# Internal Motions in Myosin†

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ABSTRACT: High-resolution proton nuclear magnetic resonance ($^1$H NMR) measurements were made on myosin, heavy meromyosin (HMM), myosin subfragment 1 (S1), light meromyosin (LMM), and actin. A strong signal from amino acid side chains undergoing motions too fast to be accounted for by simple rotations of groups on a rigid backbone was obtained from myosin. Comparison of myosin, HMM, S1, and LMM showed that the mobile region is located almost entirely in S1 and accounts for ~22% of its structure. Adenosine triphosphate (ATP) and ATP analogues had no measurable effect on the S1 spectrum. Actin, on the other hand, quenched the internal motions of S1. When S1 was titrated with actin, an association constant was obtained which was in agreement with other measured values. The actin effect was reversed by adding magnesium pyrophosphate ($MgPP_i$) or adenyl-5′-yl imidophosphate (MgAMPPNP). Quantitative treatment of the broad signals from myosin and its subfragments substantiated the existence of two flexible regions in myosin. The highly mobile portion of myosin may be located in the "swivel" between S1 and the rest of myosin or in the actin binding site or in both. These possibilities are discussed, and a new possible mechanism for muscle cross bridge elasticity is proposed.

In the current models of muscular contraction (Huxley, 1969; Huxley & Simmons, 1971; Lymn & Taylor, 1971; Eisenberg & Hill, 1978), the mechanical cycle consists of the attachment of cross bridges from the thick filament to the thin filament, reorientation of the attached cross bridges which propel the filaments by each other, and finally their dissociation to allow the cycle to be repeated. It is known that the cross bridge is part of the myosin molecule and that part of it rotates through as much as 45° with respect to the actin of the thin filament (Reedy et al., 1965). The interaction at the actomyosin interface actually produces the force generated from ATP[1] hydrolysis (Nihei et al., 1974) and rotates the cross bridge. This reorientation requires that the cross bridge provide a flexible linkage between the thick and thin filament (Huxley, 1969). Flexible regions in myosin were first inferred from its proteolytic susceptibility (Lowey, 1971; Harrington et al., 1959), since it is generally assumed that flexibility resulting from a less compact structure is required to permit the selective proteolysis which leaves intact large domains of the protein structure. Figure 1 is a scale drawing of myosin which shows its proteolytically produced fragments and the suspected flexible regions. Various physical techniques have been used to demonstrate that the hydrodynamic mobilities of myosin and its fragments are far too great for myosin to be rigid: a "swivel" has been shown to exist between S1 and the remaining rod (LMM + S2) (Mendelson et al., 1973; Thomas et al., 1975; Kobayashi & Totsuka, 1975), and a "hinge" has been shown to exist between LMM and S2 (Highsmith et al., 1977). Recent electron micrographs show myosin structures which are compatible with flexibility at these two sites (Elliot & Offer, 1978).

High-resolution NMR provides information on internal motion in proteins (Abragam, 1961; Jardetzky, 1964). It has recently been shown that a quantitative estimate of the fraction of the total structure which contributes to the motion can also be obtained (King et al., 1978). In the present study, the technique was used to determine the fraction and location of amino acid side chains showing unusually high mobility in myosin, HMM, S1, LMM, and actin. The effects of complex formation between S1 and ATP, AMPPNP, $PP_i$, and actin were also investigated.

Table I

| protein | $M_r \times 10^{-3}$ | $\epsilon_{280}{}^{1\%}$ | CaATPase [mol of OH/(g s)] |
|---|---|---|---|
| myosin | 450 | 0.570 | 4.3 |
| HMM | 320 | 0.647 | 5.6 |
| S1 | 100 | 0.770 | 7.6 |
| LMM | 130 | 0.200 | |
| actin | 42 | 1.16 | |

## Experimental Section

*Materials.* Myosin was isolated from the dorsal muscle of New Zealand rabbit by the method of Nauss et al. (1969) and purified by $(NH_4)_2SO_4$ fractionation. HMM was prepared from myosin by modified trypsin cleavage, as described earlier (Highsmith, 1978). S1 was prepared by the method of Weeds & Taylor (1975), purified as a mixture of S1 isozymes by G-200 chromatography eluting with 0.15 M KCl and 0.01 M Tes, pH 7.0, or by $(NH_4)_2SO_4$ fractionation, and resolved into S1 fractions containing only alkali light chain 1 or 2 (S1A1 and S1A2) by ion-exchange chromatography (Weeds & Taylor, 1975). LMM was made from myosin, as described by Lowey et al. (1969). Pure F-actin was prepared according to Spudich & Watt (1971).

The analytical results obtained and physical constants used for myosin, its fragments, and actin are given in Table I. The CaATPase was determined by measuring the amount of NaOH needed to neutralize the $H^+$ produced from the hydrolysis of 2 mM ATP by 0.7 μM protein in 5 mM $CaCl_2$ and

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[1] Abbreviations used: NMR, nuclear magnetic resonance; HMM, heavy meromyosin; LMM, light meromyosin; S1, subfragment 1; S2, subfragment 2; A1, alkali light chain 1; A2, alkali light chain 2; $Me_4Si$, tetramethylsilane; ATP, adenosine triphosphate; $PP_i$, pyrophosphate; AMPPNP, adenyl-5′-yl imidodiphosphate.

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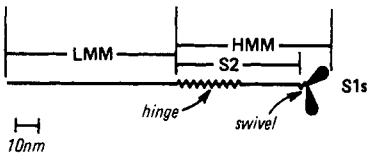


MYOSIN

FIGURE 1: Scale drawing of the myosin molecule showing the fragments which can be prepared proteolytically (LMM, HMM, S2, and S1) and the location of the regions which have been shown to be flexible on a hydrodynamic time scale (the hinge and the swivel).

0.6 M KCl, 23 °C, pH 7.5. Polyacrylamide gels in the presence of $NaDodSO_4$ showed the appropriate heavy bands. Myosin had all three light chains present. HMM had all three, but light chain 2 was partially missing. S1 had only light chains 1 or 3. KCl and $MgCl_2$ were ultrapure (Alfa), as was $(NH_4)_2SO_4$ (Sigma). All other inorganic chemicals were reagent grade. $^2H_2O$ (99.98% $^2H$) was from Bio-Rad. $NaO^2H$ was prepared from NaOH. Proteolytic enzymes were from Sigma.

Protein samples in $^1H_2O$ buffers were dialyzed against several changes of $^2H_2O$ buffers to get samples suitable for NMR measurements. This dialysis usually required 4 days and, except in the case of actin, was followed by centrifugation for 1 h at 200000*g* before a measurement was made. Protein samples were kept below 4 °C at all times. Denatured samples were prepared by adding a small aliquot of concentrated $NaO^2H$ in $^2H_2O$ to the samples.

*Methods.* Protein concentrations in $^2H_2O$ were determined by ultraviolet absorption at 280 nm without correction. The $p^2H$ was determined by using a glass pH electrode and adding 0.4 to the measured value. The sample temperature in the spectrometer was determined by using a temperature controller which had been calibrated from the chemical shift difference of methanol methyl hydrogen and hydroxyl hydrogen peaks.

$^1H$ NMR measurements were made with a Bruker HXS-360 spectrometer (Heidelberg) and a modified HXS-360 (Stanford). The computer and software packages (NTCF-T-1180) were from Nicolet Technology Corp. A good spectrum could be obtained in less than an hour. Spectra suitable for resolution enhancement required 2–4 h. Spectra used for determining total area for the broad and narrow signals were obtained without any radio frequency pulse to saturate the $HO^2H$ peak, since this irradiation has been shown to affect the intensity of the broad band signals of myosin (Akasaka et al., 1978). The dead time of the Stanford spectrometer was the lower of the two instruments and allowed more of the broad component to be measured. All quantitative determinations of spectral areas were made from data collected on that instrument. Resolution-enhanced spectra and the fraction of the hydrogens contributing to the narrow bands were obtained by the method of Gassner et al. (1978). The fraction of the hydrogens in the narrow bands was also determined by cutting and weighing traces of the spectra on paper. The results from the two methods were in agreement.

## Results

*Myosin and Its Fragments.* Figure 2 shows the 360-MHz $^1H$ NMR spectra for myosin, HMM, S1, and LMM in 0.6 M KCl and 0.010 M $PO_4^{3-}$, 4 °C, $p^2H$ 7. For the S1-containing moieties, total protein concentrations were adjusted to give equal concentrations of S1 in each sample. The concentration of LMM equals that of myosin in the same figure. The major narrow bands in the aliphatic region are centered at 0.2, 0.7, 1.0, 1.4, 1.7, 2.3, 3.5, and 3.8 ppm downfield from external $Me_4Si$ for all the species containing S1. The aromatic regions for myosin, HMM, and S1 show a common peak at 6.6 ppm (possibly His or Tyr), and myosin and HMM, but not S1 or LMM, have an additional smaller peak at 7.8 ppm (probably His). In sharp contrast, the

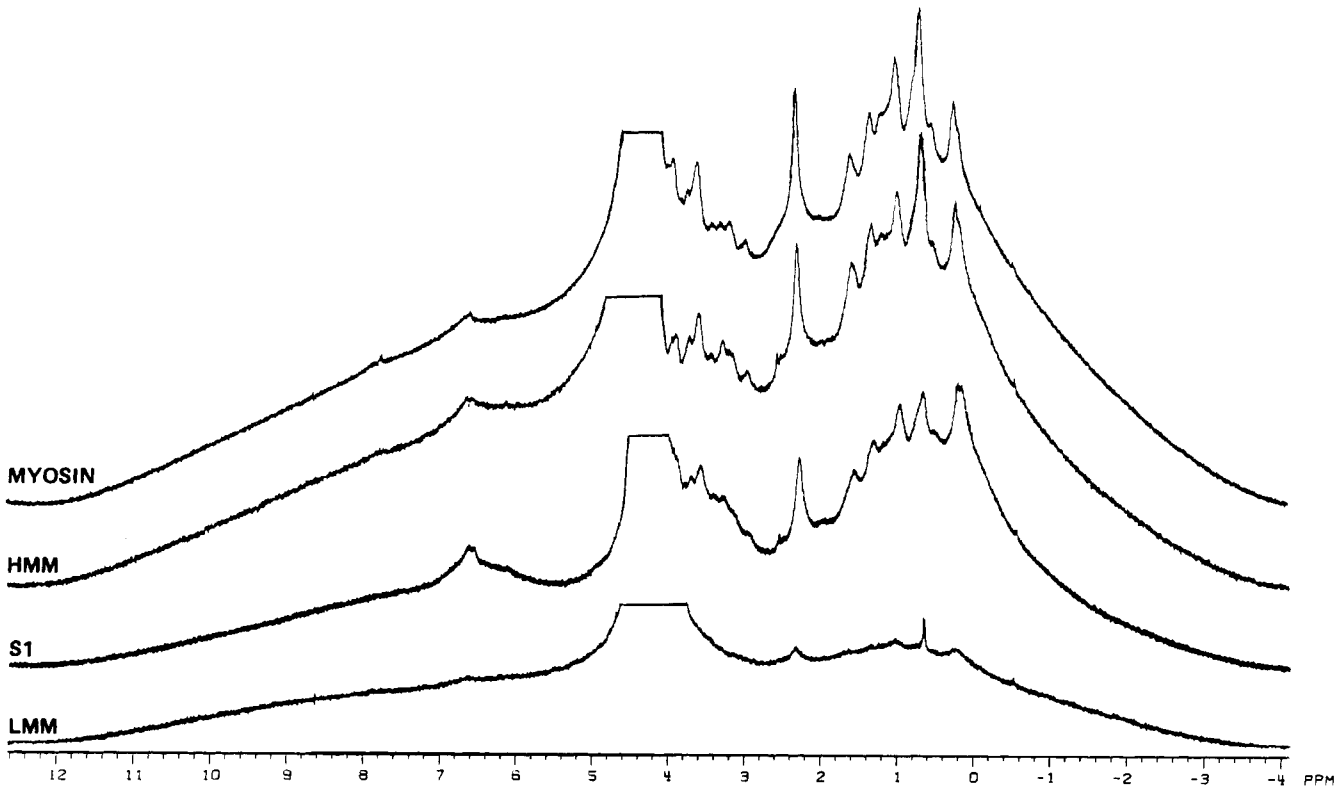


FIGURE 2: 360-MHz $^1H$ NMR spectra of myosin and its constituent fragments. The concentrations are 29 $\mu$M myosin, 29 $\mu$M HMM, 58 $\mu$M S1 (S1A1 + S1A2), and 29 $\mu$M LMM in 0.6 M KCl and 0.010 M phosphate, 4 °C, $p^2H$ 7.0, in $^2H_2O$. The shifts were calibrated to external $Me_4Si$ = 0 ppm.

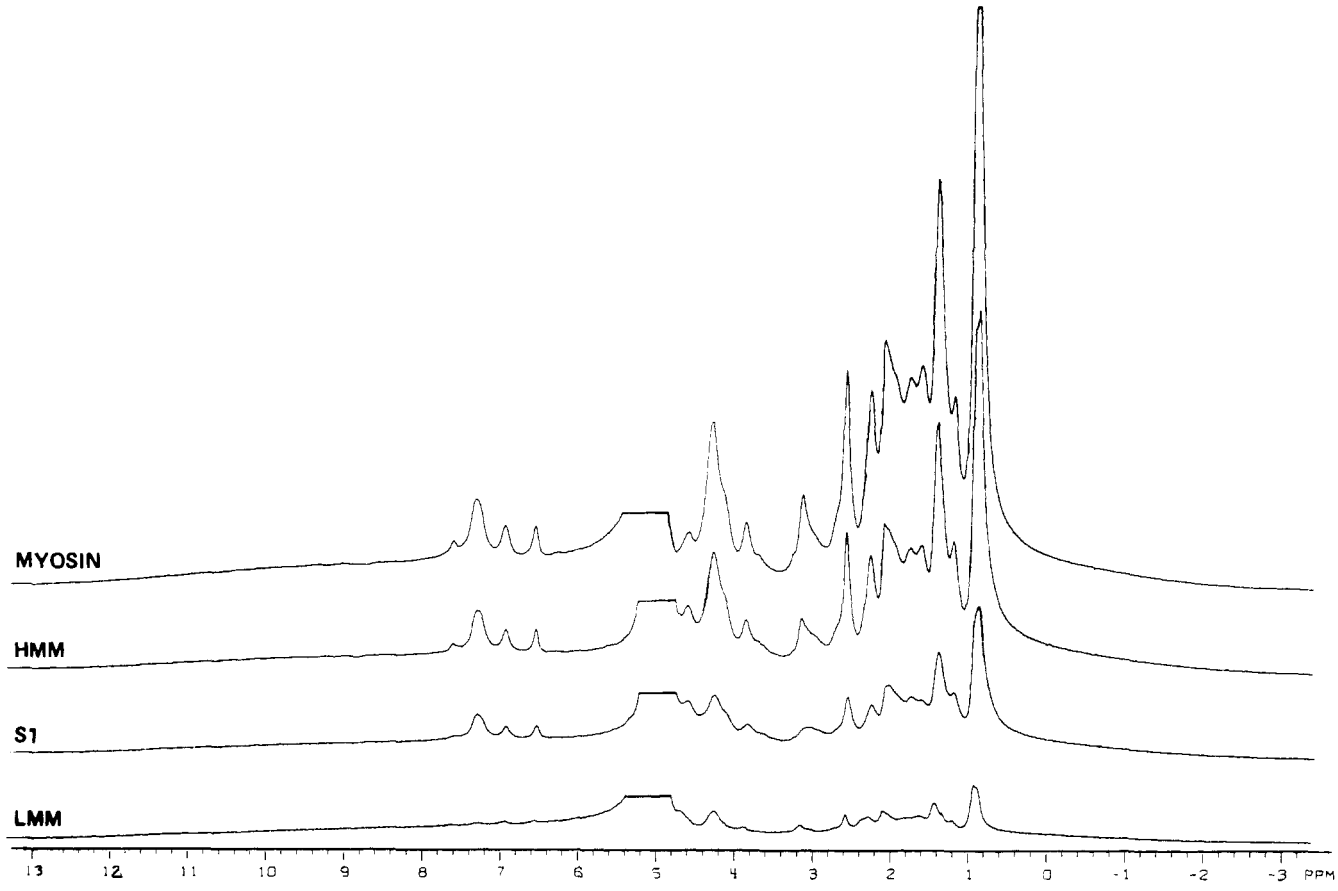


FIGURE 3: Spectra of the proteins in Figure 2 which were denatured with 4% $NaO^2H$. Other conditions are as in Figure 2.

aromatic spectrum of LMM is broad.

There are several interesting features in the spectra. (1) Strong narrow signals are observed from S1, HMM, and myosin, while practically none are observed from LMM. (2) The observed narrow components of the signals are very similar in shape to each other among S1, HMM, and myosin. (3) The narrow lines are unexpectedly sharp, particularly for myosin, considering its molecular weight of 450000. The observed narrow signals do not arise from low molecular weight impurities or from proteolytic creation of loose ends on the protein surface. As seen under Experimental Section, the preparations of all the samples involve steps which eliminate small impurities [e.g., gel filtration, equilibrium dialysis, and $(NH_4)_2SO_4$ fractionation]. Impurity peaks were occasionally seen in the spectra and could be identified by the inconsistency of their intensity for different sample preparations and from their extremely narrow line widths (a few hertz). Proteolysis is not creating the narrow signals, since myosin is not prepared proteolytically but has strong signals while LMM, which is prepared by proteolysis, does not.

The spectra of the denatured proteins were all similar to each other and quite different from those of the native proteins, as shown in Figure 3. Notice that the spectra have collapsed into all narrow bands and that their widths at half-height are comparable to those of the narrow bands in the spectra for native proteins. Comparisons of the total resonance intensity between native and denatured states for dilute solutions of protein ($\sim$10 $\mu$M) are shown in Table II. The relative areas of denatured spectra are in excellent agreement with the relative molecular weights. The areas of samples which were denatured with $NaO^2H$ were compared to the areas of the native samples. These ratios are shown in Table II. The smaller proteins, S1, LMM, and HMM, have intensities nearly equal to those when denatured. Myosin shows a substantial

Table II: Relative $^1H$ NMR Intensities of Native and Denatured Proteins[a]

| | rel denatured intensity | rel mol wt | native intensity/ denatured intensity |
|---|---|---|---|
| myosin | 1.00 | 1.00 | 0.66 |
| HMM | 0.72 | 0.71 | 0.90 |
| S1 | 0.43 | 0.44 | 0.92 |
| LMM | 0.30 | 0.29 | 0.93 |

[a] The first column contains the buffer-corrected $^1H$ NMR signal intensities for myosin, HMM, 2× S1, and LMM divided by that of myosin. The second column is the same treatment using molecular weights. The third column is the intensity of the native protein divided by that of the same sample denatured with $NaO^2H$. All values result from at least three measurements on different protein preparations, except LMM, where only two were made.

decrease in intensity. HMM was especially sensitive to aging and showed a decrease in its broad component after 5 days at 4 °C.

Convolution difference spectra (Gassner et al., 1978) of native myosin, HMM, and S1 are shown in Figure 4. Clearly, once the broad component has been removed by this treatment, the similarity between the spectra is even greater, not only in chemical shifts but also in the areas of most the peaks. Table III shows the total areas of the spectra for different values of the filter time constant. For time constants longer than 50 ms, the total areas of myosin, HMM, and S1 are nearly equal within experimental error. Extrapolation of the data to zero filtering permits our estimate of the fraction of the total signal due to the narrow bands (Gassner et al., 1978). About 22% of S1 is estimated to be contributing to the narrow components. The qualitative and quantitative similarities of the narrow aliphatic components for myosin, HMM, and S1 indicate that

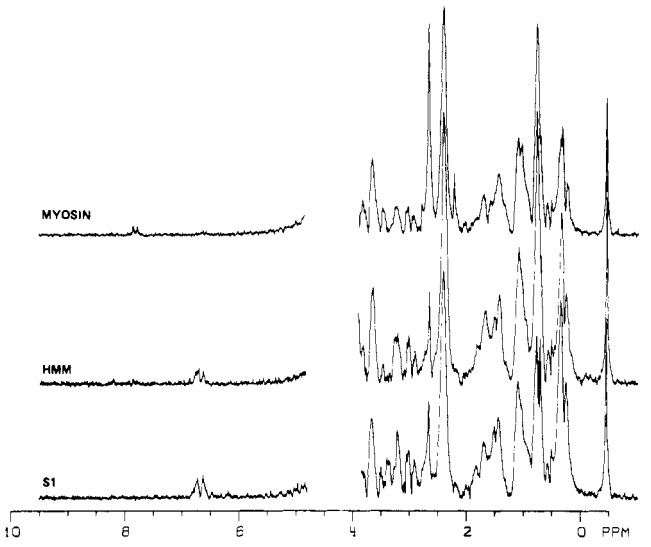


FIGURE 4: Resolution-enhanced spectra for myosin, HMM, and S1. T1 is 100 [see text and Akasaka et al. (1978)].

Table III: Relative Areas of Resolution-Enhanced Spectra[a]

| T1 | myosin | HMM | S1 |
|---|---|---|---|
| 0 | 3000 | 1990 | 2150 |
| 10 | 605 | 554 | 590 |
| 50 | 337 | 316 | 336 |
| 100 | 209 | 185 | 197 |
| 200 | 116 | 101 | 107 |
| 500 | 53 | 48 | 50 |
| 800 | 36 | 34 | 36 |
| 1000 | 31 | 30 | 32 |
| 1500 | 22 | 21 | 23 |

[a] T1 is the filter time constant in milliseconds used for trapezoidal multiplication of the FID signal. The numbers in the columns under the proteins are the relative areas in arbitrary units for the region upfield from HOD in the Fourier-transformed FID.

the portions of myosin and HMM which give the narrow resonance signal reside almost entirely in the S1 moieties. The fact that S1 has a larger narrow signal at 6.6 ppm than HMM or myosin is clear in Figures 1 and 4. This was reproducible for several protein preparations and probably reflects the presence of aromatic groups which are freed by the proteolytic production of S1. These aromatic groups could be in or near the swivel, they could be on the surface of S1 where the DTNB light chain binds, or they could be fragments of the DTNB light chains which remain bound to S1.

Resonance lines in the narrow component are composite lines consisting of differently shifted components, resulting in apparent line widths of 30–50 Hz. The actual line width of each component must be much less than the above values. From the $T_2$ decay measurements, an estimate of 8–10 Hz was obtained for the real line width. On the simple assumption that the narrow component represents methyl group rotation, typical line widths for the methyl protons can be calculated, using a Woessner model (Woessner et al., 1969), for two extreme cases: (1) $\tau_g \gg \tau_c$, the case where the methyl group has no local rotation compared to the overall rotation of the molecule, and (2) $\tau_g \ll \tau_c$, the case where the methyl group rotates much more quickly than the molecule tumbles, where $\tau_c$ is the rotational correlation time for the protein as a whole and $\tau_g$ is the correlation time for group rotation. These calculated line widths in Table IV show that the line width of the methyl protons is expected to become about one-fourth of the original line width (no group rotation) when the rapid rotation of the methyl group is considered (Woessner et al., 1969). The important point is that the expected line width is still much too broad to account for the observed line widths in the narrow components, even when the rapid group rotation of the methyl group is taken into account. This indicates that there must be motion in the molecule in addition to simple methyl rotation. The rotational correlation times obtained by fluorescence depolarization measurement (Mendelson et al., 1973) are also given in Table IV. Because the molecules are not spherical, these $\tau$ values are larger than those predicted by the Stokes–Einstein equation; thus, the argument for internal motion is a conservative one. The additional degrees of freedom may represent side-chain motions other than the methyl rotation or the mobility of a part of the main peptide chain. In proteins, these two types of motions are likely to be interdependent. The fact that the narrow component line widths are similar to those of the denatured proteins suggests that the fast motions in the native protein are comparable to those of the denatured protein.

Table IV: Calculated Line Widths for Rotating Methyl Groups[a]

| | $\tau_c$(Stokes-Einstein) (s) | expected line widths (Hz) | | $\tau_c$(fluorescence depolarization) (s) |
|---|---|---|---|---|
| | | $\tau_g \gg \tau_c$ | $\tau_g \ll \tau_c$ | |
| S1 | $3.3 \times 10^{-8}$ | 170 | 60 | $2.2 \times 10^{-7}$ |
| HMM | $9.1 \times 10^{-8}$ | 470 | 120 | $4.0 \times 10^{-7}$ |
| myosin | $1.43 \times 10^{-7}$ | 670 | 170 | $4.5 \times 10^{-7}$ |

[a] The expected line widths for a methyl group were calculated by using Stokes-Einstein correlation times and a Woessner model where the overall rotation of the molecule is much faster than the methyl group rotation ($\tau_g \gg \tau_c$) and where the group rotation is much faster than the overall rotation ($\tau_g \ll \tau_c$). The correlation times from fluorescence depolarization measurements (Mendelson et al., 1973) show that this treatment should give somewhat narrower line widths than measured; but, in fact, the calculated line widths are much broader than those obtained by experiment (~10 Hz).

On the other hand, the broad component has a line width of much more than 50 Hz. Line widths for the methyl resonances calculated with or without rapid group rotations are in the range 200–700 Hz for myosin (Table IV), which agrees reasonably well with the observation for the broad component.

*S1 and Nucleotides.* The nonhydrolyzable ATP analogues $PP_i$ and AMPPNP had no observable effect on the spectrum of S1 in 0.6 M KCl and 0.01 M $PO_4^{3-}$, p$^2$H 7, at 4 °C. $PP_i$ also had no effect in 0.15 M KCl. This was true for spectra from several hours of data accumulation on solutions with nucleotide in 1000-fold excess over the protein concentration. ATP also had no observable effect on the spectrum of S1, but, due to its reactivity, fewer data could be accumulated and the small peaks in the aromatic region were ill-defined. As shown below, there is no question that nucleotides do bind to S1 under these conditions, since they cause the dissociation of the S1–actin complex.

This observation shows that no global conformational changes in the flexible regions are induced by nucleotide binding, nor is a part of the flexible segment becoming immobilized or, conversely, a part of the rigid structure melting out. On the other hand, a local conformational change involving only a few amino acid residues may not be detectable in our NMR spectra because even a relatively sharp line represents at least 30–40 residues.

*S1 and Actin.* Pure F-actin had a spectrum with very few narrow bands, as can be seen in the lowest trace of Figure 5. As actin was added incrementally to a solution of S1 (maintaining constant [S1]), the narrow bands in the aliphatic region were reduced in increments and disappeared when actin

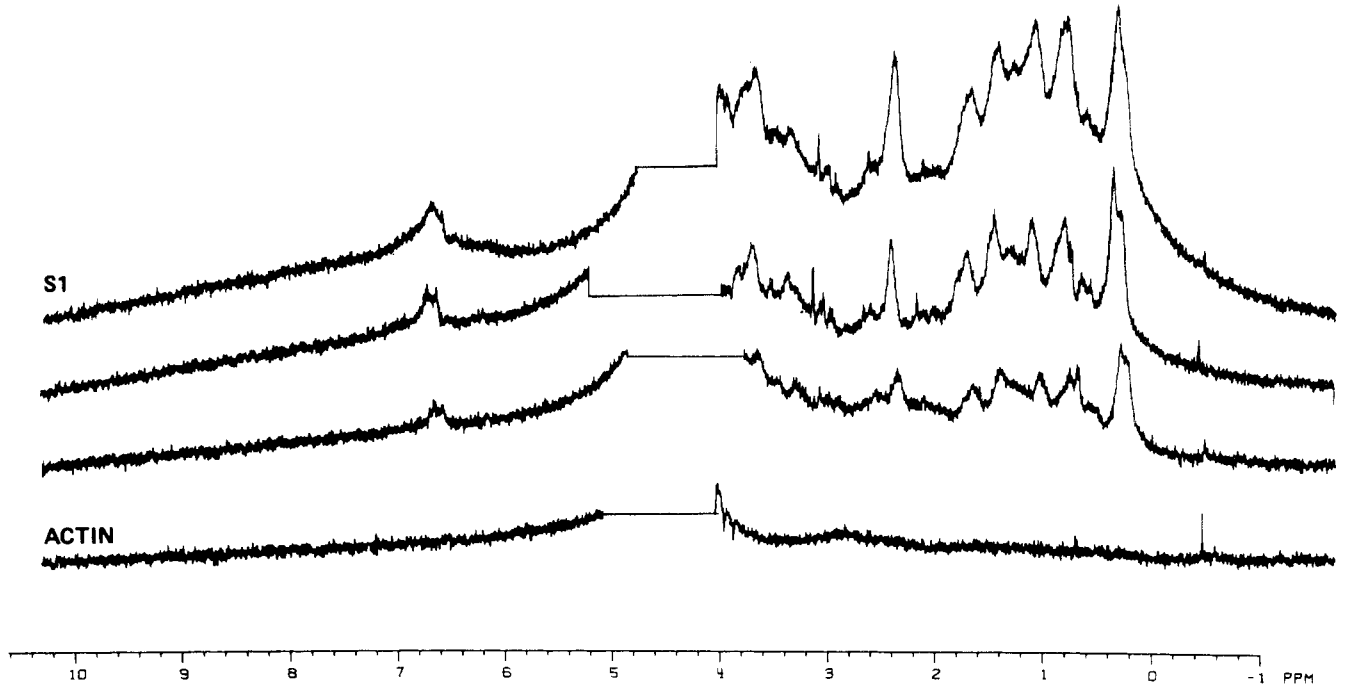


FIGURE 5: S1 titration with actin in 0.15 M KCl and 0.010 M phosphate, p$^2$H 7.0, in $^2H_2O$ at 4 °C. The upper trace is S1. Descending, the traces are for 42 $\mu$M S1 plus 38 $\mu$M actin and 36 $\mu$M S1 plus 48 $\mu$M actin. The lower trace is actin.

was in excess. Figure 5 shows two intermediate cases. If one assumes that the areas of the narrow portion of the spectra are proportional to the amount unbound S1, an association constant can be estimated from

$$K_a = \frac{a_T - a_A}{a_A[A_0 - (a_T - a_A)S_0/a_T]} \quad (1)$$

where $a_T$ and $a_A$ are the narrow band areas with no actin and with actin, respectively, and $A_0$ and $S_0$ are the total concentrations of actin and S1. Using the data shown in Figure 5 and eq 1, we calculated $K_a$ to be $(1.5 \pm 0.5) \times 10^6$ M$^{-1}$.

That the disappearance of the S1 resonance upon addition of actin is due to the formation of a molecular complex and not due to a bulk viscosity effect is evident from three observations. (1) The calculated association constant, based on the assumption that binding eliminates the narrow signals, agrees with the value obtained by other techniques. (2) The addition of $PP_i$ or AMPPNP to solutions of S1 and actin causes the narrow lines to reappear. The spectrum of free S1 can be completely restored by adding $PP_i$ to S1 in the presence of actin, which increases the viscosity substantially. (3) The change in rotational correlation time for S1 alone and in myosin (200 vs. 450 $\mu$s) should effect the narrow band areas if they are sensitive to overall rotation, but it does not.

## Discussion

The qualitative and quantitative similarities of the narrow bands due to the aliphatic protons in the $^1$H NMR spectra for myosin, HMM, and S1 and their paucity in LMM (Figure 2 and Table II) indicate without much doubt that the mobile portion of myosin is located in the S1 moiety. Since S1A1, S1A2, and myosin have the same narrow bands, the location of the mobile structure can be further limited to the heavy chain in S1 or possibly to the homologous part of the alkali light chains. The aromatic peak at 6.6 ppm is also from an amino acid located in S1. The other aromatic peak at 7.8 ppm must be from a side chain located in S2 or from the DTNB light chain since it appears only in myosin and HMM but not in S1A1 or S1A2. This latter signal may serve as an intrinsic marker for S2, which is typically difficult to distinguish in myosin or to label with an extrinsic probe.

The fact that the line widths of the narrow bands of the native proteins are similar to those of the denatured proteins suggests that the mobile structure has freedom of motion comparable to that of a random coil. A further indication that the narrow signals result from fast local motion is their apparent independence of the rate of overall molecular rotation. The rate of rotational Brownian motion of the S1 moiety varies between 450 and 200 ns for myosin, HMM, and S1 itself under conditions similar to those used here (Mendelson et al., 1973). However, the line widths are not affected by these differences. In addition, the narrow signal line widths were affected little if at all by the changes in viscosity caused by adding actin and $PP_i$.

The amount of the mobile structure is significant. Twenty-two percent of S1 corresponds to roughly 150 amino acids, assuming a molecular mass of 120 amu for an average amino acid. As a single stretch of extended chain, this could span over 50 nm, although there is no reason to assume the signals observed are from a continuous portion of the primary sequence.

The functional location of this mobile structure is of interest. A few possibilities can be eliminated immediately. It is not due to an unraveled structure or to loose ends caused by proteolysis since it exists in myosin, which was not prepared enzymatically, but does not exist significantly in LMM, which was. It also appears not to involve the nucleotide binding site since the narrow signals are not visibly affected by ATP, AMPPNP, or $PP_i$ binding. There is little doubt that these nucleotides do in fact cause a local conformational change upon binding. Spin-label mobility (Seidel & Gergely, 1971) and accessibility (Onishi & Morales, 1976), intrinsic fluorescence (Werber et al., 1972), UV absorption (Trentham et al., 1972; Yazawa et al., 1972), and of course actin binding (Szent-Györgyi, 1965; Highsmith, 1976) are all changed by nucleotide binding. On the other hand, no delocalized changes in myosin conformation due to nucleotide binding were detectable by circular dichroism (Cassin & Lin, 1975). Apparently, the

corresponding changes in the $^1H$ NMR signals for nucleotide binding are very small or are in the envelope due to the less mobile parts of myosin. Our results agree with those of Shriver et al. (1979), whose $^{31}P$ and $^{19}F$ NMR results indicate that the nucleotide binding site is nonflexible.

The two most likely locations on S1 for the mobile structure are the swivel and the actin binding site. The swivel is known to be flexible (Mendelson et al., 1973; Thomas et al., 1975; Kobayashi & Totsuka, 1975), and a large part or all of it may remain on the proteolytically prepared S1. It has been suggested that its flexibility and proteolytic susceptibility both result from a looseness of structure compared to the rest of myosin. However, it must be pointed out that the same argument for looseness is often made to explain the flexibility and proteolytic susceptibility of the hinge where LMM joins S2, but the results here indicate that only a rather small portion of the hinge could be mobile. If the swivel does contribute to the narrow signals due to its mobile structure, the mobility is lost upon binding actin. Interactions between these two sites, and/or proximity, have been reported (Shukla & Levy, 1977).

The possibility that the swivel could be a structure with freedom of motion comparable to a random coil structure allows us to suggest a new possible location and mechanism for the elastic element in the muscle cross bridge. The swivel could be an elastin-like segment where the mechanical work of the reorientation of a myosin head on actin is stored in a stretch of a disordered structure in S1 to a more ordered, elongated structure. The energy would then be converted back into work as the filaments interdigitated. Elastic elements of other types in other locations of the cross bridge have been suggested (Huxley & Simmons, 1971; Eisenberg & Hill, 1978; J. Gergely, R. Lu, and M. F. Morales, unpublished results on S2).

The other likely location of the structure contributing to the narrow bands is the actin binding site on S1. This possibility is especially plausible because binding to actin causes the narrow lines to disappear (Figure 5). The association constant for S1 and actin, $K_a = 1.5 \times 10^6$ $M^{-1}$, calculated on this assumption (see Results) is in good agreement with those determined by other methods (Margossian & Lowey, 1978; Marston & Weber, 1975; Highsmith et al., 1976). Of course the mobile portion could be different from the actin binding site but interact with it. However, the direct quenching of the internal motion of the cross bridge by actin binding is a simpler interpretation. It is also more attractive because the actomyosin interface is the site of force generation (Nihei et al., 1974) and the internal motions may be involved in the mechanism of chemomechanical energy transduction.

A comparison of the fractions of the denatured protein spectra which contribute to the areas of the native protein spectra (Table II) allows one to comment on the relative hydrodynamic mobilities of the constituents of myosin in solution. S1, HMM, and LMM are rotating rapidly enough for all of their hydrogens to be contributing to the native signal. The reduced signal for myosin, compared to the sum of HMM and LMM, indicates a loss of mobility for those constituents when they are incorporated in myosin. The myosin signal (66% of the denatured) is quantitatively close to the contribution expected from HMM. This could result from rigid LMM having lost its freedom to rotate around its long axis. If this were its dominant relaxation mode, then the flexible HMM would be the sole contributor to the broad and narrow peaks from myosin. Alternatively, the reduced mobility of S2 and LMM in myosin (compared to free in solution) may reduce both of their contributions to the broad component of myosin. In either case, the flexibility of myosin at the swivel and the hinge is substantiated by these results.

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# Dilatometric Study of Binary Mixtures of Phosphatidylcholines†

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ABSTRACT: Volumes of lipid dispersions as a function of temperature have been measured for two different kinds of binary mixtures of lecithins, (1) DMPC and DSPC and (2) DMPC and $DC_{20}PC$. Emphasis was placed on DMPC-rich compositions so as to resolve ambiguities regarding solid-phase immiscibility in DMPC–DSPC mixtures. Special attention has been paid to problems of equilibration in the low-temperature phase and to methods of mixing the lipids. We find that there is no solid–solid immiscibility in DMPC–DSPC mixtures, although this system is close to exhibiting such immiscibility, and that DMPC–$DC_{20}PC$ mixtures exhibit pronounced solid immiscibility.

The miscibility properties of phospholipids are of current interest, and, in particular, for certain binary mixtures of phosphatidylcholines, the nature of the gel phase is still an unsettled question. That is, at temperatures below those where solid and fluid domains coexist, is there a single-phase region or is there solid–solid immiscibility? An answer to this question is provided by the type of phase diagram constructed for the lipid pair under study. For example, a horizontal solidus line is indicative of monotectic phase behavior, i.e., immiscibility in the solidlike phase.

In practice, it has been somewhat difficult to determine immiscibility on this basis. A good example is the lipid pair DMPC–DSPC. A horizontal solidus line up to at least a 50:50 molar mixture has been reported by some (Phillips et al., 1970; Lentz et al., 1976; Van Dijck et al., 1977), whereas other experimenters have found a solidus line with a nonzero slope (Shimshick & McConnell, 1973; Mabrey & Sturtevant, 1976; Gent & Ho, 1978), although Shimshick and McConnell noted that their data indicated an incipient solid–solid immiscibility.

It would therefore seem useful to determine this phase diagram by another method, especially one that neither perturbs the lipid structure with a probe molecule nor departs significantly from thermal equilibrium. Since dilatometry is such a method, we feel that the present study is a worthwhile addition to the work cited above and, indeed, decides between the two types of phase behavior.

To provide comparison with the DMPC–DSPC system, we have also studied mixtures of DMPC and $DC_{20}PC$. Such mixtures, where the hydrocarbon chain length difference is six, should, we felt, display solid-phase separation and so corrobrate the analysis of the DMPC–DSPC phase diagram.

## Materials and Methods

Dimyristoylphosphatidylcholine (DMPC) and distearoylphosphatidylcholine (DSPC) were obtained from Calbiochem and used without further purification. The DMPC reported on here gave the sharpest phase transition of any lipid we have ever studied. The DSPC displayed essentially identical density properties to those of earlier samples (Nagle & Wilkinson, 1978). Dieicosanoylphosphatidylcholine ($DC_{20}PC$) was purchased from Avanti Biochemical Co.

Two methods were used for mixing the lipids. A comparison of them is presented under Results. One method, currently standard practice, consisted of dissolving the dry lipid powders in chloroform, stirring for approximately 0.5 h, removing the solvent under vacuum, and suspending the mixture in water. The other method consisted of suspending the lipids in 3 mL of water above the phase-transition temperature and then sonicating the mixture until it became nearly transparent. A Branson Model 185 sonifier operating at approximately one-third power for 5–10 min was found adequate for this. The sonicated mixture was then placed under vacuum, the water was removed, and the dried lipids were resuspended in water. The mixture then once again had the typical appearance of a multilamellar suspension.

The mixed lipid samples were degassed, and their temperature–volume relationships were measured with a differential dilatometer described previously (Wilkinson & Nagle, 1978). Scan rates of 2–5 °C/h were used. Approximately 100–150 mg of lipid in 10 mL of $H_2O$ was used for each experiment.

The absolute values of specific volume were obtained by the neutral buoyancy in $D_2O$–$H_2O$ mixtures described in a previous paper (Nagle & Wilkinson, 1978).

## Results

*Evaluation of the Cosonication Technique.* Since cosonication of lipids as a method of mixing at the molecular level has not been reported on previously, we first tried this method on a mixture where separate phase transitions for each component if present could be readily observed: 29 mol % DSPC in DMPC with a total lipid mass of 131 mg. Dilatometric scans from 18 to over 56 °C revealed only a single, broad transition whose onset and end point were close to published data on phase diagrams for these two lipids. We therefore concluded that cosonication did produce intimately mixed lipids.

In order to evaluate the cosonication method, we compared the results that it produced with those obtained using chloroform on both DMPC alone and DMPC with a small amount (4 mol %) of DSPC, a mixture where the phase transition was still expected to be quite narrow in temperature. Pure DMPC,

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0006-2960/79/0418-4244$01.00/0 