross, K.-H., & Kalbitzer, H. R. (1988) *J. Magn. Reson. 76*, 87–99.

Harris, R. K. (1983) in *Nuclear Magnetic Resonance Spectroscopy: a Physicochemical View*, pp 82–84, Pitman Books Ltd., London.

Hartshorne, D. J. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., Ed.) Vol. 1, 2nd ed., pp 423–482, Raven Press, New York.

Harvey, S. C., & Cheung, H. C. (1981) *Cell Muscle Motil. 2*, 279–302.

Henry, G. D., Trayer, I. P., Brewer, S., & Levine, B. A. (1985) *Eur. J. Biochem. 148*, 75–82.

Highsmith, S., & Jardetzky, O. (1981) *Biochemistry 20*, 780–783.

Highsmith, S., Akasaka, K., Konrad, M., Goody, R., Holmes, K., Wade-Jardetzky, N., & Jardetzky, O. (1979) *Biochemistry 18*, 4228–4244.

Hoult, R. (1976) *J. Magn. Reson. 24*, 71–85.

Ikebe, M., & Hartshorne, D. J. (1985a) *J. Biol. Chem. 260*, 13146–13153.

Ikebe, M., & Hartshorne, D. J. (1985b) *Biochemistry 24*, 2380–2387.

Ikebe, M., Hinkins, S., & Hartshorne, D. J. (1983) *Biochemistry 22*, 4580–4587.

Ikebe, M., Stepinska, M., Kemp, B. E., Means, A. R., & Hartshorne, D. J. (1987) *J. Biol. Chem. 260*, 13828–13834.

Ikebe, M., Koretz, J., & Hartshorne, D. J. (1988) *J. Biol. Chem. 263*, 6432–6437.

Kendrick-Jones, J., Smith, R. C., Craig, R., & Citi, S. (1987) *J. Mol. Biol. 198*, 241–252.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Levine, B. A., Griffiths, H. S., Patchell, V. B., & Perry, S. V. (1988) *Biochem. J. 254*, 277–286.

MacLean-Fletcher, S. D., & Pollard, T. D. (1980) *J. Cell Biol. 85*, 414–428.

Marshall, A. G., Schmidt, P. G., & Sykes, B. D. (1972) *Biochemistry 11*, 3875–3879.

Matsuda, G., Maita, T., Kato, Y., Chen, J.-I., & Umegane, T. (1981) *FEBS Lett. 135*, 232–236.

Onishi, H., Wakabayashi, T., Kamata, T., & Watanabe, S.

(1983) *J. Biochem. (Tokyo) 94*, 1147–1154.
Oswald, R. E., Bogusky, M. J., Bamberger, M., Smith, R. A. G., & Dobson, C. M. (1989) *Nature 337*, 579–582.
Perrie, W. T., & Perry, S. V. (1970) *Biochem. J. 119*, 31–38.
Prince, H. P., Trayer, H. R., Henry, G. D., Trayer, I. P., Dalgarno, D. C., Levine, B. A., Cary, P. D., & Turner, C. (1981) *Eur. J. Biochem. 121*, 213–219.
Ribeiro, A., Parello, J., & Jardetzky, O. (1984) *Prog. Biophys. Mol. Biol. 43*, 95–160.
Shriver, J. W., & Sykes, B. D. (1981) *Biochemistry 20*, 2004–2012.
Stewart, M., & Roberts, G. C. K. (1982) *FEBS Lett. 146*, 293–296.
Suzuki, H., Kamata, T., Onishi, H., & Watanabe, S. (1982) *J. Biochem. (Tokyo) 91*, 1699–1705.
Suzuki, H., Stafford, W. F., III, Slayter, H. S., & Seidel, J. C. (1985) *J. Biol. Chem. 260*, 14810–14817.
Trybus, K. M., & Lowey, S. (1984) *J. Biol. Chem. 259*, 8564–8571.
Vibert, P., & Cohen, C. (1988) *J. Muscle Res. Cell Motil. 9*, 296–305.
Walsh, M. P., Hinkins, S., Dabrowska, R., & Hartshorne, D. J. (1983) *Methods Enzymol. 99*, 279–288.
Yanagisawa, M., Hamada, Y., Katsuragawa, Y., Imamura, M., Mikawa, T., & Masaki, T. (1987) *J. Mol. Biol. 198*, 143–157.

CORRECTIONS

Structure, Multiple Site Binding, and Segmental Accommodation in Thymidylate Synthase on Binding dUMP and an Anti-Folate, by William R. Montfort, Kathy M. Perry, Eric B. Fauman, Janet S. Finer-Moore, Gladys F. Maley, Larry Hardy, Frank Maley, and Robert M. Stroud*, Volume 29, Number 30, July 31, 1990, pages 6964–6977.

Omission was made of the credits for the software used in the illustrations for Figures 3, 5, and 14 which were created by Julie Newdoll using MIDAS+ software written by Conrad Huang, Eric Petterson, and Greg Couch at the UCSF Computer Graphics Laboratory.

Fluorescence and NMR Investigations on the Ligand Binding Properties of Adenylate Kinases, by Jochen Reinstein,* Ingrid R. Vetter, Ilme Schlichting, Paul Rösch, Alfred Wittinghofer, and Roger S. Goody, Volume 29, Number 32, August 14, 1990, pages 7440–7450.

Page 7442. In eq 1, K_{EF} should read K_{EM}.

Page 7443. In the last equation in column 2, T_3 should read T_o.

Page 7447. In column 1, line 21, AP1A should read AP6A. In column 1, line 27, APkA should read AP5A. In column 1, line 29, Mg·APa5A should read Mg·AP5A. In column 2, line 4, Mg·APkA should read Mg·AP5A.