STUDIES OF MYOSIN AND ITS PROTEOLYTIC FRAGMENTS BY LASER RAMAN SPECTROSCOPY

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ABSTRACT Two bands in the Raman spectrum of myosin, at 1,304 cm^{-1} and 1,270 cm^{-1}, are attributable to α-helical structure. The first of these, also present in the spectrum of light meromyosin (LMM) but not in that of subfragment-1 (S-1), is assigned to the coiled-coil tail region of myosin; the second, seen in spectra of S-1 or heavy meromyosin (HMM), is largely absent from the spectrum of light meromyosin and is likely to correspond to the α-helical segments of the head region. When myosin or LMM aggregates, spectral bands attributable to backbone and sidechain groups sharpen suggesting a reduction in motional freedom. This sharpening is particularly apparent in the 902 cm^{-1} C—C stretching mode. Mg^{2+} broadens and shifts the peak at 1,244 cm^{-1} to 1,237 cm^{-1} and diminishes the intensity from 1,230 to 1,240 cm^{-1}, changes which appear to be associated the S-1 region. $MgPP_i$ produces changes in the 1,300 cm^{-1} region attributable to α-helical regions in coiled-coil structures suggesting that $MgPP_i$ affects not only S-1, but also some part of the myosin rod.

INTRODUCTION

Myosin is a heteropolymer consisting of two heavy (~200 kdaltons) and four light (~20 kdaltons) polypeptide chains. The NH_2-terminal regions form two distinct globular heads, while the COOH-terminal regions of the heavy chains form a coiled-coil helical rod (1–4). Myosin can be cleaved by chymotrypsin or trypsin forming several fragments: HMM, containing both heads and a short portion of the rod; LMM, containing most of the remaining portion of the rod; and subfragment-1 (S-1), corresponding to the head (5). The helical content of these fragments varies from nearly 100% for LMM to 33% for S-1 (1).

Peptides and proteins, including myosin, have been the subject of studies using Raman spectroscopy for some time (6–13). With this technique one detects frequency shifts corresponding to the vibrational modes arising from various types of chemical bonds (13). The amide I and particularly amide III vibrational modes have been found to be sensitive indicators of secondary structure. Amide I (1,650–1,675 cm^{-1}) reflects mainly C═O stretching motion and is little affected by hydrogen bonding; while amide III (1,230–1,310 cm^{-1}), originates from C—N stretch and N—H bending motions of the peptide backbone. Other modes arising from motions in the bonds of the amino acid side chains, e.g., C—C stretching of the tyrosine ring, are of potential use in analyzing sidechain motions. Analysis of the polarization of Raman scattering is a useful tool in distinguishing overlapping modes, particularly if the contributing vibrations have widely differing symmetries (14, 15). In a recent paper dealing with the application of Raman spectroscopy to myosin (10), a decrease in helicity with increasing Ca^{2+} and Mg^{2+} concentrations was reported.

In the present work we utilized differences in the spectral properties of proteolytic fragments of myosin in analyzing the myosin spectrum. We found that Raman spectra of myosin fragments arising from the head and the tail regions possessed distinguishing characteristics attributable to differences in secondary structure. These characteristics have been employed to detect the participation of various parts of the myosin molecule in changes induced by aggregation, Mg^{2+}, Ca^{2+}, and $MgPP_i$.

MATERIALS AND METHODS

Raman spectra were taken on a Spex Ramalog 4 system (Spex Industries, Inc., Metuchen, NJ) using an Ar^+ laser (model 164; Spectra-Physics Inc., Mountain View, CA). The response of the thermoelectrically cooled photomultiplier (RCA-31034; RCA Electro-Optics & Devices, RCA Solid State Div., Lancaster, PA) (GaAs photocathode) is rather flat

between 400 and 700 nm. The laser output was passed through a Claesen filter to eliminate plasma lines, and a quartz-wedge polarization scrambler placed before the entrance slit of the spectrometer served to assure equal instrumental response to the scattered light regardless of its polarization. The samples were held in Kimax glass capillary tubes (inner diameter 1.6 mm) mounted perpendicularly to the scattering plane. Incident power levels of 100 to 500 mW and spectral resolutions of 4 to 6 cm^{-1} were used. All Raman frequencies are calibrated to the vibrations of CCl_4.

The spectra shown in Figs. 1–5 have been recorded with a strip chart system (Linear Instruments Corp., Reno, NV). The spectra shown in Figs. 5 and 6 have been obtained with the same instrument except that the monochromator drive was controlled by a Nicolet 1180 computer (Nicolet Instrument Corp., Madison, WI) programmed to signal-average and perform digital filtering functions, such as fast Fourier transform, in order to remove high and low frequency noise from the spectrum. Polarized spectra were obtained by inserting an analyzer into the path of the scattered beam between the condenser lens and the entrance slit. Heights obtained by standard methods (16) were taken as a measure of intensity and an indicator of relative change in conformation.

Myosin, tryptic HMM, and tryptic S-1 were prepared as described by Nauss et al. (17). Tropomyosin was prepared by the procedure of Bailey (18, 19). Tryptic LMM was prepared by ethanol fractionation of a myosin digest (20).

RESULTS

Secondary Structure of Myosin and Subfragment-1: Amide I (Largely C=O Stretch) and Amide III (Largely NH Bending)

Comparison of the spectra of myosin, HMM, S-1, and LMM makes it possible to assign certain spectral features to various portions of the myosin molecule. On the basis of published spectral assignments (Table I), the major peak in the myosin spectrum at 1,244 cm^{-1} may be attributable to disordered structures, while the broad shoulder at 1,230 cm^{-1} is within the β-pleat range (Fig. 1). The peak in the 1,270 cm^{-1} region can be attributed to helical structures in globular proteins. In addition there is a peak at 1,304 cm^{-1}, a position in which Raman lines have been observed in spectra of helical homopolymers of amino acids. A 1,304 cm^{-1} shoulder is also recognizable in the spectrum of HMM, which contains part of the rodlike portion (S-2), but not in that of S-1, which does not contain any part of the rod. The presence of this peak in the spectrum of LMM and tropomyosin (Fig. 2) suggests that it may be characteristic of coiled-coil helices, which account for essentially 100% of these proteins. Tropomyosin, which also has a coiled-coil α-helical structure, exhibits a spectrum that very closely resembles that of LMM, showing higher intensities in the 1,300–1,400 cm^{-1} region and low intensities between 1,220 and 1,180 cm^{-1} (Fig. 2). The almost total absence in the spectrum of LMM of activity in the 1,200 to 1,280 cm^{-1} region (Fig. 2) suggests that the main contribution to this region of the myosin spectrum must come from HMM. The spectra of HMM and S-1 reveal substantial intensities in the 1,220–1,280 cm^{-1} region, including overlapping peaks at 1,230 and 1,244 cm^{-1} corresponding to disordered structure and random coil, respectively, and a peak at 1,274 cm^{-1} arising from α-helical structures (Fig. 1).

TABLE I
ASSIGNMENTS FOR MYOSIN, TROPOMYOSIN, AND MYOSIN FRAGMENTS

	α-helix		Disordered structure		β-pleat	
Amide III assignments (cm^{-1})						
Myosin	1,304	1,265	1,244		1,232	
LMM, TM	1,304	—	—		—	
HMM	1,304	1,265	1,244		1,232	
S-1	—	1,265	1,244		1,232	
Globular protein averages*	—	1,268	1,247		1,236	
Model peptide averages*	—	1,299	1,248		1,231	
Amide I and I' assignments (cm^{-1})	I	I'	I	I'	I	I'
Myosin	1,654, 1,650	1,642, 1,632	1,668	1,655	1,678	1,663
HMM	1,654, 1,650	1,640, 1,636	1,656	1,655	1,672	1,663
LMM, TM	1,650	1,635	—	—	—	—
S-1	1,654	—	1,663	—	1,672	—
Globular protein averages*	1,659	—	1,669	1,664	1,672	—
Model peptide averages*	1,652	1,639	1,660	1,658	1,661	1,657

*Averages have been determined for eleven globular proteins and nine model peptides using data from references 6, 16, 23, 28–42.

Deuteration experiments serve to resolve amide III activity at 1,304 cm^{-1} from overlapping CH_2 intensity. Upon deuteration of myosin, HMM, LMM, or tropomyosin (Figs. 1, 2), the intensity in the 1,300 cm^{-1} (amide III) region, and particularly the 1,304 cm^{-1} peak, is greatly reduced accompanied by a corresponding increase at ~950 cm^{-1} (amide III'). The virtual disappearance of the 1,304 cm^{-1} peak is most striking in the spectra of LMM and tropomyosin. Since the 1,320 cm^{-1} and 1,342 cm^{-1} peaks, which are superimposed on the 1,304 cm^{-1} intensity, remain after deuteration, they are likely to be due to aliphatic side chains (see reference 21). This assignment is further supported by spectra obtained with polarized radiation. The amide III modes show a highly polarized character, whereas the peaks corresponding to CH_2 modes show little or no response to polarization (Fig. 1, inset).

The amide I band (Table I) is a less sensitive indicator of structural differences between the head and tail portions of myosin, in part because of the large H_2O peak that overlaps the 1,645 cm^{-1} band. The peak for D_2O is shifted to 1,200 cm^{-1} and upon deuteration the amide I peak for LMM is clearly seen centered at 1,635 cm^{-1}, while the peaks for myosin, HMM and S-1 are at 1,645 cm^{-1} with side bands at 1,635 cm^{-1}. Note that the 1,635 cm^{-1} peak has been observed in D_2O solutions of α-helical homopoly-

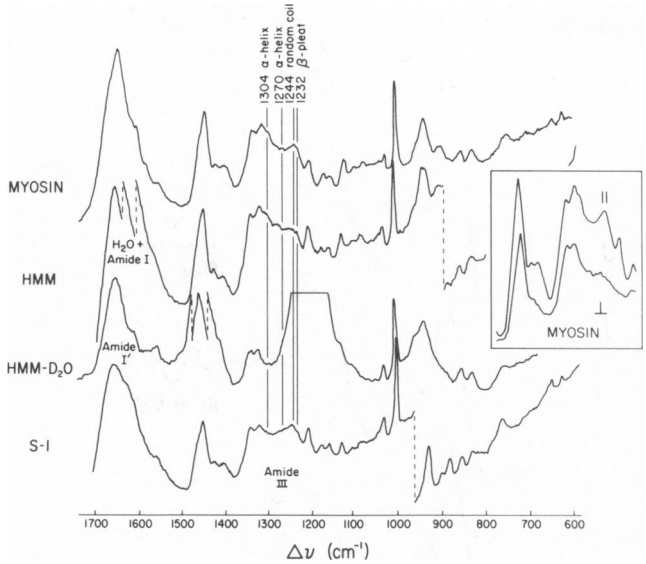

FIGURE 1 Raman spectra of myosin, HMM, and S-1. Protein concentrations were between 14 and 20 mg/ml in a solution containing 1 mM imidazole, pH 7.4, and 0.5 M KCl. Amide III and amide I modes related to secondary structures are indicated. *Inset*, polarization of Raman scattering in amide III and CH-bending regions of the spectrum. Spectral resolution, 5 cm^{-1}; scanning speed, 30 cm^{-1}/min full-scale sensitivity, 3 × 10^3 cpm; laser power, 250 mW; excitation at 514.5 nm.

peptides while the 1,645 cm^{-1} band is characteristic of α-helical structures in deuterated globular proteins (Table I). The sums of the spectra of LMM and HMM in the amide region III when appropriately scaled correspond closely to that of myosin (not shown).

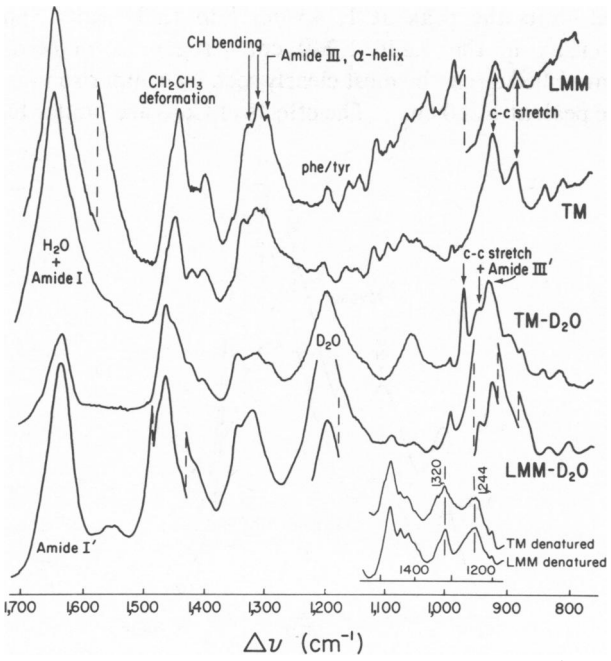

FIGURE 2 Raman spectra of LMM and tropomyosin. Conditions as described in the legend to Fig. 1.

Amide III and Other Sidechain Modes as Detectors of Protein Conformation and Intermolecular Interactions

With the above detailed information available on the contribution of various portions of the myosin molecule to Raman spectra, it seemed worthwhile to use Raman spectroscopy to study the aggregation of myosin and LMM. These processes are of interest since the structurally important self-assembly of myosin into thick filaments depends on the packing of LMM portions into the core of the thick filaments.

The aggregation of myosin monomers, by dialysis against 0.15 M KCl, is accompanied by sharpening of a number of resonances attributable to C—C stretch frequencies, especially those of the sidechain modes at 902 cm^{-1}, which are particularly sensitive to conformational changes in model peptides and proteins. The 902 cm^{-1} peak increases substantially upon aggregation of LMM with a small increase upon aggregation of myosin (Fig. 3). The 940 cm^{-1} peak, also a C—C sidechain mode, is enhanced by 60% on myosin aggregation but only by 20% on aggregation of LMM, suggesting that the 940 cm^{-1} mode may reflect aggregation-related conformational changes in the S-1 or S-2 regions while the intensity at 902 cm^{-1} reflects changes only in the myosin tail. The doublet at 853 and 830 cm^{-1} (Fig. 3) is more apparent in myosin

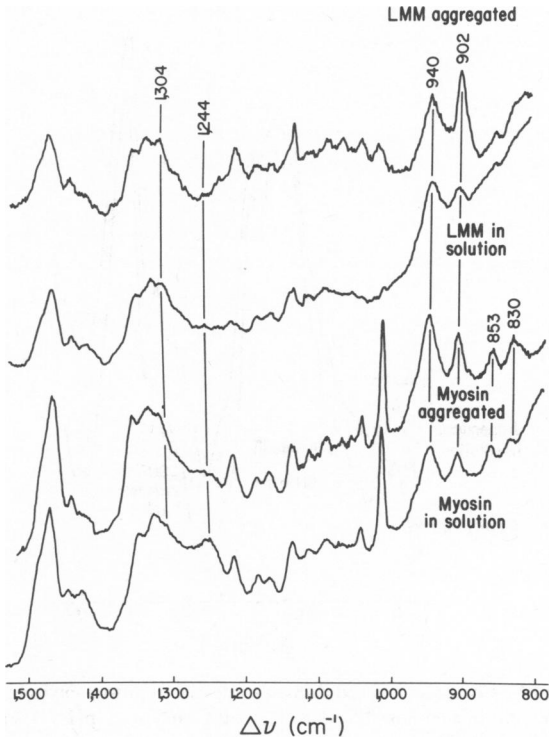

FIGURE 3 The effect of KCl on Raman spectra of myosin and LMM aggregated in 0.15 M KCl or solubilized in 0.5 M KCl. Other conditions as described in legend to Fig. 1.

than in LMM owing to the higher tryosine content in S-1. Aggregation of myosin results in an increase of the intensity of the 830 cm^{-1} peak relative to the 853 cm^{-1} peak. In both myosin and LMM there is a similar sharpening of peaks in the region between the 1,004 cm^{-1} phenylalanine peaks and the combined phenylalanine/tryosine peak at 1,210 cm^{-1}. The 1,300–1,400 cm^{-1} region of the LMM spectrum is largely unchanged on aggregation while the comparable amide III band centered at 1,244 cm^{-1}, which corresponds to the head region of myosin, loses intensity.

The coiled-coil structure of the tail portion of myosin is similar to the coiled-coil structure of tropomyosin (22). On comparing the spectrum of LMM with that of tropomyosin, the similarity in the 1,304 cm^{-1} region is striking, as is the low activity in the 1,220–1,300 cm^{-1} region. Spectra of aggregates of LMM (Fig. 3) and tropomyosin (Fig. 4) at low ionic strength, however, show differences, presumably owing to different sidechain interactions and modes of aggregation. The sharpening of peaks (1,000–1,200 cm^{-1}) arising from sidechain modes in aggregated LMM is not found in tropomyosin (not shown). Aggregation of tropomyosin (Fig. 4) leads to sharpening of the 1,304 cm^{-1} amide III peak, and there is enhanced intensity in the 1,410 cm^{-1} region attributable to COO^- stretch (21).

Conformational Changes in Myosin and LMM Induced by Mg^{2+}, Ca^{2+}, and $MgPP_i$

We have carried out some preliminary experiments to illustrate the potential usefulness of the Raman technique

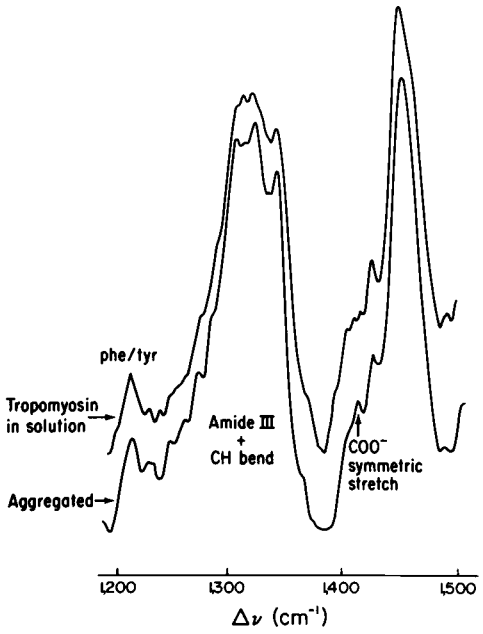

FIGURE 4 Computer assisted Raman spectroscopy of tropomyosin. Key: tropomyosin in solution: 0.5 M KCl, 1 mM imidazole, pH 7.4; aggregated, 1 mM imidazole, pH 7.4. A series of nine scans at 30 cm^{-1}/min were averaged; the resulting spectrum was then subjected to a Fourier transform routine to remove high frequency noise and the baseline rotated to flatten the background between 1,190 and 1,380 cm^{-1}. Other conditions were the same as described in legend to Fig. 1.

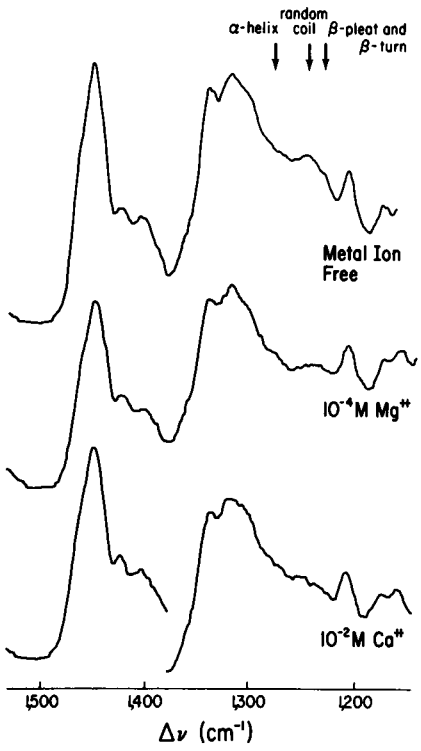

FIGURE 5 Effect of 10^{-2} M $CaCl_2$ and 10^{-4} M $MgCl_2$ on the spectra of myosin. Other conditions are the same as described in the legend to Fig. 1.

in studying the interaction of myosin with ligands. Both Ca^{2+} and Mg^{2+} bring about changes in the spectral region of myosin associated with S-1 (Fig. 5), which are, however, much smaller than those on aggregation. Mg^{2+} broadens and shifts the peak at 1,244 cm^{-1} to 1,237 cm^{-1}. The intensity in the 1,230–1,240 cm^{-1} region is markedly diminished as can be most clearly seen by comparison with the peak at 1,210 cm^{-1}. The effects of Ca^{2+} are similar but

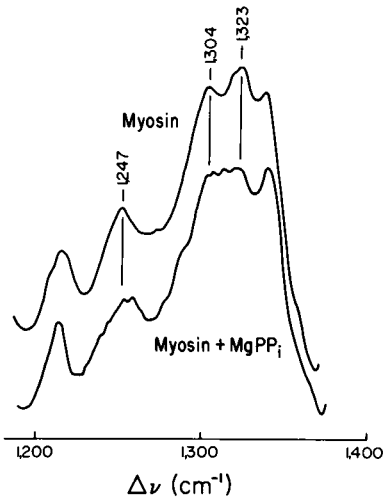

FIGURE 6 Effect of 5 mM $MgPP_i$ on myosin. Other conditions are the same as described in the legend to Fig. 1.

the spectra are broadened between 1,230 cm^{-1} and 1,300 cm^{-1} to a greater extent than on addition of Mg^{2+}. Note that divalent cations have no effect on the 800–1,150 cm^{-1} region although changes are apparent on aggregation of myosin or LMM (Fig. 3). This suggests that the change produced by divalent cations occurs chiefly in the S-1 regions of the molecule.

As shown in Fig. 6, addition of $MgPP_i$ to myosin alters the 1,244 cm^{-1} peak, attributable to disordered structure, and the 1,304 cm^{-1} region, attributable to α-helical regions in coiled-coil structures. These changes suggest that $MgPP_i$ affects not only S-1 but also some part of the rod adjacent to it.

DISCUSSION

By comparing the Raman spectra of myosin, HMM, S-1, and LMM it appears that certain parts of the myosin spectrum are almost exclusively ascribable to the coiled-coil, helical tail while others are attributable to the heads. Notably, the amide III peak at 1,304 cm^{-1} can be unequivocally assigned to LMM. The 1,304 cm^{-1} band occurs at essentially the same wave number as the α-helical peak in polyglutamate, polylysine, and other homopeptides. At present it is not clear what precise structural features in these compounds are responsible for the similarities. Fasman et al. (23), in a recent study of polyglutamate, concluded that a 1,295 cm^{-1} peak, which shifts on deuteration, most likely represents a superposition of amide III and CH vibrational modes. The 1,304 cm^{-1} peak, which is not completely resolved from the CH_2-deformation bands in H_2O, completely disappears upon deuteration of LMM. Thus, the 1,304 cm^{-1} peak is distinct from the CH_2 bands. While the 1,304 cm^{-1} peak can be attributed to coiled-coil structures, the spectral intensities between 1,220 and 1,300 cm^{-1} seem to be ascribable to the S-1 moieties, the globular heads of myosin. In this spectral region the α-helical, disordered, and β-structures, described in the literature for various globular proteins, can be recognized.

Differences between monomeric and aggregated myosin in the ratio of the 853 and 830 cm^{-1} peaks suggest differences in the hydrogen bonding of tyrosine (24). The fact that the 853 and 830 cm^{-1} peaks are present in myosin, HMM, and S-1, but not in LMM, also suggests that portions of myosin other than LMM are affected by the association process. The finding of Sutoh et al. (25) that in aggregated myosin the S-1 and S-2 domains remain close to the core, may in part, account for this observation.

Barrett et al. (10) have associated intensity changes in the strong 902 cm^{-1} peaks with an enhancement in β-structure following addition of divalent metal ions to myosin. We have found the 902 cm^{-1} peak to be a particularly good indicator of aggregation in myosin although it is insensitive to metal ions at or below millimolar concentrations. There is no obvious explanation for the fact that the broad sloping background in the spectra of solutions of LMM in the 900–1,000 cm^{-1} region essentially disappears on aggregation, accompanied by an extensive sharpening of the peaks and an increase in intensity of the C—C sidechain peak at 902 cm^{-1}. In the case of monomers, sidechains exposed to the solvent may have a broad distribution of C—C modes, which become narrow in the aggregated state, owing to reduced sidechain-solvent interaction.

The spectral changes observed on the addition of Ca^{2+} and Mg^{2+} indicate a considerably more subtle structural change than that which accompanies aggregation. The dramatic modification in secondary structure reported by Barrett et al. (10), but not found by us, may perhaps be accounted for by the fact that they used up to 1 M $CaCl_2$ and $MgCl_2$, substantially higher concentrations than those used in this work.

We suggest that changes apparent in the amide III region of our spectra indicate slight increases in order in some portions of the protein molecule (see reference 26). The spectral changes in the 1,270 cm^{-1} region produced by addition of $MgPP_i$ to myosin imply that the substrate analogue may induce local changes in the head portion without increasing its α-helical content (27). Comparable changes in the 1,304 cm^{-1} region, characteristic of the α-helices of the rod, imply that $MgPP_i$ binding may also influence order within the α-helical regions of the myosin molecule other than the head. This effect seems consistent with an earlier report (28) showing a destabilizing effect of ATP and various analogues, including PP_i on the filament core.

Supported by grants from the National Institutes of Health (HL5949, HL15391, HL23249), the National Science Foundation, and the Muscular Dystrophy Assocation.

Received for publication 20 May 1982 and in final form 14 June 1983.

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