It appears from the work cited above that the most stable region of paramyosin is the N-terminal segment.

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Studies on the "Hinge" Region of Myosin†

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ABSTRACT: The structural stability of the myosin rod and its smaller segments light meromyosin and subfragment II (isolated by papain digestion of myosin) has been examined to obtain more insight into the nature of the trypsin-sensitive region of the myosin rod. Although optical rotatory dispersion studies show that these rod segments are highly helical at ambient or lower temperatures, the kinetics of their proteolysis by trypsin suggest the presence of regions differing in accessibility to the protease. This conclusion is supported by thermal denaturation studies which demonstrate that the loss in helical structure of these rod segments occurs in cooperative phases. Tryptic digestion of myosin to the completion of the fast reaction results in a light meromyosin which exhibits only a single phase melt, indicating that the more heat labile phase may be correlated with the trypsin-sensitive region. Evidence is presented to show that the rod exhibits a substantial amount of flexibility under conditions where no discernible loss in helix content occurs. The possible significance of the trypsin labile helical region of the myosin rod to the contractile process is discussed.

A variety of proteolytic enzymes, including trypsin, chymotrypsin, and subtilisin, rapidly cleave the myosin molecule into two relatively homogeneous, high molecular weight fragments, light meromyosin and heavy meromyosin. Hydrodynamic and electron microscope studies have demonstrated that cleavage occurs predominantly within the helical rod segment in a region 700–1000 Å from the tail end of the molecule. More recently, Kominz *et al.* (1965) and Lowey *et al.* (1969) have shown that papain, which also cleaves the molecule in the rod region, has a slightly greater preference for attack at a second site near the globular (subfragment I) segments. The reason for the increased susceptibility of the polypeptide chains to proteolytic fission in the rod segment is unclear at the moment. Segal *et al.* (1967) observed a rapid release of proline-containing peptides in the early stages of

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tryptic digestion of myosin, and suggested that proline residues, localized in the trypsin-sensitive belt, were responsible for disruption of the α-helical conformation in this segment, thus providing a more open and susceptible structure for enzymatic attack. This proposal was challenged by Lowey *et al.* (1969), who found no analytical evidence for the presence of proline in the myosin rod isolated following papain treatment of the parent molecule. Results to be presented below support this conclusion. The proline containing peptides released in the early stages of proteolysis must therefore arise from very rapid cleavage within or near subfragment I. Nevertheless, the significance of the trypsin-sensitive region of the rod in the organization of the thick filament of muscle and in the mechanism of contraction has been emphasized by several authors. Huxley (1969) proposed that this region may act as a hinge enabling the heavy meromyosin portion of myosin to move away from the thick filament surface during contraction. Pepe (1967) has suggested that this region provides a flexible coupling to the cross bridge, allowing the globular heads of myosin molecules, whose light meromyosin segments lie in the interior of the filament core, to be arranged systematically along the surface of the filament. A recent theory of muscular contraction (Harrington, 1971) has invoked a helix–coil transition within this region as a means for producing tensile force in the I filaments.

In the present study we have examined the proteolytic kinetics and the thermal and pH stabilities of the myosin rod and have compared these properties with those of light meromyosin prepared by papain treatment and subfragment II. Our results indicate a region in the predominantly helical rod section of myosin which is intrinsically less stable than the remainder of the structure. Evidence is provided demonstrating that this section is, in fact, the proteolytic-labile region.

Materials and Methods

All water used was glass distilled. Bovine serum albumin, ovalbumin, and cytochrome *c* were purchased from Pentex, Worthington, and Sigma, respectively. Iodoacetamide-*1*-^{14}C was purchased from New England Nuclear, Boston, Mass. Blue Dextran 2000 was a product of Pharmacia. Bio-Gel A-15M (4% agarose) was purchased from Bio-Rad Laboratories (Control No. 97813, mesh 100–200). Urea was recrystallized from hot filtered solutions of ethanol after removal of activated charcoal; guanidine hydrochloride (Gdn·HCl) was prepared by the method of Anson (1941). All other reagents were of analytical grade. Experiments were carried out at 5° unless otherwise stated.

The preparation of myosin and the papain proteolytic fragments including the rod segment of myosin, light meromyosin, and subfragment II has been reported in detail elsewhere (Kielley and Bradley, 1956; Godfrey and Harrington, 1970; Lowey *et al.*, 1969; Harrington and Burke, 1972).

Protein concentrations were usually determined by fringe count from synthetic boundary experiments in the ultracentrifuge on the concentrated stock solutions (Harrington and Burke, 1972). Absorbance measurements at 280 nm were also employed in the case of myosin (Godfrey and Harrington, 1970). All dilutions were made gravimetrically.

Optical Rotation and Circular Dichroism Procedures. A Cary 60 spectropolarimeter was used for the optical rotatory dispersion (ORD) and circular dichroism (CD) measurements. Jacketed quartz cuvets were used. Temperature control was monitored by measuring the temperatures of the inlet and outlet water circulating through jacketed quartz cells and was found to vary between ±0.3°. The instrument was first calibrated against solvent to give a constant base line by adjustment of the "multipot" controls over the desired wavelength range 350–600 nm. The ORD of the sample was then obtained; the data were analyzed by both the Moffitt–Yang (1956) and the Shechter–Blout (1964) procedures. In the former analysis, a value for the parameter $b_0 = -700$ was taken as representative of a 100% α helix.

The effect of temperature on the optical rotation of the rod segments was investigated by following the change in rotation at the Cotton trough (233 nm) as a function of temperature. Because of their low thermal pH coefficient, phosphate buffers were routinely used at pH values of 7.0 and 7.5. A solvent blank was determined using the same procedure, and this "blank" value was subsequently subtracted from the reading of the protein at the corresponding tempe ature. The temperature was elevated through 2° intervals within the cooperative transition phases of the melt curve. Since small changes in rotation are discernible over considerable lengths of time, it was inconvenient to obtain true equilibrium values, so instead, the value of the rotation 15 min after reaching the desired temperature was employed in this study. It has been shown recently (Harrington and Burke, 1972) that light meromyosin and the myosin rod undergo rapid, reversible dimerization and for this reason we have purposely utilized low protein concentrations (of the order of 0.008%), where the equilibrium is shifted in favor of monomer. A mean residue weight of 115 has been assumed.

In the analysis of the thermal melting behavior of myosin rod, light meromyosin prepared by papain treatment, and subfragment II, the linear portion of the $[m']$ *vs.* temperature plot (5–25°) was extrapolated to high temperatures assuming this line to represent the thermal coefficient of the mean residue rotation $(d[m']/dT)_{233}^{helix}$ for the specific two-stranded helical structure under study. A value of 61°/°C for $(d[m']/dT)_{233}^{helix}$ was found for both paramyosin and its proteolytic fragment light paramyosin (Halsey and Harrington, 1973). Since the Moffitt parameters for these two particles ($b_0 \sim -670$) indicate them to be virtually 100% α helical, this thermal coefficient would appear to be a characteristic parameter for an intact, two stranded α rope. We find an identical value for $(d[m']/dT)_{233}^{helix}$ of myosin rod (see Figure 1), but the thermal coefficients of light meromyosin (prepared by papain treatment) and subfragment II are somewhat lower (50°/°C and 43°/°C, respectively) suggesting some perturbation in the helix geometry as a result of the proteolytic treatment. Nevertheless, both particles have a high helix content as judged from their Moffitt parameters ($b_0 = -660$ and -640, respectively) and in the present treatment we assume them to be 100% helical at 6°. The value of $[m']_{233}$ at 80° was taken to be that of the random coil at this temperature. For the purposes of the present investigation, the thermal coefficient $(d[m']/dT)_{233}^{coil}$ of the random coil was assumed to be zero. Riddiford (1966) has measured $[m']_{233}$ of paramyosin as a function of temperature in 7 M Gdn·HCl and has found that $[m']_{233}$ is invariant with temperature. Thus, at any temperature, T, the fraction of structure (helix) present, f_H, can be represented as

$$f_H{}^T = \left(\frac{[m']_e - [m']_r}{[m']_h - [m']_r}\right)_T$$

where $[m']_e$ is the value of the reduced mean residue rotation obtained from the experimental curve and $[m']_h$ and $[m']_r$

are values of the mean residue rotation of the helix and coil, respectively, all at temperature T.

Figure 1 presents a typical optical rotation *vs.* temperature profile for rod together with the extrapolations expected from the thermal coefficients of $[m']_{233}$ for 100% double-stranded α helix and 0% helix. The data shall subsequently be presented as f_H *vs.* temperature profiles. Derivative plots where df_H/dT is plotted as a function of temperature have also been employed since this procedure yields accurate assignments of the transition temperatures (T_m) and the fraction of structure associated with each cooperative transition. A smooth curve was drawn through the experimental data and the value of df_H/dT was obtained as a function of temperature by the smoothing method of Savitzky and Golay (1964) employing a sliding fit on five data points equally spaced at 1° intervals.

pH-Stat Digestions. The pH-Stat kinetics have been analyzed by procedures described elsewhere (Mihalyi and Harrington, 1959; Segal *et al.*, 1967). The kinetic parameters obtained for myosin and its fragments can be compared if the conditions of proteolysis are normalized as follows. The amount of enzyme employed in each case was a constant fraction of the total number of bonds split. This number of bonds split was obtained by employing normal ratios of enzyme to protein and allowing the reaction to proceed for sufficient time to characterize the slow reaction precisely. An alternative procedure employing relatively high enzyme to protein ratios and shorter times was also used. The total number of bonds per 10^5 g of each segment can be reliably obtained by utilizing both these methods. In the present investigation we have employed a ratio of 90:1 (protein to enzyme on a weight basis) corresponding closely to 100 μmol of bonds/mg of trypsin for rod, light meromyosin (papain treated), and subfragment II. This ratio is comparable to values employed in earlier studies on myosin.

Viscosity Determinations. An Ostwald viscometer with a moderately long capillary (90 cm) and an average shear gradient of 158 sec^{-1} was employed for the viscosity measurements. The kinetic energy correction was found to be negligible. Temperature variation was monitored and was found not to exceed ±0.02°. All protein stock solutions were centrifuged at 20,000 rpm for 60 min to remove particulate matter, and the supernatant was carefully withdrawn. All dilutions were made gravimetrically. Viscosity measurements were done on 2.0-ml samples and were repeated four–five times on each solution.

Determination of Structural Integrity of Myosin Rod. Since myosin rod is obtained by limited papain digestion of myosin it is necessary to establish the extent of main chain degradation produced by its isolation. This has been examined by the experimental procedures of Fish *et al.* (1969) and Weber and Osborn (1969). A typical elution profile obtained by gel filtration in 6 M Gdn·HCl–0.1 M mercaptoethanol is shown in Figure 2a. Myosin and the myosin rod always eluted as single, slightly asymmetric peaks, which in the case of the latter corresponded to a weight of 105,000 (±10,000). The sodium dodecyl sulfate gel electrophoretic method yielded a molecular weight of 100,000 (±10,000) for the major band which comprised greater than 85% of the total protein as measured by densitometery. The remaining material gives bands corresponding to 70,000 ± 5000 and 24,000 ± 6000 (Figure 2b). We believe that these lower molecular weight bands arise by sodium dodecyl sulfate denaturation of a small proportion of rod molecules containing a few internal peptide cleavages, since high-speed sedimentation equilibrium experiments on the rod in nondenaturing solvents show no evidence

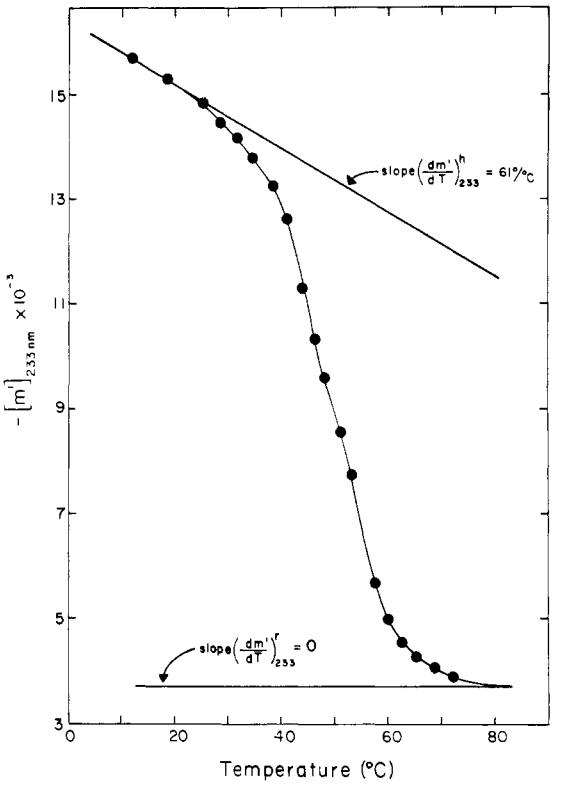

FIGURE 1: The effect of temperature on the reduced mean residue rotation of the myosin rod. Protein concentration 0.01 g/dl in 0.5 M KCl–0.005 M PO_4^{2-}, pH 7.2. The extrapolated lines represent the thermal coefficients for the reduced mean residue rotations of a double-stranded helix and random coil.

of heterogeneity and yield a molecular weight of 198,000 for rod (Harrington and Burke, 1972). The studies, therefore, demonstrate that the major portion of the rod preparation is free of internal bond cleavage and that these preparations provide a reasonable model for investigating the structure and stability of this section of the myosin molecule.

Amino acid analyses on the rod were performed by the method of Spackman *et al.* (1958) using normal and high loading concentrations. In neither case was proline detected, in agreement with the findings of Lowey *et al.* (1969).

Results

Kinetics of Tryptic Proteolysis of Rod, Light Meromyosin (Prepared by Papain Treatment), and Subfragment II. Earlier kinetic analyses of the tryptic digestion of myosin revealed the presence of two parallel first-order reaction classes with rate constants differing by approximately an order of magnitude. Although the rate of cleavage of the parent molecule into the two fragments light meromyosin and heavy meromyosin appeared to correlate with the faster of these two reactions, the size of the trypsin-sensitive belt digested during production of these fragments has been difficult to establish experimentally as a result of concomitant cleavage in the folded, globular region of the molecule. The recent report of Lowey *et al.* (1969) that relatively undegraded rod, light meromyosin, and subfragment II segments of myosin can be isolated following brief treatment of the native protein with insoluble papain suggests that these particles can be utilized to investigate the size and stability properties of the trypsin-sensitive "hinge" region of the rod.

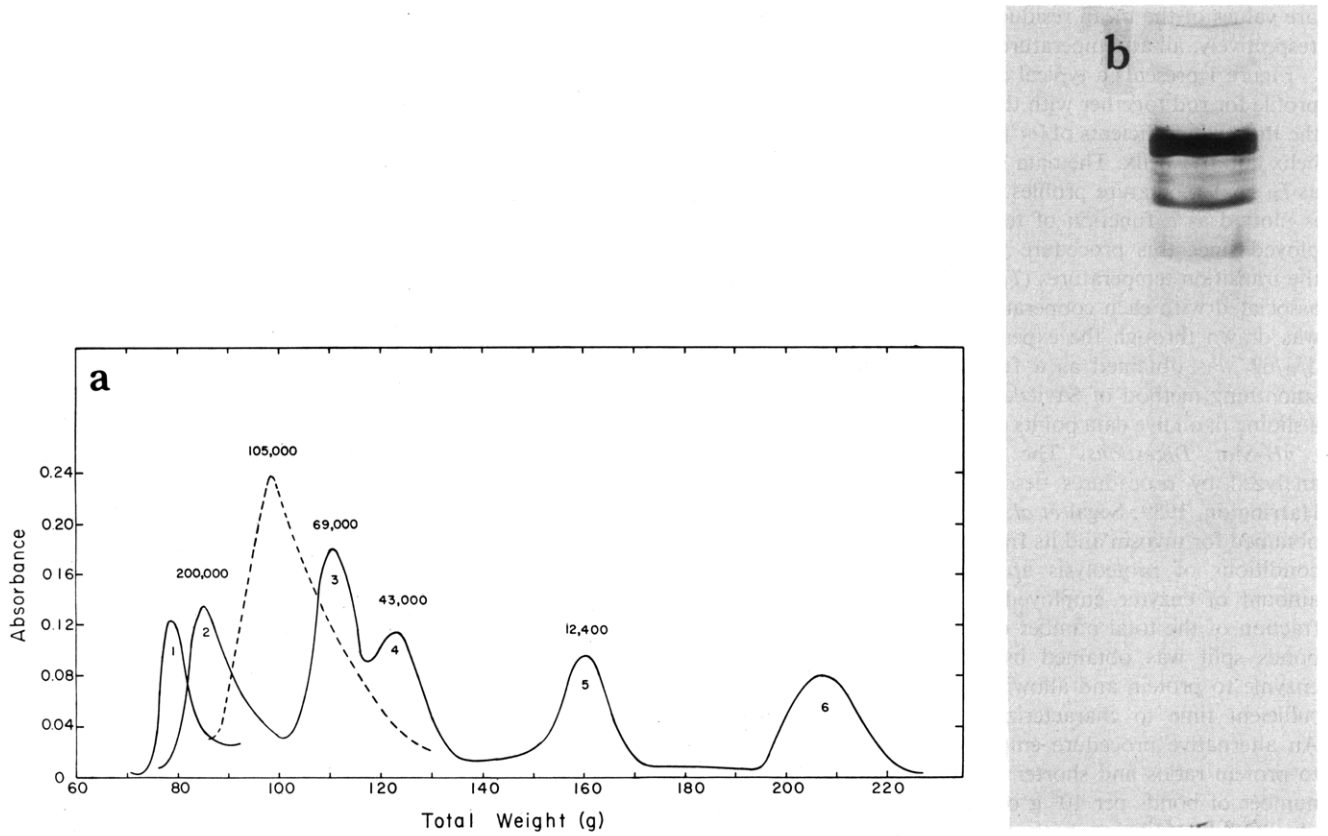

FIGURE 2: (a) Elution pattern of proteins by gel filtration on 4% agarose in 6 M Gdn·HCl–0.1 M mercaptoethanol. Peaks 1–6 are Blue Dextran myosin, bovine serum albumin, ovalbumin, cytochrome *c*, and DNP alanine, respectively. The dashed line represents the elution profile of the myosin rod. (b) Sodium dodecyl sulfate gel electrophoresis of myosin rod on a 10% acrylamide gel employing half the normal concentration of cross-linking reagent (see Materials and Methods).

TABLE I: Summary of Kinetic Data from the pH-Stat Studies (25°).

Fragment	Mol Wt (g/mol) $\times 10^{-5}$	Bonds/Mol: Very Fast (I)	Bonds/Mol: Fast (II)	Bonds/Mol: Slow (III)	First-Order Rate Constants[a]: k_I	First-Order Rate Constants[a]: k_{II}	First-Order Rate Constants[a]: k_{III}
Rod	2.0	38	96	99	200	31	5.5
Light meromyosin[b]	1.4	21	74	65	150	29	6.5
Subfragment II	0.6	10	13	49	350	83	7.8

[a] First-order velocity constant ($\times 10^3$) calculated with natural logarithms and time in minutes. [b] Prepared by papain treatment.

Table I presents the results of the kinetic analyses of tryptic bond cleavage for the three papain fragments according to the procedure described under Materials and Methods. Although the kinetics of tryptic proteolysis of native myosin invariably shows the two discrete reaction classes described above, proteolysis of the papain fragments exhibits a more complicated cleavage process. Two reaction classes with rate constants comparable to those estimated from the time dependence of myosin proteolysis are observed, but an additional reaction class with much higher rate constant is required to fit the data in the early stages of the reaction. Since the first-order rate constants, corresponding to the three parallel classes of the overall reaction (very fast, fast, and slow), are quite reproducible and are comparable for each particle, it seems likely that they reflect the presence of common structural features in all three rod segments.

Assuming that the origin of the very fast reaction class stems from a modification of the structure introduced by the papain cleavage (possibly a local destabilization or perturbation of the structure in the vicinity of the terminus of the fissioned peptide bond), it is clear that the number of fast bonds in the rod (96 per 198,000 g/mol) is in fair agreement with the fast tryptic reaction of myosin (86 per 460,000 g/mol). This information indicates that the fast reaction class of bonds observed in tryptic digestion of native myosin resides mainly in the interior of the rod section. The total number of slow bonds cleaved in the rod amounts to 112 ± 18/198,000 daltans and accounts for only 37% of the total slow bonds cleaved in myosin; the remaining slow bonds must, therefore, arise from cleavage in the head region or in the region connecting the rod to the globular subfragment I sections. It will also be seen from Table I that the fast tryptic bonds are

TABLE II: Second-Order Rate Constants Calculated from Pseudo-First-Order Rates.

pH-Stat Data	k_I[a]	k_{II}[a]	k_{III}[a]
Present data (rod)	92	15	3.4
Mihalyi and Harrington (1959) (myosin)		29	4.9
Segal *et al.* (1967) (myosin)		33	3.1

[a] Second-order velocity constant ($\times 10^{-3}$) k values are in mol^{-1} min^{-1}.

TABLE IV: Summary of Optical Rotatory Dispersion on Rod Fragments of Myosin.

Particle	b_0	% Helix M-Y[a]	% Helix H_{193}[c]	% Helix H_{225}[c]
Rod	−680,[a] −660[b]	97	98	100
Light meromyosin[d]	−660,[a] −630[b]	95	96	98
Subfragment II	−640,[a] −610[b]	92	94	95

[a] Moffitt–Yang procedure, present data at 2°. [b] Moffitt–Yang procedure at 20° (Lowey *et al.*, 1969). [c] Shechter–Blout (1964) procedure at 5°. [d] Prepared by papain treatment.

partitioned between light meromyosin (prepared by papain treatment) and subfragment II, with about 85% of this class remaining with the papain-prepared light meromyosin particle. We infer that this class of bonds has its origin in a region common to both particles.

It is possible to obtain the true second-order rate constant (k_2) from the pseudo-first-order rate constant (k_1) since $k_1 = k_2[E]$, where [E] is the molar concentration of the enzyme. This parameter has been calculated from the data on rod (Table I) and in myosin (Mihalyi and Harrington, 1959; Segal *et al.*, 1967) and the estimated values are presented in Table II. Clearly, these data show that the k_2 values for the fast and slow reactions for rod are of the same order of magnitude as those obtained from the myosin data, suggesting that the lysine and arginine residues cleaved in the various rate classes reside in similar structural environments in myosin and the rod. Thus, the fraction of bonds split in each class can as a first approximation be assigned to the amount of structure associated with these rate classes (Table III).

Rotation Melt Experiments. Optical rotatory dispersion studies of the rodlike subfragments indicate that rod, papain-prepared light meromyosin, and subfragment II have an extremely high helical content (see Table IV and also Lowey *et al.*, 1969) which would apparently rule out the existence of an extensive disordered region within the tail segment of the native protein. Nevertheless, the clear indication of partitioning of the cleavage reaction into reaction classes may still reflect the presence of an easily perturbable helical region. In this connection, Segal and Harrington (1967) reported tritium exchange data on myosin suggesting the presence of an exchanging class sensitive to changes in pH from 6.5 to 7.0. This class is not observed in the proteolytic fragments light meromyosin and heavy meromyosin.

An alternative way of approaching this problem is to examine the behavior of a parameter of structure such as optical rotation as a function of temperature, since the "melting" behavior of the two-chain α-helical rod would be expected to be highly cooperative. Figures 3, 4, and 5 present fraction helix (f_H) *vs.* temperature profiles (see Materials and Methods) for the three rodlike fragments from which it is clear that there are two distinct cooperative transitions. The biphasic nature of these "melting" plots is more clearly seen in the derivative profiles shown in the inserts of Figures 3–5. Areas corresponding to the first and second cooperative phases are designated I and II, respectively, and are proportional to the amount of structure associated with each. The temperatures at the maxima represent the transition temperatures of the cooperative phase. These data have been summarized in Table V. We have also examined the helix → coil transition of light meromyosin prepared by tryptic digestion of myosin through 90% of the fast reaction. This product yields a single transition with T_m of 45° (Figure 6). The fact

TABLE III: Estimate of Ordered Structure from pH-Stat Kinetics.

Particle	% Structure of Reaction Class I	II	III
Rod	16	41	43
Light meromyosin[a]	13	46	41
Subfragment II	14	18	68

[a] Prepared by papain treatment.

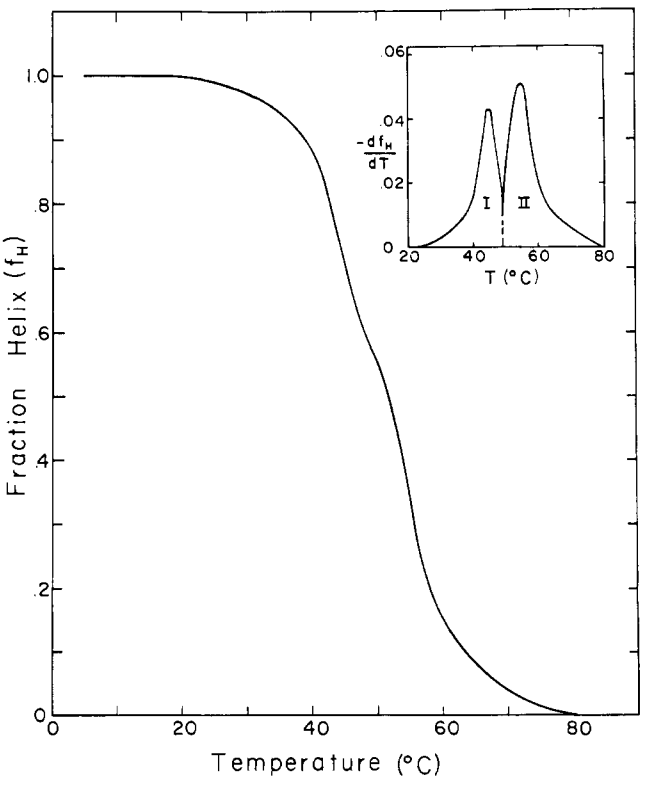

FIGURE 3: Amount of structure (f_H) as a function of temperature for the myosin rod; insert, derivative plot as a function of temperature.

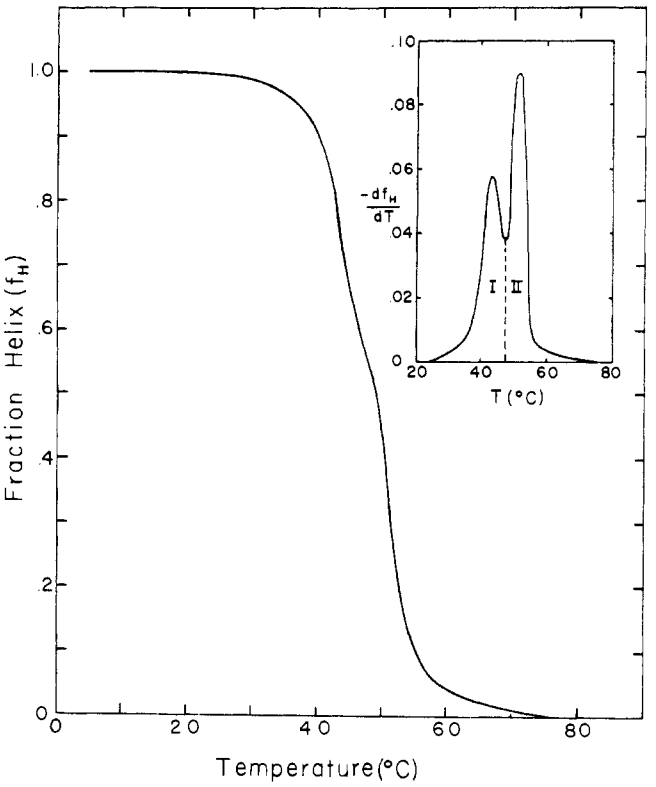

FIGURE 4: Fraction of helix (f_H) of papain-prepared light meromyosin as a function of temperature; insert, derivative plot with respect to temperature.

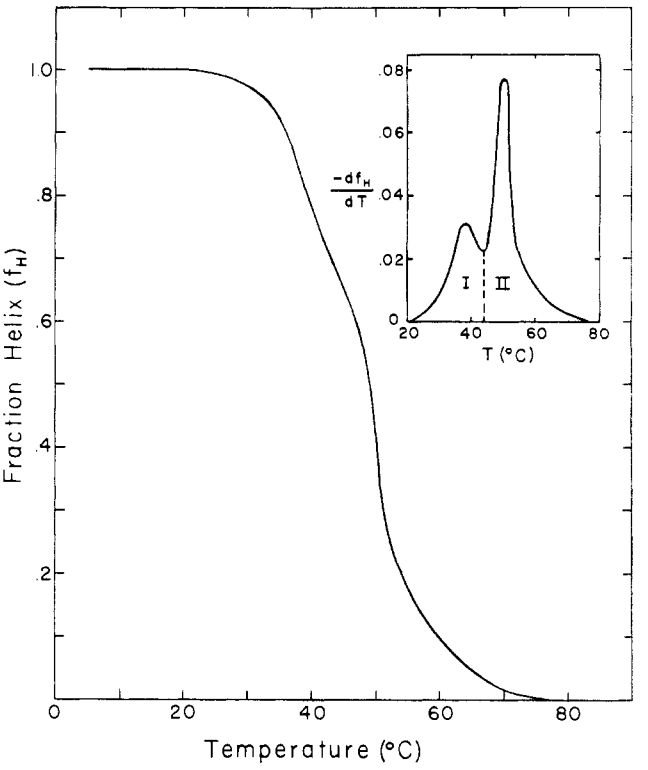

FIGURE 5: Fraction of helix (f_H) of subfragment II as a function o temperature; insert, derivative plot as a function of temperature.

that the "melting" profile of this particle is monophasic suggests that the segment of low thermal stability of the rod (T_m = 44°) has been eliminated by the extensive proteolysis employed in the isolation of this light meromyosin fragment. A significant number of slow bonds (about 20%) will be cleaved in light meromyosin during the period of time associated with the fast reaction phase and it seems likely that the random clips introduced in the interior of the light meromyosin segment will result in some destabilization of this structure leading to a lowered T_m. In the case of paramyosin, Halsey and Harrington (1973) observed that digestion through the fast reaction stage results in complete elimination of the first transition phase of the melting curve. In this case, the reaction rates of fast and slow classes differ by a factor of about 20 and very few bonds are cleaved in the interior of the structure during the isolation of paramyosin and light meromyosin.

Evidence for Flexibility from Viscosity Studies. The melting behaviors of myosin rod and light meromyosin have also been investigated by measurement of the reduced viscosity as a function of temperature. Reduced viscosity–temperature plots of rod (see Figure 7) show a viscosity drop on increasing temperature which is distinctly different from that observed for papain-prepared light meromyosin. Over the range 5–35° a significant decrease (about 25% of the total helix → coil viscosity change) in reduced viscosity occurs while this parameter for papain-prepared light meromyosin shows virtually no change over the same temperature span. A dramatic fall in asymmetry of both particles is seen over the temperature range associated with the first cooperative transition of the optical rotation melt curves. In the case of the rod an 80% drop occurs in reduced viscosity, compared to a loss of 42% in helix content. This finding is consistent with the unfolding of a region in the interior of the rod. The reduced viscosity of light meromyosin (prepared by papain treatment) falls about 50% for a comparable loss in helix content, and this change would appear to be compatible with the suggestion advanced earlier, that the residual segment of the lowest melting region of the myosin rod occurs at one end of the papain-prepared light meromyosin particle.

The behavior of the viscosity profiles at temperatures below 35° is deemed to be of special significance. Arguments have already been presented (see Methods) to support our contention that the helical structure remains virtually intact up to the temperature initiating the first cooperative transition. Yet it seems clear from the drop in reduced viscosity that the rod particle becomes significantly more flexible with increasing temperature. Since no comparable change is observed in the highly asymmetric papain-prepared light meromyosin particle, it seems reasonable to infer that the observed flexing in the rod stems from a localized perturbation within the interior, trypsin-sensitive region of the particle.

In view of this finding and its possible relevance to con-

TABLE V: Analysis of Thermal Dependence of Optical Rotation of Rod Segments.

Particle	T_{m_1}	T_{m_2}	% Total Structure I	% Total Structure II
Rod	44	55	42	58
Light meromyosin[a]	44	52	45	55
Subfragment II	39	50	34	66

[a] Prepared by papain treatment.

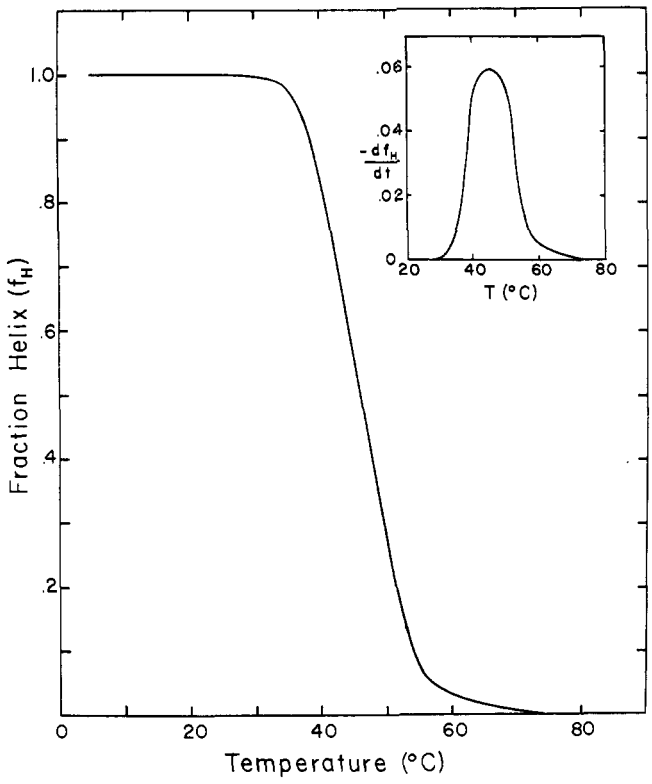

FIGURE 6: Fraction of helical structure (f_H) of long digested light meromyosin as a function of temperature; insert, derivative plot as a function of temperature.

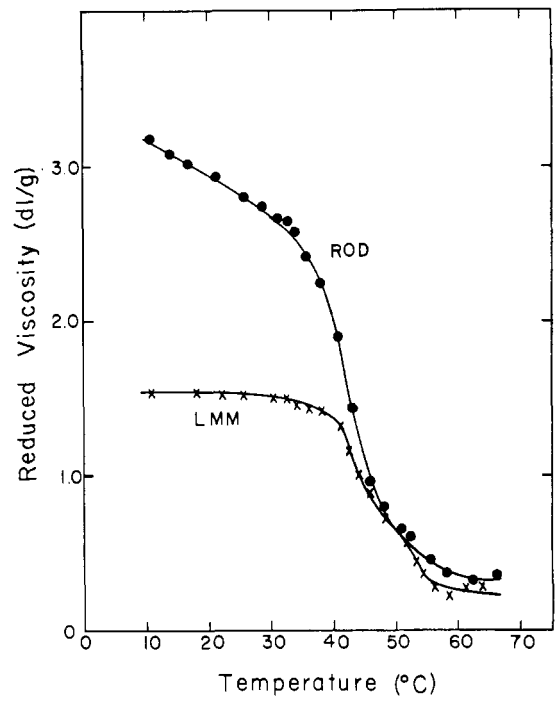

FIGURE 7: The effect of temperature on the reduced viscosity of myosin rod and papain-prepared light meromyosin. Solvent is 0.5 M KCl–0.02 M PO_4^{2-}–0.001 M EDTA, pH 7.0.

traction (see Discussion) a search was initiated for other environmental conditions which would induce hinging. To amplify the effect, the reduced viscosity of rod at high ionic strength was examined at temperatures just below T_m (40°) in the presence of a variety of low molecular weight substances believed to be relevant to contraction. These included Ca^{2+}, Mg^{2+}, ATP, and ADP to a concentration of 10^{-2} M.

No significant changes were observed in the reduced viscosity of rod under any of the ionic conditions employed with the notable exception of low pH. We found that the intrinsic viscosity of rod at pH 2.0 is (at 5°) substantially lower (~25%) than that observed at pH 7.0. Figure 8 presents a reduced viscosity *vs.* concentration plot (upper curve) for rod in a neutral, high salt medium (0.5 M KCl–0.2 M PO_4^{2-}–0.01 M EDTA, pH 7.0) showing upward curvature with increasing concentration consistent with the monomer–dimer chemical equilibrium in this solvent system (Burke and Harrington, 1972). The reduced viscosity *vs.* concentration plot of rod at pH 2 (0.5 M KCl) shows a significant drop in intrinsic viscosity and the increasing slope of this plot with protein concentration suggests the presence of some type of particle–particle interaction in this system as well. The decrease in reduced viscosity in acid is completely reversible; dialysis of the pH 2 solution against the neutral pH, high salt buffer results in an elevation of η_{sp}/c to its original value. Quite different results are obtained for light meromyosin. The upward curvature of the η_{sp}/c *vs.* c plot of this particle, signifying the monomer–dimer association in the neutral pH, high salt environment is damped out at pH 2, but there is no decrease in intrinsic viscosity in the acid medium.

In spite of the large viscosity change observed in the rod, ORD and CD measurements show no significant differences in the conformational pattern at pH 7 and 2. Thus the results are comparable to those observed in the temperature studies of viscosity and are consistent with a local perturbation of the structure in the vicinity of the rod center.

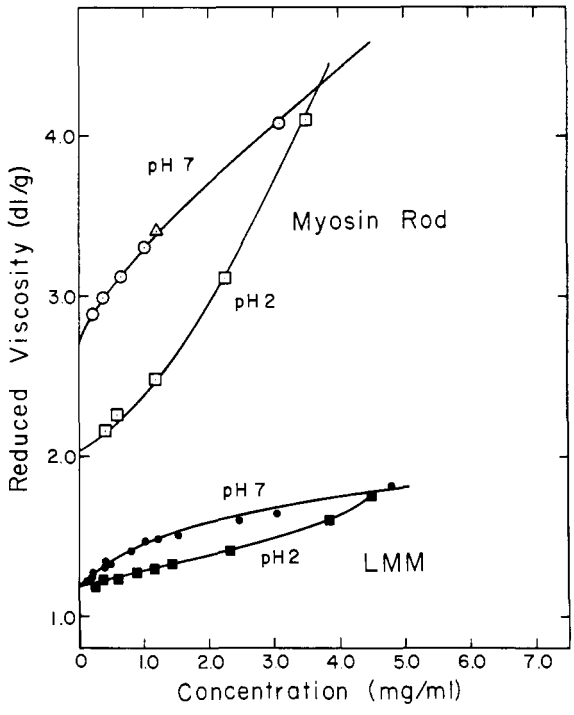

FIGURE 8: The effect of pH on the reduced viscosity *vs.* concentration behavior of myosin rod and papain-prepared light meromyosin. pH 7.0 solvent is 0.5 M KCl–0.2 M PO_4–0.01 M EDTA, and pH 2.0 solvent is 0.5 M KCl–0.01 M HCl. The open triangle refers to the reversal experiment (pH 7.0 → pH 2.0 → pH 7.0); temperature, 5°.

Discussion

Although the three physical methods employed in the present study are rather crude techniques for probing the internal structure of the myosin rod, the assembled pH-Stat, optical rotation and viscosity results, taken together, provide rather compelling evidence for the presence of a structural element of low thermal stability within the interior of the coiled coil tail segment of the myosin molecule. Our optical rotatory dispersion studies indicate, in agreement with the previous work of Lowey *et al.* (1969), that the myosin rod and its smaller fragments light meromyosin (prepared by papain-treatment) and subfragment II have an extremely high α-helical content at temperatures below ambient (see Table IV). These parameters, together with the respective intrinsic viscosities, indicate that these particles are rigid, coiled coil rods (Lowey *et al.*, 1969; Harrington and Burke, 1972). On the other hand, the heat denaturation studies (Figure 3 and Table V) show that the helical structure of the rod is not of uniform stability since complete unfolding of the molecule occurs through two cooperative transitions with T_m of 44 and 53°. Optical rotation *vs.* temperature profiles of papain-treated light meromyosin and subfragment II exhibit two cooperative melting transitions with very similar T_m values to that of rod, whereas light meromyosin produced by prolonged tryptic digestion through 90% of the fast reaction yields a single, broad transition. Woods (1969) has examined the thermal denaturation of light meromyosin, formed by brief tryptic proteolysis of myosin, under acidic (pH 2.0) and alkaline (pH 9.0) conditions and has also observed that the transition occurs in two steps. The transition midpoint of the light meromyosin fragment isolated at the end of the fast reaction phase of proteolysis ($T_m = 45°$) as well as the width of the transition profile (see Figure 6) suggest that some destabilization of the structure occurs as a result of interior peptide bond cleavage.

Analysis of the helix content *vs.* temperature plots (Table V) reveals that about 42% of the total coiled coil structure of the rod is involved in the cooperative transition with $T_m = 44°$. We estimate, therefore, that a segment of the rod comprising about 84,000 g/mol or about 730 amino acid residues melts in this first cooperative transition. In a previous study (Segal *et al.*, 1967) the average size of the peptides released in the rapid phase of myosin proteolysis was estimated to be 680 g/mol. Thus, the size of the trypsin-sensitive belt deduced from the present pH-Stat kinetics is 96 × 680 or ~65,000 g/mol, a value in reasonably good agreement with the size of this segment previously estimated from the kinetics of proteolysis of native myosin (60,000–74.000 g/mol based on a monomeric weight of 4.6×10^5 for myosin.)

The evidence that the segment of low thermal stability occupies a region adjacent to the center of the rod is compelling. Limited papain proteolysis of myosin results in formation of rod with an average length of 1450 Å and mol wt 200,000 g/mol (Harrison *et al.*, 1971; Harrington and Burke, 1972), while more extensive papain proteolysis results in the production of papain-prepared light meromyosin and subfragment II. The molecular weights of these particles, 140,000 and 60,000 g/mol, respectively, have been obtained by high-speed sedimentation equilibrium studies (Lowey *et al.*, 1969). It appears that very little mass is lost during papain cleavage, suggesting that the number of bonds split in the fast and slow reactions should, when summed for papain-prepared light meromyosin and subfragment II, total closely those obtained by tryptic proteolysis of the rod. The results given in Table I are in reasonable agreement with this prediction. According to the optical rotation results presented in Table V the residual labile segment represents about 45% of the papain-prepared light meromyosin structure, whereas the remainder of this segment is retained in subfragment II and represents ~34% of its mass. The size of the trypsin-sensitive belt deduced from this data (~80,000 g/mol) is consistent with its size estimated from the melt profile of the rod.

Although none of the size measurements of the labile helix region can be considered definitive, we conclude from the present study that this segment comprises a significant fraction of the myosin tail (~450 ± 50 Å). Our results would suggest, in general agreement with earlier studies (Woods *et al.*, 1963; Segal *et al.*, 1967), that this region spans a segment lying 700–1100 Å from the tail end of the molecule, that is, it comprises a portion of both papain-prepared light meromyosin and subfragment II.

Tritium exchange studies of Segal and Harrington (1967) have demonstrated that 500–650 (per 460,000 daltons) slowly exchanging hydrogens (identified as arising from the trypsin-sensitive belt) become rapidly exchanging on raising the pH from 6.5 to 7.0; elevating the temperature to 25° produces similar results. The comparatively high availability of the peptide hydrogen atoms of this region for exchange with solvent is consistent with its susceptibility to enzymatic attack and low thermal stability. An average standard free energy of 3.0 kcal/mol is required to open the exchanging units in this region, whereas in other sections of the myosin rod the exchanging units tend to be more stable with ΔF values of 3.2–6.0 kcal/mol. The primary structural basis for the instability of this segment is suggested from the composition of peptides released at various times during the fast phase of tryptic proteolysis. Halsey and Harrington (1973) have shown that the α-helix forming power of peptides (see Robson and Pain, 1971) released in the early stages of the fast reaction of myosin is extremely low; this parameter increases monotonically during the course of the fast reaction approaching the value characteristic of the native myosin molecule. Clearly, both the tritium exchange and the peptide amino acid data suggest that the trypsin-sensitive region is a less stable helical structure than the remaining light meromyosin segment.

The recent physical chemical studies of Lowey *et al.* (1969) indicate that the secondary structure of the rod segment of myosin is virtually 100% α helical and complementary hydrodynamic studies demonstrate that the solution properties of the tertiary structure are consistent with those expected for a rigid, coiled coil rod. Lowey (1971) has summarized the evidence for and against the existence, within the myosin tail, of a unique region which could act as a flexible hinge. She has concluded that there is no compelling evidence for such a region.

The rigidity of the myosin rod may, however, be altered under certain experimental conditions. Indeed, the viscosity results of the present paper indicate that the rod does hinge under conditions where the pH is lowered to 2.0 or the temperature is raised to 35°. Further, this induced flexibility is not accompanied by a significant change in the helix content as measured by optical rotation (minimum at 233 nm) or by circular dichroism (minima at 208 and 222 nm). The fact that no significant change in the viscosity or helix content of papain-prepared light meromyosin occurs under identical conditions strongly suggests that a segment of the structure adjacent to the light meromyosin section of the rod is the site of bending with increasing temperature or decreasing pH. We conclude, therefore, that the rod portion of the myosin

molecule can indeed undergo flexing by a local perturbation of the structure in the vicinity of the trypsin-sensitive region.

The question immediately raised is whether this induced flexibility, together with the cooperative, temperature-labile helical segment in the rod section of the myosin molecule, has any physiological significance. It will be recalled that the model of the thick filament proposed by Pepe (1967) requires side by side alignment of the light meromyosin sections of the myosin molecules such that they lie parallel to the long axis of the thick filament. In order for all the globular heads (cross bridges) of the myosin molecules to project to the surface of the filament, Pepe (1967) has proposed that the myosin molecule is deformed in the trypsin-sensitive region.

Any mechanism for muscle contraction based on the sliding filament–swinging cross-bridge theory must be able to account satisfactorily for cross-bridge movement and for the way in which the cross bridges adapt to the variable interfilament spacings caused by changing sarcomere lengths. Huxley's model (1969) has relied strongly on the postulate that flexibility arises in the regions of the molecule which are sensitive to proteolytic attack and proposes that this flexibility can account for movement of the cross bridges over a wide span of interfilament spacings. Our findings demonstrate that flexibility in the myosin rod arises by perturbation of the trypsin-sensitive region by changes in the solution environment or by an increase in temperature. Further, the dimensions of the trypsin-sensitive region elucidated from the present work indicate that it extends from ~700 to 1100 Å from the light meromyosin terminus of the molecule. This suggests that it may contribute significantly in length to the nonassociating or cross-bridge portion of the molecule.

Huxley and Simmons (1971) have measured the transient responses occurring when striated muscle under isometric tension is subjected to sudden changes (<1 msec) in length (±0.1–1.5%). The tension response occurs in two stages. The initial phase is very fast and occurs concomitantly with the step change in length (<1 msec), while the second stage requires a few milliseconds for completion. They have interpreted these observations in terms of two structural elements in series comprising the cross bridge. The instantaneous tension response is assumed to arise from an elastic element in the cross bridge and has been assigned to the region between the light meromyosin portion and the myosin head. The quick tension recovery is thought to result from rotation of the myosin head through binding states of decreasing potential energy while it remains attached to the actin filament. A requirement of their theory is that the linkage between light meromyosin and the myosin head (subfragment II) be elastic and capable of being extended as much as ~100 Å beyond its isometric length. Since subfragment II is an α-helical, two-stranded rope it is highly unlikely that this segment of the myosin rod could suffer an elastic deformation of this magnitude. However, the element of instantaneous elasticity in the cross bridge may reside anywhere within the myosin molecule not involved directly in association within the core of the thick filament and may, therefore, include a major portion of the trypsin-sensitive region.

In view of our findings that the trypsin-sensitive segment can undergo a cooperative phase change at low temperature, and the likelihood that the opening of this segment will show a high degree of sensitivity to applied tension (Flory, 1956), we offer an alternative interpretation of the length–tension measurements of Huxley and Simmons (1971). Under conditions where the cross bridge is attached to actin during isometric tension we postulate that the trypsin-sensitive region has undergone a helix to coil transition resulting in tension generation (Harrington, 1971). This set of circumstances can be looked upon as representing the equilibrium situation for a random coil under tension. Should the length of the fiber be now suddenly increased or decreased by a small increment, this will result in a situation analogous to a relaxation or temperature-jump experiment.

Consider the situation where the fiber under isometric tension is instantaneously (<1 msec) extended by a small increment. We would expect an immediate resistance to stretch by the random coil resulting in an instantaneous increase in tension. This would be accompanied or followed by a partial crystallization of the coil, as a consequence of the applied tension increment, resulting in a rapid decay in tension (see Flory, 1956). If, on the other hand, the length of the fiber under isometric tension is instantaneously decreased by a small increment the length change would be expected to result in a simultaneous decrease in tension. This would be followed or accompanied by an *increase* in tension brought about by additional "melting" as the partially crystalline structure rapidly readjusts to the new decreased length.

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Hemoprotein–Ionic Detergent Interaction. Volume Effects Produced by Reaction with Sodium Dodecyl Sulfate†

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ABSTRACT: The volume effects produced by the reaction of sodium dodecyl sulfate with sperm-whale ferrimyoglobin, human oxyhemoglobin, and methemoglobin are determined by the proteins' composition, three-dimensional structure, association state, the type and ligand state of the heme moiety. The reaction of proteins with sodium dodecyl sulfate produces volume changes which are the resultant of water–protein, water–detergent, and protein–detergent interactions. The volume changes were determined dilatometrically at 30.0 ± 0.001°. The data were converted to a δV function which is the measure of the volume effect resulting from protein–sodium dodecyl sulfate interaction (Katz, S., Shaw, M. E., Chillag, S., and Miller, J. E. (1972), *J. Biol. Chem. 247*, 5228). The δV isotherm for myoglobin is characterized by a sharp volume rise at low sodium dodecyl sulfate concentrations reaching a value of 200 ml/10^5 g of myoglobin at 0.005 M sodium dodecyl sulfate. This parameter decreased to a minimum of −450 ml/10^5 g of protein at 0.07 M sodium dodecyl sulfate and then exhibited a positive nonlinear functional dependence on sodium dodecyl sulfate reaching a value of 10 ml/10^5 g of myoglobin at 0.05 M sodium dodecyl sulfate concentration. The δV isotherms for oxyhemoglobin and methemoglobin were characterized by abrupt volume decrease at low sodium dodecyl sulfate concentrations, *i.e.*, 0.005–0.07 M, with values for the minima being −725 and −875 ml per 10^5 g of protein at about 0.09 M sodium dodecyl sulfate, respectively. As the sodium dodecyl sulfate concentration increased to 0.5 M there was a gradual increase of the δV isotherm with values of −550 and −750 ml per 10^5 g of protein found at 0.5 M sodium dodecyl sulfate. While kinetic effects were observed for myoglobin at a restricted range of concentrations, 0.005–0.02 M, the hemoglobins exhibited time-dependent volume changes over the entire range of detergent concentrations. These isotherms are the composite of detergent binding, conformational changes, disruption of quaternary structure, and the type and ligand state of the heme group.

It has been established that the reaction of sodium dodecyl sulfate with protein produces volume effects which are a function of the protein's composition, charge and three-dimensional structure (Katz *et al.*, 1972). This investigation was extended to hemoproteins because of their physiological significance and also since they provide useful models to examine the influence of protein's composition, quaternary structure, and prosthetic groups on this parameter. Myoglobin is a single-chain hemoprotein, mol wt 17,800, whereas hemoglobins, mol wt 64,500, differ not only with respect to size but also because they consist of four subunits maintained by noncovalent forces. The heme iron exists in the ferric state in metmyoglobin and methemoglobin but is in the ferrous state in oxyhemoglobin. Acrylamide gel electrophoresis reveals two categories of protein–dodecyl sulfate complexes: one type whose electrophoretic mobility increased with increasing sodium dodecyl sulfate concentration, while the other's mobility exhibited little dependence on the detergent's concentration. The first type predominated at detergent concentration <0.05 M and is considered responsible for the large negative volume effects. The second type which dominated at sodium dodecyl sulfate concentration ≥0.07 M is associated with the positive slope of the volume isotherm; the protein under these conditions undergoes drastic conformational change (Reynolds *et al.*, 1967; Reynolds and Tanford, 1970).

Experimental Section

Methods. The dilatometric procedure for studying the reaction of dodecyl sulfate with protein has been described (Katz *et al.*, 1972). The 8:2 mixing protocol involves the mixing of 8.00 ml of detergent with 2.00 ml of 10% protein. The detergent concentrations cited are the concentrations after mixing; these ranged from 0.001 to 0.5 *M*.[1] Oxyhemoglobin was reoxygenated for 0.5 hr and then evacuated for 5 min with a Cenco Hyvac vacuum pump before use. Water was saturated with oxygen for 0.5 hr and evacuated for 5 min; the P_{O_2} for both systems was 70–80 mm. The dilatometric experiments were performed at 30.0 ± 0.001° (Katz, 1963).

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[1] This convention is used in preference to estimating the amount of sodium dodecyl sulfate bound because the volume data were determined 5–60 min after mixing, whereas binding values are obtained from equilibrium data which generally are determined several days after the initial contact.