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Thermal Transitions of Myosin and Its Helical Fragments. Regions of Structural Instability in the Myosin Molecule†

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ABSTRACT: The structural stabilities of all the familiar proteolytic fragments of myosin have been investigated in melting studies over the pH range 5.5–7.0 in 0.5 M KCl. All fragments except subfragment 2 undergo a melting transition manifested by the cooperative uptake of protons in the temperature range 34–47 °C, and these fragments experience an increase in transition temperature, T_m, as the pH is increased. Subfragment 2 undergoes a melting transition in the 43–55 °C range, manifested by the dissociation of protons, and it experiences a decrease in T_m as the pH is increased. These results suggest that pH changes can modulate the relative stabilities of the light meromyosin, subfragment-1, and subfragment-2 regions of the myosin molecule.

Most current theories of muscle contraction require that the myosin molecule possess sufficient flexibility to allow it to bend away from the thick filament in order to make contact with the thin filament (see, for example, Huxley, 1969; Huxley and Simmons, 1971; Harrington, 1971). The results of electron microscopy, x-ray diffraction, and biochemical studies suggest that such flexing actually does occur during contraction (Reedy et al., 1965; Huxley and Brown, 1967; Pepe, 1967; Huxley, 1968; Young et al., 1972; Pollard, 1975). Studies on mechanical transients of muscle fibers further support this view (Huxley and Simmons, 1972; Podolsky et al., 1969; Julian et al., 1972). Since myosin is bound into the thick filament by its rod-like tail moiety, it is generally supposed that this portion of the molecule is the site of the flexing phenomenon. The results of Lowey et al. (1969) and Burke et al. (1973) show that the rod portion of the molecule consists of a supercoil of two polypeptide chains, each of which contain 98–100% α-helical secondary structure. Although this helical structure confers appreciable rigidity to the polypeptide chains (Flory, 1956), flexibility is still possible in small regions of imperfect or unstable helicity. The existence of one or several such "hinge regions" is suggested by studies using ORD,[1] viscosity, proteolytic digestion, and pH as the observables (Burke et al., 1973; Jacobsen and Henderson, 1973; Goodno and Swenson, 1975a,b).

In order to examine the myosin molecule for these regions of structural instability, we have carried out melting studies on all the well characterized proteolytic fragments of myosin. Since the characteristic melting temperature (T_m) of each fragment provides an index of its structural stability (Goodno and Swenson, 1975a), it is possible to use the results to make a primitive assignment of the relative stabilities of various regions of myosin structure.

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[1] Abbreviations used: ORD, optical rotary dispersion; HMM and LMM, heavy and light meromyosins, respectively; EDTA, ethylenediaminetetraacetic acid.

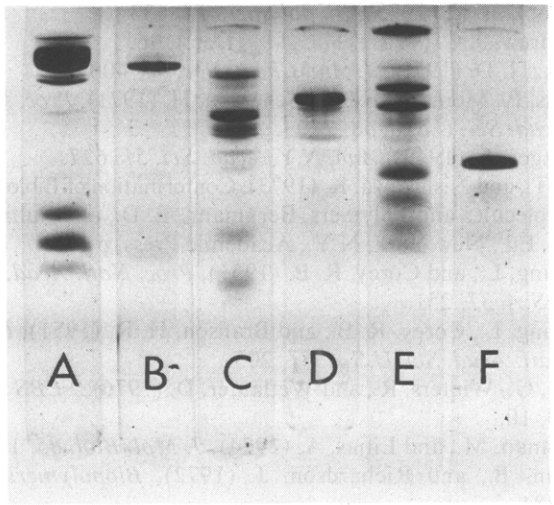

FIGURE 1: Sodium dodecyl sulfate gels of myosin and its fragments. (A) Myosin; (B) myosin rod; (C) heavy meromyosin; (D) light meromyosin; (E) subfragment 1; (F) subfragment 2. The gels were run in different series and are presented in order to show the purity of the fragments.

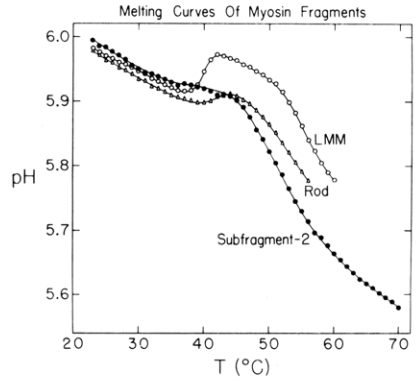

FIGURE 2: Typical melting curves of helical fragments of myosin. (○) Light meromyosin; (△) myosin rod; (●) subfragment 2. The protein concentration was 2 mg/ml and the solvent was 0.5 M KCl. Initial pH was approximately 6.0.

Methods

Myosin and light meromyosin were prepared by methods described earlier by Goodno and Swenson (1975b). HMM was prepared by a modification (Goodno and Swenson, 1975c) of the procedure of Lowey and Cohen (1962), in which myosin was digested for 90 s at 23 °C and digestion was terminated by addition of a fourfold excess of trypsin inhibitor. Myosin rod and subfragment 1 were prepared from myosin by papain digestion according to the procedures of Bálint et al. (1975), using a 2-min digestion. Subfragment 2 was prepared from myosin by the procedure of Bálint et al. (1972) and by two alternate procedures. (1) Myosin rod was digested with trypsin under the same conditions that were used in the preparation of HMM from myosin. (2) HMM was digested with papain under the same conditions that were used for the preparation of myosin rod from myosin. All three types of subfragment-2 preparation were purified according to the procedure of Bálint et al. (1972).

The Ca^{2+} and EDTA–ATPase activities of myosin, heavy meromyosin, and subfragment 1 were assayed under the conditions of Kielley and Bradley (1956) and Kielley et al. (1956). All preparations of myosin and its fragments were checked for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Essentially homogeneous preparations of myosin, myosin rod, light meromyosin, and subfragment 2 were obtained, while the usual spectrum of peptides was obtained from HMM and subfragment 1 (Figure 1). Although subfragment 2 migrated as a single band, it consistently gave an apparent subunit molecular weight of 43 000, as compared with 38 000 obtained by Biró et al. (1972) and 32 000 obtained by Lowey et al. (1969). We noted that the molecular weight of this fragment was dependent on digestion time and, although our times of digestion were similar, our enzyme activity may have been lower than that of Bálint et al. (1972). In view of this factor and the inherent error of ±2000 on the molecular weights determined by gels, we feel that we are in substantial agreement with the more recent work of Biró et al. (1972). The identity of this fragment was subsequently verified by preparing it from myosin rod and heavy meromyosin, as well as from myosin.

Protein melting experiments were carried out by the method of Bull and Breese (1973), as modified by Goodno and Swenson (1975a,b). Before melting, protein solutions were exhaustively dialyzed to remove residual buffers. Melting was performed on 5.0-ml samples of each fragment at a protein concentration of 2 mg/ml in 0.5 M KCl. Protein concentrations had only a small effect on the melting temperatures. Likewise the difference in melting behavior in 0.5 M KCl and more physiological ionic strengths, 0.2–0.25 M, was noted to be small (Goodno and Swenson, 1975b). Studies were performed on several batches of each fragment under several pH conditions. As previously described (Goodno and Swenson, 1975a), the melting temperature was assigned to the center of the cooperative transition in the plot of pH vs. temperature. The center of this transition was sometimes located by finding the center of the peak or trough that was obtained by plotting $(dpH/dT)_T$ vs. temperature. In usual practice, the melting temperature was easily located from the plot of pH vs. temperature.

Results

Figure 2 compares the melting curves of the helical fragments of myosin at pH 6. As observed earlier (Goodno and Swenson, 1975b) myosin rod and LMM yield qualitatively similar curves with almost identical T_m values. Subfragment 2, however, shows a unique melting curve and yields a considerably higher T_m value. Although rod and LMM absorb protons during their melting transition, subfragment 2 releases them. The monotonic baseline portions of the rod, LMM, and subfragment-2 curves all have a negative thermal coefficient of pH; however, that for subfragment 2 generally has a smaller value.

Cursory examination of Figure 2 suggests that myosin rod and LMM might have two pH transitions, of which the second is similar to the transition of subfragment 2. Analysis of the rod and LMM curves (see Goodno and Swenson, 1975b) suggests that what appears to be the second transition (downturn) in pH is actually a gradual return of the curve to the original baseline slope. In further support of this analysis, it is found that the downturn exhibits the same pH dependence found for the T_m of myosin rod and LMM. This pH dependence is opposite from the pH dependence of the T_m of subfragment 2. It is possible that more transitions are contributing to the experimental curves; however, at present they have not been resolved.

Since the melting properties of subfragment 2 were somewhat unexpected, and since the subunit molecular weight we observed for it was high in comparison with reported values, we questioned whether we had a proper preparation of this

fragment. Consistent melting results were obtained on several standard preparations, and previous experience in preparing myosin fragments suggested a possible explanation for this unusual behavior. Biró et al. (1972) have prepared a series of proteolytic fragments from LMM, which they have denoted "LF" fragments. We have found that prolonged papain digestion of *soluble* myosin produces a mixture of helical, water-soluble fragments in the same molecular weight range as the LF fragments. Furthermore, these fragments are isolated under the same conditions used for isolation of subfragment 2. For this reason, we were concerned that our subfragment 2, although prepared from *precipitated* myosin, might actually consist of the LMM fragment that Biró et al. (1972) have denoted "LF-1". To test this possibility, we prepared subfragment 2 by papain hydrolysis of HMM and by tryptic hydrolysis of myosin rod. All these preparations, however, showed identical electrophoretic and melting behavior, so we must conclude that the observed properties are intrinsic to genuine subfragment 2.

Figure 3 depicts the melting of myosin, HMM, and subfragment 1 under the same conditions. All three molecules show similar melting profiles, melting temperatures, and thermal coefficients. A graded increase in the size of the pH shift across the melting transition seems to occur from myosin to HMM to subfragment 1, with subfragment 1 giving an unusually large shift.

Figure 4 summarizes the melting results for myosin and its fragments as a function of pH. Native myosin and all the fragments except subfragment 2 melt with an absorption of protons and show a decline in T_m with decreasing pH. The most striking observation is that the behavior of subfragment 2 is opposite to that of all the other fragments. It melts with a release of protons, rather than an absorption, and the melting temperature increases with decreasing pH. While myosin rod and LMM melt partially reversibly, the melting of subfragment 1, HMM, and subfragment 2 is irreversible. In most cases an increase in the turbidity of the protein solution accompanies melting. As we have earlier discussed (Goodno and Swenson, 1975a,b), the aggregation that gives rise to this turbidity is distinct from the melting of protein structure that is observed by the pH method.

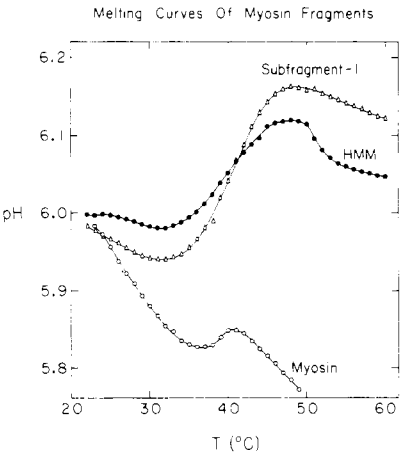

FIGURE 3: Melting curves of myosin, heavy meromyosin, and subfragment 1. (○) Myosin; (△) subfragment 1; (●) heavy meromyosin. The protein concentration was 2 mg/ml and the solvent was 0.5 M KCl. The initial pH was approximately 6.0.

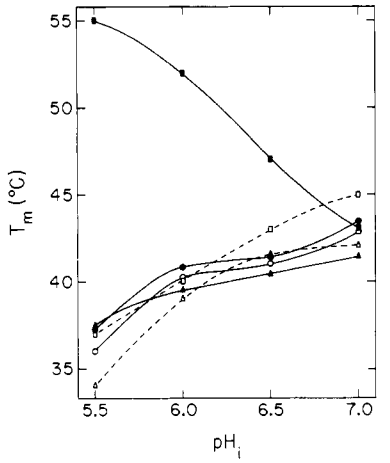

FIGURE 4: The effect of pH on melting temperatures of myosin and its fragments. (A) (—○—) Myosin; (- - □ - -) subfragment 1; (- - △ - -) heavy meromyosin. (B) (—▲—) Light meromyosin; (—●—) myosin rod; (—■—) subfragment 2. The protein concentration was approximately 2 mg/ml and the solvent was 0.5 M KCl. T_m is defined as the midpoint of the thermal transition as shown in Figures 2 and 3.

Discussion

The present study is a continuation of our attempts to map out the conformational stability of various regions of the myosin molecule. The overriding difficulty in this work is that myosin and its fragments undergo a thermally induced aggregation (Burke et al., 1973) that prevents the quantitative measurement of the optical and hydrodynamic properties of their solutions over a range of pH and temperature. We have used pH for the observable in these studies because it is insensitive to changes in aggregation (Goodno and Swenson, 1975a; Bull and Breese, 1973). A comparison between the melting results obtained by pH, viscosity, and ORD methods can be made, however, under solvent conditions where aggregation is minimal (high pH and high ionic strength). To our knowledge, the results for subfragment 1 and HMM are the only structural melting results for these fragments. The T_m values for these species, however, are found to fall in the general temperature range where subfragment 1 and HMM lose their ATPase activity (Kawakami et al., 1971). As observed earlier and confirmed here, our results for myosin, rod, and LMM are consistent with the monophasic viscosity transitions that were observed by Burke et al. (1973) and Jacobson and Henderson (1973). By contrast, when Burke et al. (1973) monitored the melting of rod and LMM by ORD, they found a biphasic transition, the first phase of which roughly parallels their viscosity transition and our pH transition. Thus, pH and viscosity seem to respond to the same sorts of changes in myosin structure, but ORD responds to a rather different set of changes.

In partial confirmation of the results of Burke et al. (1973), subfragment 2 shows an elevated melting temperature relative to the other fragments. Burke et al. (1973) observed by ORD a biphasic melting curve similar to that of LMM, but involving a larger change in helicity in the higher temperature transition. The monophasic transition in our pH study roughly parallels this high-temperature transition in ORD. Since the low-temperature transition characteristic of LMM and rod is totally absent in our subfragment-2, pH melting curve, it is surprising that this transition is present in the ORD melting curve. A possible explanation for this discrepancy is found in our observation that the digestion of soluble myosin by soluble papain under the conditions of Lowey et al. (1969) yields a mixture of fragments, whereas digestion by the procedure of Bálint et al. (1972) yields a single fragment. Burke et al. (1973) used

subfragment 2 prepared from soluble myosin by the method of Lowey et al. (1969), and we are unable to evaluate the purity of their preparation. The three batches of subfragment 2 used in these studies were prepared under the conditions of Bálint et al. (1972) and purified to a single band on sodium dodecyl sulfate gel electrophoresis. In addition, subfragment 2 was prepared from HMM and myosin rod. Since all preparations gave the same electrophoretic and melting behavior, we can be reasonably sure of the identity of this fragment. It is still possible, however, that subfragment 2 undergoes a biphasic melting transition whose lower phase is not observed by pH. Further studies by tandem ORD, viscosity, and pH experiments on the same preparation will be required to resolve this question.

All the fragments except subfragment 2 yield qualitatively similar melting curves and strikingly similar T_m values. Descending baselines indicate an aggregate positive heat of ionization (about +3 kcal/mol), which is characteristic of the dissociation of a mixture of carboxyls and histidine residues in the pH range studied (Wyman, 1939). The proton absorption upon melting is consistent with a normalization of the pK values of histidines as they transfer from a buried to an exposed environment upon disruption of the protein structure (Timasheff and Rupley, 1972). In addition, T_m for all these fragments varies inversely with pH. As previously discussed, these effects are characteristic of the disruption of a region of structure where histidines are the predominant residues ionizing near pH 6.0 (Goodno and Swenson, 1975b).

The melting curve of subfragment 2, on the other hand, suggests the unfolding of a region of structure where glutamic and aspartic acids are the major ionizable residues. The descending baseline is indicative of a small heat of ionization (+1.5–2.0 kcal/mol), which is typical of the dissociation of carboxyl groups (Wyman, 1939), and the abrupt release of protons during melting is consistent with the exposure of buried carboxyls. These features are, moreover, consistent with the high glutamate and aspartate and low histidine content of subfragment 2 (Lowey et al., 1969).

The view of myosin structure at pH 6 that emerges from these studies is one of a rather low structural stability ($T_m \sim$ 40 °C) throughout the molecule, with the exception of the subfragment-2 region, which is relatively more stable. It is highly informative to consider this picture as a function of pH, however, for the T_m of subfragment 2 varies inversely with pH, while the T_m of the rest of the molecule varies proportionally. As pH is increased, the stability of subfragment 2 is depressed to the level of the rest of the molecule (Figure 4). Specifically this means that at pH 7 subfragment 2 is relatively more stable than the rest of the rod portion of the molecule; however, as the pH is increased above 7, the molecule's stability decreases and it becomes relatively more flexible than the rest of the molecule. Thus pH changes appear to modulate the relative stabilities of various portions of the myosin molecule. This may be relevant to the proposal of an elastic element by Huxley (Huxley and Simmons, 1972) and to the models of muscle contraction proposed by Harrington (1971) and Huxley and Simmons (1972).

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