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Thermal Stability of Myosin Rod from Various Species†

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Received November 3, 1986; Revised Manuscript Received August 24, 1987

ABSTRACT: The radius of gyration and fraction helix as a function of temperature have been determined for myosin rod from four different species: rabbit, frog, scallop, and antarctic fish. Measurements from sodium dodecyl sulfate gel electrophoresis indicate that all particles have the same molecular weight (~130K). All fragments are nearly 100% α-helical at low temperatures (0–5 °C). The melting profiles for each are qualitatively similar in shape, but their midpoints are shifted along the temperature axis in the following order: antarctic fish (T_m = 33 °C), scallop (T_m = 39 °C), frog (T_m = 45 °C), and rabbit (T_m = 49 °C). Corresponding radius of gyration vs temperature profiles for each species are shifted to lower temperatures (approximately 5–8 °C) with respect to the optical rotation melting curves. From plots of radius of gyration vs fraction helix, we find a marked drop in the radius of gyration (from 43 to ~34 nm) with less than a 5% decrease in fraction helix for rabbit, frog, and antarctic fish rods, whereas the radius of gyration of scallop rod never exceeds 34 nm. Results indicate hinging of the myosin rod of each species. The thermal stabilities of the myosin rods shift in parallel with the working temperature of their respective muscles.

In the preceding paper (Rodgers & Harrington, 1987), we have combined light-scattering and optical rotation measurements to provide evidence for a flexible region, the light meromyosin–heavy meromyosin hinge (LMM–HMM hinge),[1] in rabbit myosin rod. Hinges in myosin rod have also been clearly demonstrated in smooth muscle myosin using both electron microscopy and analytical centrifugation (Trybus et

† Supported by National Institutes of Health Grant AM-04349 (to W.F.H.) and by a Muscular Dystrophy Association postdoctoral fellowship (to M.E.R.). This is Contribution No. 1387 of the McCollum-Pratt Institute.
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[1] Abbreviations: S-1, myosin subfragment 1; LMM, light meromyosin; HMM, heavy meromyosin; TLCK, tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; IAA, iodoacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; R_g, radius of gyration; f_h, fraction helix; SDS, sodium dodecyl sulfate.

0006-2960/87/0426-8703$01.50/0

al., 1982; Trybus & Lowey, 1984). If the LMM–HMM hinge does exist in skeletal myosin, and if it is relevant to the contractile mechanism, then it should be a common property of all myosins. Furthermore, since hinges must result from a disruption of the coiled-coil structure of the rod, one would expect the thermal stability of the hinge to show some relationship to the working temperature of the muscle. In this study, we examine the temperature dependence of both the secondary and the tertiary structure of myosin rod isolated from four different species, three vertebrates which function over the temperature range from 37 to −1 °C and one invertebrate which functions around 4 °C. As in our preceding paper (Rodgers & Harrington, 1987), the secondary structure has been determined by using optical rotation. The global tertiary structure was monitored by the angular dependence of light scattering.

Materials and Methods

Reagents. All enzymes used in this study were purchased from Worthington Biochemicals (Freehold, NJ). α-Chymotrypsin (3× recrystallized) was further treated with TLCK according to the procedure of Shaw et al. (1965) to eliminate any trypsin contaminant. The TLCK-treated chymotrypsin was stored frozen in a solution of 0.001 N HCl. Papain (Cooper Biomedical) was activated immediately prior to use according to the method described in the Worthington manual. Iodoacetic acid and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma Chemicals. Ultrapure ammonium sulfate was purchased from Schwarz/Mann. Hydroxylapatite (Bio-Gel HT) was obtained from Bio-Rad. Sephacryl S-300 and S-400 and DEAE-Sephadex A50 were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

The rabbits used in this study were New Zealand white young adults. Frogs (*Rana temporaria*) were obtained from Charles D. Sullivan, Nashville, TN. Deep sea scallops (*Placopectin magellanicus*) were obtained from the Woods Hole Oceanographic Institute. The antarctic fish (*Pagothenia borchgrevinki*) were collected from the McMurdo Sound, Antarctica. The white fast twitch muscles from this species were cut into small pieces and stored at −18 °C in 50% glycerol suspension prior to use.

Preparation of Myosin Rod Subfragments. (1) Rabbit Rod. Rabbit rod was prepared as described in the accompanying paper (Rodgers & Harrington, 1987). Myosin rod was also prepared by papain digestion according to the procedure of Hvidt et al. (1982). No difference was observed between papain and chymotryptic rod in these experiments. This was expected since the digestion sites for papain and chymotrypsin are only two residues apart in the primary sequence (Lu & Wong, 1985).

(2) Frog Rod. Frog rod was obtained from *Rana temporaria* myofibrils according to the procedure of Lovell and Harrington (1981).

(3) Scallop Rod [See Stafford et al. (1979) and Wallimann and Szent-Györgyi (1981)]. Deep sea scallop (*Placopectin megellanicus*) myofibrils (2 mg/mL) were digested with papain (enzyme:substrate ratio equals 1:3000 w/w) at 15 °C for 20 min in a solvent consisting of 0.1 M NaCl, 20 mM sodium phosphate, 2 mM EDTA, and 1.5 mM DTT (pH 7.0). Digestion was quenched by addition of iodoacetic acid (IAA; 5 mM). Digested myofibrils were collected by low-speed centrifugation and washed several times with a dilute salt solvent (5 mM sodium phosphate, 40 mM NaCl, and 1 mM IAA, pH 7). The rod subfragment (and residual myosin) was extracted from the resulting myofibrillar debris with 0.5 M NaCl, 5 mM ATP, and 5 mM sodium phosphate (pH 7) and precipitated by addition of ammonium sulfate (45–70% saturation). Following dissolution of the precipitate in high salt [0.6 M NaCl, 5 mM sodium phosphate, and 0.5 mM IAA (pH 7)], the rod was fractionated from the residual myosin by treatment with 3 volumes of ethanol and then dissolved in the high-salt buffer and again precipitated by dialysis vs low salt [40 mM NaCl, 5 mM sodium phosphate, 0.1 mM DTT, and 0.5 mM IAA (pH 6.0)]. The resulting rod preparation was further purified by chromatography on Sepharose 4B in 0.6 M NaCl and 5 mM sodium phosphate buffer (pH 7.0).

(4) Antarctic Fish Rod. Antartic fish (*Pagothenia borchgrevinki*) myofibrils (5–10 mg of protein/mL), obtained from the fast, white (pectoral) muscle of this species, were digested with α-chymotrypsin (0.2 mg/mL) in 80 mM NaCl, 40 mM cacodylate, and 2 mM EDTA (pH 7) at 0 °C for 40 min to prepare rod subfragment. Digestion was quenched by addition of PMSF (1 mM), and the residual myosin and rod were precipitated by methanol (3 volumes). The precipitate was collected by low-speed centrifugation and homogenized in 0.6 M NaCl (pH 7) to extract the rod subfragment. The supernate was exhaustively dialyzed vs 30 mM sodium phosphate buffer (pH 6.0) at 4 °C. The resulting rod aggregates were collected by centrifugation (10000 rpm, 10 min), dissolved in 40 mM sodium pyrophosphate (pH 7.4), and further purified by column chromatography on a DEAE-Sephadex A-50 column as described for rabbit rod.

Solvents. Most experiments in this study were carried out in a solvent of 0.6 M NaCl, 20 mM sodium phosphate, 0.25 mM EDTA, and 0.1 mM DTT, pH 7.0 (buffer M). In some experiments, EDTA was increased to 0.5 mM and DTT was increased to 1 mM.

Optical Rotation and Light-Scattering Measurements. The methods used and analysis of the data have been described in detail in our preceding paper (Rodgers & Harrington, 1987). Protein concentrations were in the range of 0.3–0.5 mg/mL for the optical rotation experiments and 0.1–0.3 mg/mL for the light-scattering experiments.

Protein Concentrations. Concentrations were routinely determined from absorption measurements at the spectral peak near 275–280 nm for each species. These values were corrected for scattering by measuring the absorbance at 320 nm and assuming a λ^{-4} dependence of scattering. Extinction coefficients were determined on the basis of concentrations obtained from C_0 runs in the analytical ultracentrifuge.

C_0 runs were carried out according to the procedure of Chervenka (1973) in a Beckman Model E analytical ultracentrifuge. Samples of ~5 mg/mL protein were dialyzed exhaustively into solvent M described earlier. The sample and solvent were placed in a 12-mm double-sector synthetic boundary cell. The centrifuge was run at ~8000 rpm until the boundary was formed and then slowed to 3000 rpm to allow the boundary to diffuse. Initial and final photographs were taken by using interference optics, and the total fringe shift was determined by measuring the photographs on a Nikon projection microcomparater. The concentration was calculated by assuming a value of 0.185 mL/g for the specific refractive index increment (dn/dc).

Electrophoresis. The method of Weber and Osborne (1969) was used for gel electrophoresis of myosin rod samples in SDS-containing gels. Samples were denatured by boiling in SDS and reduced by the addition of 2-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue G-250 (0.25% w/v in a solvent comprising MeOH/HOAc/HOH 5:1:5) and destained with aqueous 5% MeOH/7.5% HOAc. Destained

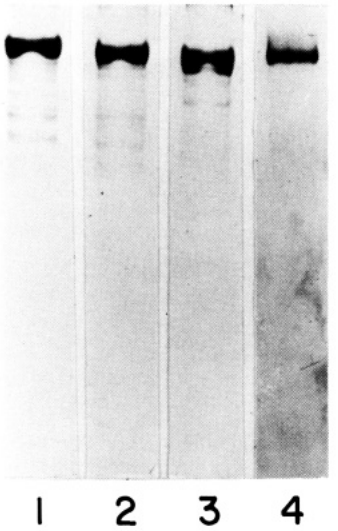

FIGURE 1: SDS gels of myosin rod samples. SDS–polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborne (1969). Samples are myosin rod isolated from (1) rabbit, (2) frog (*Rana temporaria*), (3) scallop (*Placopectin magellanicus*), and (4) antarctic fish (*Pagothenia borchgrevinki*).

gels were scanned by using an LKB densitometer with integrator in order to estimate the purity of the samples.

Results

Characterization of Myosin Rod Fragments. SDS gel electrophoresis patterns of each of the four myosin rod species are shown in Figure 1. All species have nearly identical molecular weights which we estimate to be 130K ± 5K per chain. No observable difference was noted in the chain weights of fragments prepared by papain digestion as compared to those prepared by α-chymotryptic digestion. Estimates of the purity of each sample were obtained by densitometric scanning of Coomassie Blue stained gels. Typical purities were in the range of 87–95% with the principal contaminants having molecular weights of 60K–80K. Samples used in light-scattering studies were further purified by chromatography on Sephacryl S-300 and S-400 columns, and the selected fractions were 95% pure or better.

Extinction coefficients for each rod species were determined at the absorption peak near 280 nm using concentrations measured by fringe counting in the analytical ultracentrifuge as a primary standard. We found values of $E^{0.1\%}$ to be 0.196 (278 nm), 0.185 (278 nm), and 0.202 (277 nm) for frog, scallop, and antarctic fish, respectively. A value of 0.21 (278 nm) was used for $E^{0.1\%}$ for rabbit rod (Hvidt et al., 1982).

Optical Rotation Melting Curves. Thermal melting profiles for each of the four rod species in the same solvent system (buffer M) are shown in Figure 2 where the specific mean residue rotation, $[m']_{231.4}$, is presented as a function of temperature over the range 0–80 °C. All melting curves were qualitatively similar in form but varied markedly in the observed midpoints (*Tm*) of the transitions. At the lowest temperatures, each fragment exhibits an $[m']_{231.4}$ of about −16000. On elevation of the temperature, the magnitude of $[m']_{231.4}$ falls continuously, leveling off at a value of ∼−3000. The striking difference in the midpoints of the overall transitions is indicative of large differences in the thermal stabilities of these structures even though each is believed to be a coiled-coil of α-helices. The order of decreasing thermal stability of these fragments is rabbit > frog > scallop > antarctic fish. This trend parallels the working temperatures of the various species. It is also clear from derivative plots of the data in Figure 2

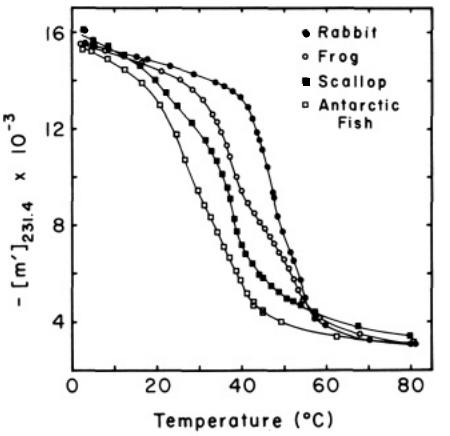

FIGURE 2: Optical rotation thermal melting profiles of myosin rod. Melting profiles of myosin rod isolated from four species were obtained by using optical rotation at 231.4 nm. $-[m']_{231.4}$ vs temperature values are plotted for each sample. Solid lines are smooth curves drawn through the data points. The solvent in each case was 0.6 M NaCl, 20 mM PO_4, 0.25 mM EDTA, and 0.1 mM DTT, pH 7.0. Symbols are as indicated on the figure.

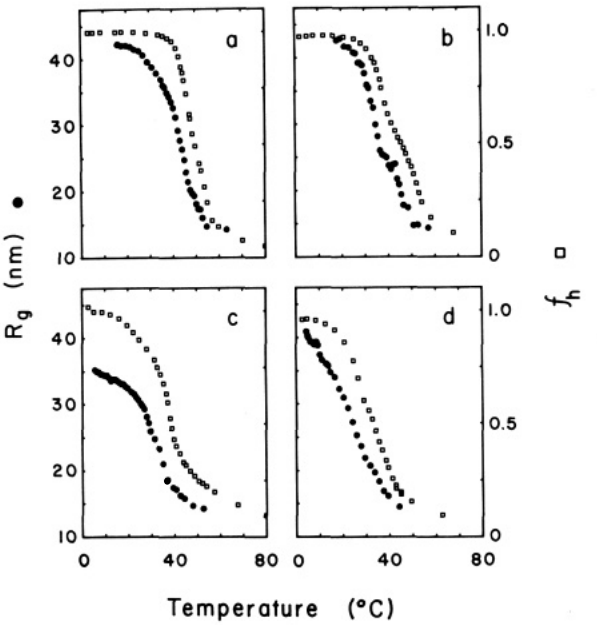

FIGURE 3: Comparison of radius of gyration (R_g) and fraction helix (f_h) for various myosin rod species vs temperature. Plots of R_g (●) and f_h (□) vs temperature are shown for each species of myosin rod. R_g's were determined from elastic light-scattering measurements as described under Materials and Methods. f_h's were estimated from the optical rotation measurements of Figure 2 using the method of Hvidt et al. (1985). (a) Rabbit; (b) frog; (c) scallop; and (d) antarctic fish. The solvent for the light-scattering measurements was the same as in Figure 2 except that EDTA was 0.5 mM and DTT was 1 mM.

that the melting transition for each species is multiphasic. Similar behavior has previously been observed in thermal melting studies of other coiled-coil proteins (Burke et al., 1973; Potekhin et al., 1979; Privalov, 1982), suggesting the existence of quasi-independent melting domains within the structure. In addition, we found that the unfolding process was highly reversible. Nearly 100% of α-helical structure was re-formed on standing overnight at room temperature following the termination of a melting experiment. From the $[m']_{231.4}$ vs temperature data, we can estimate the fraction of the total structure in the α-helix conformation (f_h) at any temperature taking into account the temperature dependence of the reference states (Hvidt et al., 1985). Plots of f_h vs T for each rod fragment are shown as the open squares in Figure 3. The

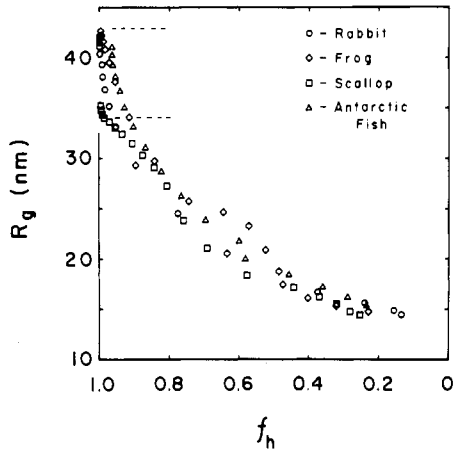

FIGURE 4: Radius of gyration vs fraction helix for each myosin rod species. The data in Figure 3 have been replotted as R_g vs f_h to eliminate the temperature axis. Points were obtained by interpolation at 2 °C increments between the lowest and highest temperatures. Symbols are as indicated on the figure. The upper dashed line indicates the expected R_g for a rigid rod 150 nm in length, and the lower dashed line is the calculated R_g for a rigid rod of the same length but having a universal hinge at its midpoint.

midpoints of the transitions (T_m) are defined as the points where $f_h = 0.5$. These values are characteristic of the overall thermal stability of the proteins and were found to be 49, 45, 39, and 33 °C for rabbit, frog, scallop, and antarctic fish myosin rod, respectively.

Light Scattering as a Function of Temperature. Elastic light-scattering measurements were also made on each sample in the same solvent (buffer M) as a function of temperature. The radius of gyration (R_g) at each temperature was obtained from plots of the scattered light intensity [$I(\theta)$] vs $\sin^2(\theta/2)$ as described in Rodgers and Harrington (1987). The plots have been scaled so that the low- and high-temperature regions of f_h and R_g roughly coincide. It is clear that in each rod species, the major decrease in R_g occurs at a significantly lower temperature (5–8 °C) when compared to the f_h vs T plot. In three cases (rabbit, frog, and antarctic fish), the R_g at low temperatures is ~43 nm. If one assumes a rigid rod model for the structure ($R_g^2 = L^2/12$), then this corresponds to a length of ~150 nm and is consistent with the lengths observed for rabbit myosin rod in electron microscopic studies (Elliott & Offer, 1978; Walker et al., 1985) and is only slightly shorter than the calculated length assuming 1100 residues per chain (Strehler et al., 1986) for a 100% α-helical structure. Scallop rod (the only invertebrate included in this study) had an R_g = 34 nm even at the lowest temperature examined (10 °C). This corresponds to an apparent rigid rod length of 117 nm. Since this value is significantly shorter than the calculated rigid rod length above, it is clear that unlike the other three rod fragments, scallop rod does not behave like a rigid rod even at low temperatures. The correlation between changes in secondary structure and changes in radius of gyration for each rod species are shown more clearly in Figure 4. In this figure, the data from Figure 3 have been replotted as R_g vs f_h, thus eliminating the temperature axis. As the fraction of helix decreases from 1.0 to ~0.95, R_g shows a marked drop to a value of about 34 nm. Further decreases in f_h down to ~0.4 are accompanied by a more gradual decline in R_g. For f_h values below 0.4, the R_g in each case is ~15 nm and remains essentially constant. Thus, it appears that although myosin rods isolated from the various muscle species have vastly different thermal stabilities, the melting behavior of their α-helical structures shows a common set of properties. A summary of the midpoints of the overall melting transitions for the various rod species is presented in Table I.

Table I: Apparent Midpoints of Overall Melting Transitions of Myosin Rod from Four Species[a]

	$T_{1/2}(f_h)$	$T_{1/2}(R_g)$
rabbit	49	43
frog	45	36
scallop	39	32
antarctic fish	33	24

[a] Note that the melting transitions of these structures are multiphasic. All measurements were made in solvent M. All temperatures are in degrees Celsius.

Discussion

The myosin rod fragments investigated in the present study were isolated from the muscles of species covering a broad span of the evolutionary scale. Three of these species operate within confined but distinctly different temperature ranges (rabbit, 37 °C; deep sea scallop, 4 °C; and antarctic fish, −2 °C) while one species can readily adapt to a broad range of temperatures (frog, 5–25 °C). Our results show that the melting temperatures of the α-helical coiled-coil structures of the myosin rods decrease roughly in parallel with the working temperature of their respective muscles.

The idea of hinging within the myosin rod stems from some of the earliest observations on isolated myosin. Myosin is readily cleaved by proteolytic enzymes to form LMM and HMM subfragments. The relatively high proteolytic susceptibility of the center of the rod as compared to the flanking regions suggests that this region may have lower thermal and structural stability (Mihalyi & Harrington, 1959). Such a region could behave as a mechanical hinge within the myosin rod. The physiological significance of a hinge in myosin rod is not clear, but it has been incorporated into both the rotating cross-bridge model (Huxley, 1969) and the helix–coil model (Harrington, 1971, 1979) for muscle contraction. Nevertheless, the existence of the LMM/HMM hinge has been somewhat controversial [see Harvey and Cheung (1982)].

Proteolytic digestion studies of isolated myosin and myosin rod clearly show that the region between short S-2 and LMM is highly labile (Mihalyi & Harrington, 1959; Weeds & Pope, 1977; Sutoh et al., 1978; Ueno & Harrington, 1984; Stafford, 1985). Further, the proteolytic susceptibility is very dependent on solvent conditions and temperature. More recent studies on intact myofibrils and muscle fibers under relaxed, rigor, and activating conditions have shown that the "hinge" region is also protease sensitive in the organized muscle system (Ueno & Harrington, 1986a,b). The cleavage rate of activated systems was found to be ~100-fold greater than in rigor or relaxed psoas fibers, and these data have been taken to indicate helix melting during the active cross-bridge cycle. Other indications of a lower stability of the "hinge" region have come from analysis of the amino acid sequence of rabbit myosin rod (Lu & Wong, 1985) and rat myosin rod (Strehler et al., 1986). These authors find that a region of amino acids between short S-2 and LMM has significantly lower helix-forming probability than the flanking regions. Although the lower stability of the hinge regions seems well established, these studies are not per se measures of hinging.

Physical studies which can directly measure hinging have yielded mixed results. Electron microscopy studies of isolated myosin have shown localized sharp bends in the myosin rod within the hinge region (Elliott & Offer, 1978; Walker et al., 1985; Takahashi, 1978). Electric birefringence (Highsmith et al., 1977; Bernengo & Cardinaud, 1982) and viscosity

(Burke et al., 1973) studies on rod and LMM have also provided evidence for significant flexibility of the myosin rod. On the other hand, fluorescence depolarization studies (Harvey & Cheung, 1977) have shown no bending of myosin rod, and viscoelastic studies (Rosser et al., 1978) indicate only limited flexibility. Additionally, Hvidt et al. (1984) reported only slight flexibility of myosin rod over the range of 1–43 °C based on both electric birefringence and elastic light scattering.

Most of the studies above have been carried out on rabbit myosin and its subfragments using a wide variety of experimental conditions. Recent studies on smooth muscle myosin have provided very clear hydrodynamic and electron microscopic evidence for hinging depending on the state of phosphorylation of the myosin and the ionic strength of the solvent (Trybus et al., 1982; Trybus & Lowey, 1984).

In the present study, each myosin rod species exhibits a similar multiphasic optical rotation melting profile, but the midpoints of these transitions differ markedly in temperature and shift roughly in parallel with the working temperature of their respective muscles. Measurements of the radius of gyration as a function of temperature for each species showed similar behavior, but in each case, the R_g vs T curve was shifted to a lower temperature than the respective f_h vs T curve. Thus, a significant decrease in the radius of gyration from the expected rigid rod value occurred with only a small change in f_h.

Yu and Stockmayer (1967) have calculated the expected R_g for a rod with a universal hinge at its center to be $(5/8)^{1/2}$ times the R_g of an unhinged rod. At low temperatures, rabbit rod, frog rod, and antarctic fish rod all show an R_g of ~43 nm, consistent with an elongated rigid rod structure. As the temperature is increased, both rabbit rod and antarctic fish rod exhibit a marked drop in R_g to a value of nearly $(5/8)^{1/2}R_{g,max}$ with only a few percent change in f_h (Figure 4). Frog rod shows a less dramatic change in R_g vs f_h, but even in this case, the radius of gyration is consistent with a hinged structure when less than 5% of the α-helix has been melted. Scallop rod behaves quite differently since the radius of gyration of this rod species is only 34 nm, even at the lowest temperatures examined, suggesting that it never behaves as a rigid rod. At higher temperatures, all four rod species show more gradual changes in R_g vs f_h. It is difficult to interpret the results in this region of the melting curve since it is likely that several domains are undergoing melting at higher temperatures (Potekhin et al., 1979; Privalov, 1982). Taken together, our findings are consistent with hinging of the myosin rod in all four species examined.

Previous studies have shown a striking relationship between the thermostability of fish myofibrillar ATPase and the temperature at which the species live (Johnston et al., 1973, 1975; Johnston & Goldspink, 1975). At 37 °C, the rates of thermal denaturation of the myosin ATPase of 22 species of fish increase in the order African equatorial lakes (35–38 °C) < Indian Ocean (23–25 °C) < Mediterranean (18–21 °C) < North Sea (5–14 °C) < Antarctica (0–2 °C). The half-life of inactivation varied about 350-fold between the two temperature extremes. In these species, the activation enthalpy, $\Delta H^{\ddagger}$, decreases monotonically from 33.3 kcal mol^{-1} (African lakes) to 6.9 kcal mol^{-1} (Antarctic) while the activation entropy, $\Delta S^{\ddagger}$, changes from +49 to −42 eu over the same temperature range. Thus, the activation free energy, $\Delta G^{\ddagger}$ remains virtually constant at 18.7 ± 0.5 kcal mol^{-1} and provides a high specific ATPase activity at all environmental temperatures (Johnston & Goldspink, 1975). It appears from the present study that the thermal stability of the α-helical coiled-coil structure of the myosin rod follows a pattern similar to that of the observed ATPase of the myosin head.

ACKNOWLEDGMENTS

We are grateful for the excellent technical assistance of Michael Koval and Howard Flaxman. We are also indebted to Dr. Søren Hvidt for many helpful discussions and to Dr. Dennis Powers for his gift of antarctic fish muscle.

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