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Melting of myosin rod as revealed by electron microscopy. II. Effects of temperature and pH on length and stability of myosin rod and its fragments

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Myosin rod — helix-coil transition — length — stability — pH and temperature effects

Effects of temperature and *p*H on intact rabbit and chicken myosin, isolated myosin rods, rabbit subfragment-2 (61 kDa, 53 kDa, and 34 kDa) and chicken light meromyosin (LMM) fragments were tested to induce a phase transition [4, 19] from α-helix to coil conformation, within the hinge region. The influence of temperature and *p*H were studied directly with length determination by electron microscopy. An increase of temperature to 50 °C yielded a shortening of 16 nm, 8 to 9 nm and 7 to 11 nm for intact myosin, isolated rods and long S-2 fragments, respectively. The length of the 34 kDa short S-2 and LMM fragments were unchanged. An increase of *p*H from neutral to *p*H 8.0 yielded values that were somewhat smaller, e.g. 12 nm, 6 nm and 6 to 8 nm for intact myosin, isolated rods and long S-2 fragments, respectively, whereas the 34 kDa short S-2 LMM fragments were also unaffected. Thus, melting and subsequent shortening is confined to the region between LMM and short S-2 segment, that is the hinge region. Alteration of temperature had a stronger shortening effect than alteration of *p*H, and shortening of long S-2 was more pronounced under physiological salt conditions as compared with high (0.3 M) salt. The shortening of rods in intact myosin amounted to twice the value observed with isolated rods. The amount of contraction was somewhat smaller in rods than in the 61 kDa and 53 kDa long S-2 fragments.

Introduction

According to the model for force-generation in muscle proposed by Huxley and collaborators (for references see [7]), some type of structural transition in the myosin head while it is attached to actin in the cross-bridge cycle is believed to be the origin of the contractile force. This process results in a change in the effective angle of the head and extension of a spring-like elastic component provisionally located within the subfragment-2 region of the myosin rod. Retraction of the elastic element during the power stroke acts to slide thin filaments past thick filaments. In the helix-coil model by Harrington and collaborators (for review see [5]) release of the actin-attached cross-bridge from its resting state environment, where the S-2 fragment is a part of the thick filament core, results in a transition from helix to random coil in a region of the S-2 lever arm, that is the light meromyosin — heavy meromyosin (LMM-HMM) hinge domain. The proposed phase transition in that region leads to a contraction of the polypeptide chains as a result of the increased number of conformational states or rotatable bonds [3]. This shortening of approximately 10 nm of the long S-2 fragment is caused by a helix-coil melting of about 165 residues per polypeptide chain within the hinge region [5].

Thermal melting and temperature-jump experiments demonstrate that the LMM-HMM hinge domain of S-2 is a segment of lower thermal stability [1, 5, 6, 15] as compared to the flanking LMM and short S-2 segments of the myosin rod. In addition, sequence analysis of the hinge regions of rabbit psoas myosin [10] and nematode myosin [11] showed that this domain has a substantially larger number of charged amino acid residues than the LMM or short S-2 fragment at positions near the coiled-coil interface normally occupied by hydrophobic residues [11]. This is leading to a destabilization of the hinge region. Furthermore, enzyme-probe studies of the α-helical rod structure using chymotrypsin, trypsin and papain also reveal a common pattern of local melting in the hinge region which is dependent on temperature [5, 13, 18, 19]. In addition, when muscle is activated or when the *p*H of glycerinated rigor fibers is slightly elevated (*p*H 8.5), the S-2 segments are believed to be released and swing away from the thick filament surface during each cross-bridge cycle, for activation of glycerinated psoas fibers is accompanied by an abrupt increase in chymotryptic susceptibility within the

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hinge region [1, 16]. These data are consistent with the idea that a helix-coil transition and thus contraction of the polypeptide domain within the long S-2 fragment may play a fundamental role in the mechanism of muscle contraction. We tried to directly examine by electron microscopy under controlled conditions [22, 23] the effects of temperature and *p*H on the length of intact myosin, isolated rod and rod fragments in an attempt to test whether α-helix melting near physiological temperature and salt conditions is truly accompanied by a measurable shortening of these structures.

Materials and methods

Chicken myosin and myosin rod

Chicken pectoralis myosin and myosin rod was isolated, processed, stored and prepared for electron microscopy as described in the preceding paper [23]. Immediately before spraying, samples were diluted to 10 μg/ml into solutions indicated in Tables I to III. The *p*H was adjusted from 7.0 to 8.0 by the addition of 0.3 M ammonium carbamate.

Rabbit myosin, myosin rod, rod subfragments and LMM

Rabbit skeletal myosin was a gift of Dr. M. C. Schaub and myosin rod was provided by Dr. S. Hvidt and Ms. C. Licalsi. Rabbit myosin rod prepared by papain digestion of myofibrils [8] and further purified on hydroxyapatite, was dialyzed against 5 mM pyrophosphate (*p*H 8.0), lyophilized, and then diluted to 2 mg/ml in 0.7 M ammonium acetate, *p*H 7.0. Tryptic rod fragments, long S-2 of 61 kDa and 53 kDa, and short S-2 of 34 kDa (Fig. 1) were prepared from isolated rabbit rod [19]. Lyophilized samples of 61 kDa, 53 kDa and 34 kDa subfragment S-2 were dissolved at 1 mg/ml in 0.7 M ammonium acetate at *p*H 7.0, dialyzed overnight against the same buffer and stored at −20 °C. Tryptic LMM from chicken myosin rod [9] was stored at −20 °C.

Variation of pH

Samples were diluted to 10 μg/ml into appropriate volatile buffers containing 50% glycerol (Tab. III) after the *p*H had been adjusted by addition of 0.3 M ammonium carbamate, sprayed onto freshly cleaved mica at 20 °C, air dried at 20 °C in vacuo and shadowed by Pt/C at 20 °C [22, 23]. As a control, samples (500 μg/μl) that were exposed for 10 min at *p*H 8.0 were diluted back into *p*H 7.0 buffer at 10 μg/μl and immediately sprayed. The temperature was kept at 20 °C at all steps.

Variation of temperature

Samples were sprayed always in *p*H 7.0 buffer either at 20 °C and then transferred onto the temperature controlled (20 °C–70 °C) specimen holder (Balzers/Liechtenstein) and shadowed under controlled temperature conditions varying from 20 °C to 70 °C (Tab. II); or the samples themselves were preheated to 50 °C for 2 to 4 min and sprayed onto mica on the preheated specimen holder at 50 °C. Spraying was performed in a small lamp-heated box enclosing the preheated Balzers stage (Tab. I, 50°/50 °C). As a control, samples preheated for 2 to 4 min at 50 °C were cooled down to 4 °C and sprayed at 20 °C (Tab. I, 50°/20°).

Electron microscopy

Heavy metal shadowing, recording of micrographs and length measurements were done as described [22, 23].

Results

Morphology

The overall morphology of rabbit and chicken intact myosin was as expected, but at high temperature (> 50 °C), myosin heads showed a tendency to clump together (not shown). When myosin was prepared in solutions adjusted to *p*H 8.0 before spraying, an additional kink was often found at 16 to 17 nm distant from the tip of the rod (not shown). In the other experiments, rods S-2 fragments and LMM were more or less straight or slightly curved structures (Fig. 2).

Measurements

Length measurements on intact myosin, isolated rods, S-2 fragments (61 kDa, 53 kDa, 34 kDa) and LMM (Fig. 1)

Tab. I. Dependence on temperature jumps (20° to 50 °C) of the lengths of myosin rod in intact myosin and of isolated myosin rods as well as S-2 subfragments (61 kDa and 53 kDa = long S-2; 34 kDa = short S-2) and light meromyosin (LMM). Dispersion medium containing either 0.15 or 0.3 M ammonium acetate with 50% glycerol at *p*H 7.0. 20°/50 °C = samples sprayed at 20 °C, heated to 50 °C while on mica and shadowed at 50 °C; 50°/50 °C = samples preheated to 50 °C for 2 to 4 min, sprayed on 50 °C preheated mica and shadowed at 50 °C; 50°/20 °C = samples preheated to 50 °C sprayed and shadowed at 20 °C.

	Length nm ± S.D.							
	Chicken intact myosin	Isolated rod	Rabbit intact myosin	Isolated rod	Rabbit S-2 fragments			Chicken LMM fragment
					61 kDa	53 kDa	34 kDa	
20°/20 °C								
0.3 M	149.4 ± 6.5 (62)	139.0 ± 6.8 (38)	155.9 ± 4.8 (33)	145.8 ± 5.8 (31)	69.5 ± 3.1 (26)	59.5 ± 3.5 (34)	40.1 ± 2.5 (22)	74.4 ± 3.8 (0)
0.15 M					71.1 ± 3.3 (28)	59.7 ± 3.0 (35)	38.5 ± 3.3 (81)	
20°/50 °C								
0.3 M	138.5 ± 3.5 (15)	133.4 ± 6.8 (21)	—	139.1 ± 8.0 (21)	65.6 ± 5.6 (25)	52.5 ± 3.3 (30)	42.6 ± 3.3 (11)	71.6 ± 5.3 (47)
0.15 M					68.5 ± 3.5 (25)	52.6 ± 3.3 (81)	39.2 ± 2.2 (20)	
50°/50 °C								
0.3 M	133.7 ± 7.2 (13)	130.4 ± 7.1 (25)	—	137.9 ± 10.1 (35)	64.2 ± 3.7 (19)	52.7 ± 2.9 (19)	40.9 ± 2.9 (29)	73.8 ± 8 (17)
0.15 M					58.5 ± 4.3 (15)	48.8 ± 5.2 (68)	38.2 ± 5.4 (31)	
50°/20 °C								
0.3 M	149.7 ± 6.0 (40)	146.6 ± 4.4 (47)	—	150.7 ± 7.4 (43)	71.4 ± 3.2 (77)	57.2 ± 3.7 (45)	42.4 ± 2.6 (87)	
0.15 M					67.9 ± 4.4 (46)	55.6 ± 3.3 (21)	39.8 ± 4.2 (70)	

S.D. Standard deviation. — Values in brackets: number of molecules measured.

prepared at 20° under standard conditions (0.3 M or 0.15 M ammonium acetate, 50% glycerol, *p*H 7.0) are summarized in Table I (20°/20 °C). Rod lengths in intact myosin (chicken: 149.4 nm, rabbit: 155.9 nm) were about 10 nm longer compared to that of isolated rod of chicken (139.0 nm) and rabbit (145.8 nm). Intact and isolated rod from rabbit were each about 6 to 7 nm longer than those of chicken. The lengths of rod S-2 fragments (61 kDa, 53 kDa, 34 kDa) prepared under physiological conditions were similar to those under high salt conditions, that is in 0.3 M buffer solvents: 69.0 nm, 59.5 nm, 40.1 nm; in 0.15 M buffer solvents: 71.1 nm, 59.7 nm, 38.5 nm respectively. The length of LMM was 74.4 nm all at 20°.

Effects of temperature on intact myosin rod, isolated rods and rod fragments

The lengths of intact myosin, isolated rods, S-2 fragments (61 kDa, 53 kDa, 34 kDa) and LMM after air-drying in vacuo from 0.3 M or 0.15 M ammonium acetate, containing 50% glycerol at *p*H 7.0, but at different temperatures are summarized in Tables I and II.

In the first set of experiments the samples were prepared and sprayed onto mica at 20 °C and heated to 50 °C *after* spraying and transfer to vacuum (Tab. I, 20°/50 °C). Prepared in such a way, intact myosin rod and isolated rods

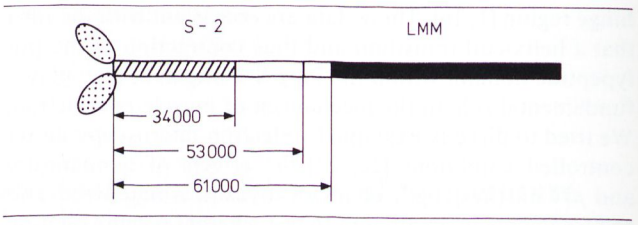

Fig. 1. Schematic drawing of a myosin molecule with light meromyosin (LMM), long subfragment S-2 (M_r 53000 and 61000) and short S-2 (M_r 34000).

shortened by about 10 nm and 6 nm, respectively upon elevation of temperature to 50 °C. The 61 kDa and 53 kDa S-2 fragments in 0.3 M buffer were shortened at 50 °C by 4 nm and 7 nm, respectively. By contrast, the 34 kDa S-2 fragment seemed to be even somewhat longer at 50° than at 20 °C. At 50 °C in 0.15 M buffer, the 61 kDa and 53 kDa S-2 fragments were about 3 nm and 7 nm shorter, respectively, compared to controls at 20 °C, whereas the 34 kDa S-2 fragment did not seem to shorten at all at this temperature. LMM fragments had an average length of 71.6 nm, a value that was not significantly different from that at 20 °C (Tab. I).

In a next step, samples were preheated to 50 °C *before*

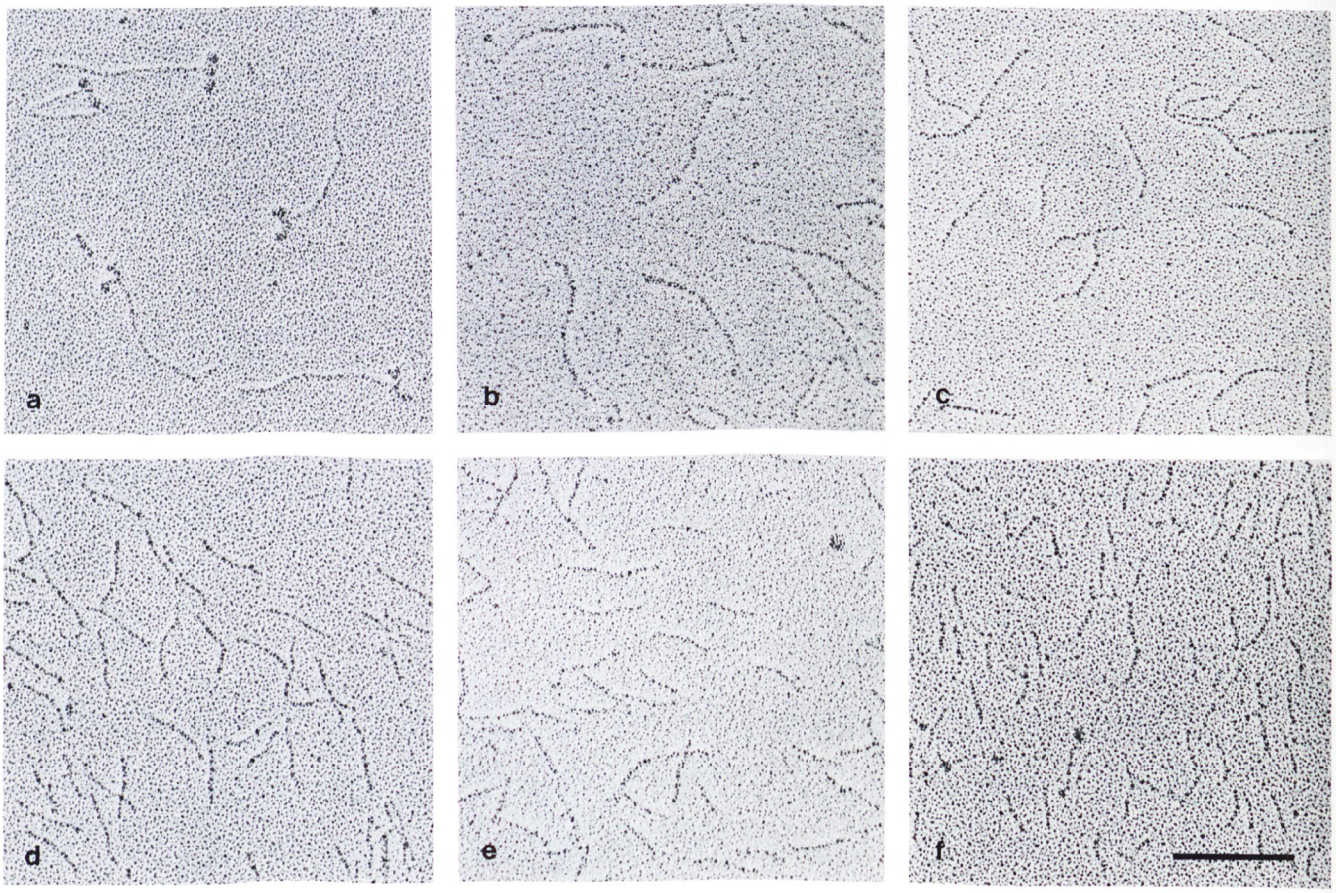

Fig. 2. Rabbit myosin (**a**), isolated rod (**b**), and rod fragments, LMM (**c**), 61 kDa S-2 (**d**), 53 kDa S-2 (**e**) and 34 kDa S-2 (**f**), prepared for electron microscopy after air-drying in vacuo from 0.3 M ammonium acetate, containing 50% glycerol at *p*H 7.0, and room temperature, rotary shadowed with Pt/C. — Bar 100 nm.

Tab. II. Dependence on temperature increments (20°, 30°, 40°, 50° and 70 °C) of the lengths of myosin rod in intact myosin and of isolated myosin rods as well as S-2 subfragments (61 kDa and 53 kDa = long S-2; 34 kDa = short S-2) in 0.15 M or 0.3 M ammonium acetate containing 50% glycerol at *p*H 7.0.

	Length nm ± S.D.				
	Chicken isolated rod	Rabbit isolated rod	Rabbit S-2 fragments		
			61 kDa	53 kDa	34 kDa
20 °C	139.4 ± 5.4 (26)	145.8 ± 5.8 (31)	70.9 ± 4.4 (22)	59.6 ± 3.2 (28)	38.8 ± 2.8 (55)
30 °C	138.4 ± 9.5 (25)	142.6 ± 6.6 (26)	65.4 ± 2.9 (28)	55.6 ± 3.7 (29)	37.3 ± 3.7 (41)
40 °C	133.8 ± 9.5 (25)	138.8 ± 8.3 (20)	63.1 ± 4.0 (31)	52.7 ± 2.9 (49)	38.2 ± 2.9 (30)
50 °C	130.4 ± 7.1 (25)	137.8 ± 7.4 (12)	62.4 ± 3.8 (36)	51.7 ± 4.1 (49)	37.5 ± 3.6 (23)
70 °C	119.7 ± 8.0 (21)	129.7 ± 7.3 (22)	53.1 ± 4.0 (31)	45.3 ± 2.6 (20)	33.8 ± 3.3 (15)

S.D. Standard deviation. — Values in brackets: number of molecules measured.

spraying onto mica and kept at 50 °C during the whole drying and shadowing procedure (Tab. I, 50°/50 °C). Prepared in such a way, intact myosin and isolated rods from chicken and rabbit were shorter by about 16 nm and 8 to 9 nm, respectively, compared to the controls at 20 °C. In 0.3 M buffer solvents at 50 °C the 61 kDa and 53 kDa S-2 fragments were shorter by 5 nm and 7 nm, respectively, in 0.15 M buffer solvents by 13 nm and 11 nm, respectively, compared to controls at 20 °C. Again, the length of the 34 kDa S-2 fragment did not change significantly when heated to 50 °C (0.3 M: 40.9 nm, 0.15 M: 38.2 nm) neither did LMM. In addition, rods of intact myosin seemed to be more sensitive to the heating than the isolated rods. When long S-2 fragments (61 kDa and 53 kDa) were prepared in 0.3 M salt, the difference in length between the 20 °C and 50 °C preparations was 5 to 7 nm, whereas in 0.15 M salt the difference was more pronounced, being 11 to 13 nm. Thus, long S-2 fragments were more sensitive to heating (50 °C) at physiological than at high salt.

In a next step, the samples were preheated to 50 °C for 2 to 4 min and cooled to 20 °C before spraying (Tab. I, 50°/20 °C). Since obviously the differences between the length of these myosin subfragments were not significantly different from those at 20 °C short heating of myosin rod and fragments thereof does not irreversibly damage the α-helical double-coiled structure.

In another set of experiments (Tab. II), the samples were air dried at 20 °C in vacuo and shadowed after the temper-

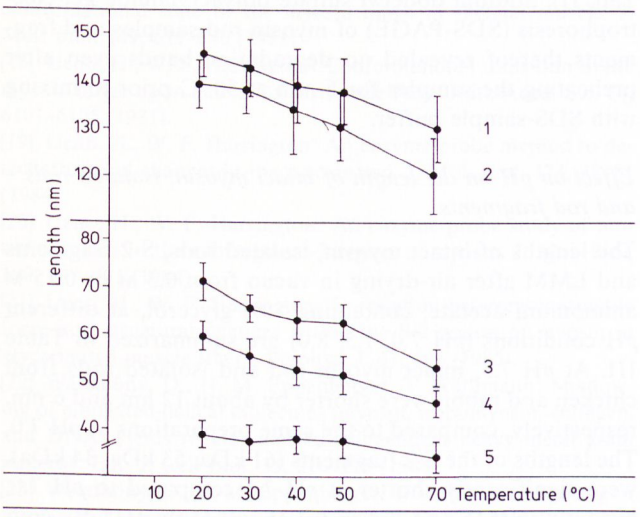

Fig. 3. Isolated rabbit (1) and chicken (2), and rabbit S-2 fragment (61 kDa (3), 53 kDa (4), and 34 kDa (5)) were air-dried in vacuo from 0.3 M ammonium acetate, containing 50% glycerol, *p*H 7.0, at stepwise increased temperatures (20 °C, 30 °C, 40 °C, 50 °C, or 70 °C) and rotary shadowed with Pt/C. The lengths of individual molecules were plotted against the corresponding temperatures. — Note the marked decrease in length between 20 °C and 40 °C, and the small decrease in length between 40 °C and 50 °C among the isolated rods and long S-2 fragments of 61 kDa and 53 kDa whereas the short S-2 fragment (34 kDa) did not significantly change between 20 °C and 50 °C.

Tab. III. Dependence on the *p*H value (*p*H 7.0, 7.5, 8.0) of the lengths of myosin rod in intact myosin and of isolated rods as well as S-2 subfragments (61 kDa, 53 kDa = long S-2; 34 kDa = short S-2) and light meromyosin (LMM) in 0.15 or 0.3 M ammonium acetate at 20 °C. The *p*H value of the dispersion medium was adjusted by addition of 0.3 M ammonium carbamate.

	Length nm ± S.D.							
	Chicken intact myosin	Isolated rod	Rabbit intact myosin	Isolated rod	Rabbit S-2 fragments			Chicken LMM fragment
					61 kDa	53 kDa	34 kDa	
*p*H 7.0								
0.3 M	149.4 ± 6.5 (62)	139.0 ± 6.8 (38)	155.9 ± 4.8 (33)	145.8 ± 5.8 (31)	69.0 ± 3.1 (26)	59.5 ± 3.5 (34)	40.1 ± 2.5 (22)	74.4 ± 3.8 (60)
0.15 M					71.1 ± 3.3 (28)	59.7 ± 3.0 (35)	38.5 ± 3.3 (81)	
*p*H 7.5								
0.3 M	137.9 ± 6.7 (31)	133.6 ± 9.1 (15)	—	139.0 ± 9.9 (21)	66.2 ± 2.3 (39)	54.5 ± 3.1 (46)	37.1 ± 2.5 (64)	73.3 ± 4.5 (38)
0.15 M					65.2 ± 2.7 (37)	46.2 ± 6.2 (19)	38.3 ± 2.2 (36)	
*p*H 8.0								
0.3 M	137.6 ± 8.1 (31)	137.0 ± 6.4 (17)	—	139.5 ± 10.9 (31)	64.0 ± 3.5 (30)	53.4 ± 2.7 (34)	42.6 ± 4.6 (13)	74.6 ± 5.5 (32)
0.15 M					61.6 ± 3.5 (25)	51.3 ± 3.8 (62)	38.8 ± 2.6 (91)	
*p*H 8.0/7.0								
0.3 M	151.5 ± 5.4 (43)	141.6 ± 4.4 (40)	—	150.0 ± 5.2 (54)	70.2 ± 4.2 (78)	61.3 ± 3.4 (46)	40.2 ± 2.3 (80)	—
0.15 M					67.9 ± 4.1 (32)	57.4 ± 3.6 (32)	38.5 ± 2.7 (35)	

S. D. Standard deviation. — Values in brackets: number of molecules measured.

ature of the stage had been adjusted stepwise to increasing temperatures (20°–70 °C). Table II and Figure 3 show that between 20° and 40 °C there was a remarkable decrease in lengths among rabbit and chicken rod and long S-2 fragments (61 kDa and 53 kDa). No significant contraction of these structures took place between 40° to 50 °C, but additional shortening, possibly due to irreversible damage to the helical structure, was measured upon further heating to 70 °C. However, most importantly, the 34 kDa short S-2 fragment did not shorten at all as the temperature was brought up to 50 °C. Only further increasing the temperature to 70 °C led to some 5 to 6 nm shortening of short S-2 corresponding to 10 to 15% of its original length (Fig. 3, Tab. II). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of myosin rod samples and fragments thereof revealed no degradation bands even after preheating the samples for 5 min at 50 °C prior to mixing with SDS-sample buffer.

Effect on pH on the length of intact myosin, isolated rods and rod fragments

The lengths of intact myosin, isolated rods, S-2 fragments and LMM after air-drying in vacuo from 0.3 M or 0.15 M ammonium acetate, containing 50% glycerol, at different *p*H conditions (*p*H 7.0, 7.5, 8.0) are summarized in Table III. At *p*H 7.5, intact myosin rod and isolated rods from chicken and rabbit were shorter by about 12 nm and 6 nm, respectively, compared to the same preparations at *p*H 7.0. The lengths of the S-2 fragments (61 kDa, 53 kDa, 34 kDa), were significantly shorter at *p*H 7.5 compared to *p*H 7.0, but only in 0.15 and not in 0.3 M salt (Tab. III). By contrast, the 34 kDa short S-2 fragment displayed the same or even a slightly increased length at *p*H 7.5 compared to *p*H 7.0. The length of the LMM did not change either. Intact myosin and isolated rods were shorter at *p*H 8.0 by 12 nm and 2 to 6 nm, respectively, compared to controls at *p*H 7.0. The long S-2 fragments prepared in 0.3 M buffer were shorter by about 5 to 6 nm at *p*H 8.0, whereas the length of the 34 kDa S-2 fragments was unchanged by *p*H as was the length of LMM. The *p*H-induced shortening of the 61 kDa and 53 kDa long S-2 fragments was more pronounced in 0.15 M salt (8–10 nm) as compared to high salt.

In control experiments, in which the samples were incubated first in buffer solvents at *p*H 8.0 for 5 min and then diluted in buffer solvents of *p*H 7.0 (Tab. III, *p*H 8.0/7.0) the lengths of all samples favorably compared to those prepared in standard solutions at *p*H 7.0.

Discussion

The results presented demonstrate that the lengths of myosin rod and rod fragments, except for the short S-2 and LMM fragments, underwent shortening by 7 to 15 nm upon raising of temperature and/or raising of *p*H. The shortening observed was a reversible process as shown by control experiments and not due to irreversible damage of the rod structure by heat or alkaline *p*H. Also, the decrease in lengths of rods and long S-2 fragments was not a result of proteolysis induced by warming up of the samples, since control experiments with samples preheated to 50 °C for 2 to 4 min showed only single bands on SDS-gels that comigrated with the original polypeptides. Since the lengths of LMM and short S-2 fragments were not decreased on raising the temperature to 45 to 50 °C or the *p*H to 8.0 (Fig. 3, Tab. I–III) we believe that the region sensitive to temperature and *p*H resides between the LMM and the short S-2 segment of the rod (Fig. 1). Length determination by electron microscopy used here can directly demonstrate shortening of the rod resulting from a phase transition, but cannot yield data on the exact temperature or *p*H where the transitions took place. However, it was possible to deduce that only those fragments which contained the flexible hinge region were able to shorten at temperatures below 50 °C. The melting temperature and thus the stability of coiled-coil α-helical structures is expected to be elevated significantly in the presence of 50% glycerol [16] or ethylene glycol both known to displace the melting profile of isolated hinge fragments by about 17 °C [15]. Thus, the fact that one can directly observe shortening of the flexible hinge in the presence of 50% glycerol at temperatures between 20 to 50 °C and the fact that this shortening is enhanced at physiological salt conditions versus higher salt strengthens the importance of our finding.

Recently, Cross et al. [2] using differential scanning calorimetry, were unable to detect significant melting in the rod of smooth muscle myosins at temperatures below 50 to 56 °C. They concluded from these studies that the development of contractile force in smooth muscle does not result from helix melting but more likely from structural transitions in subfragment-1.

Based on our results, however, it does seem probable that the isolated rods and rod fragments of skeletal myosin, with the exception of LMM and short S-2, have the ability to shorten as a result of helix melting even when the heads are removed by digestion. Moreover, recent enzyme-probe studies of glycerinated psoas fibers [20] suggest that Ca^{2+}-induced activation results in a striking shift (by about 20 °C) in the cleavage rate constant versus temperature profile within the hinge domain toward lower temperatures. Thus, it seems possible that the activation process itself may amplify melting [17] within the hinge region of the organized contractile apparatus by reducing its thermal stability into the physiological temperature range where the experimentally well documented α-helix-coil transition [12, 14, 15, 16, 18, 20] indeed may be of physiological importance for contraction.

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