Thermal Transitions of Myosin and Its Helical Fragments. I. Shifts in Proton Equilibria Accompanying Unfolding[†]

Charles C. Goodno* and Charles A. Swenson

ABSTRACT: The thermal transitions of myosin and its helical fragments have been studied with pH as the observable. Heating unbuffered solutions of these proteins near their pI values causes an abrupt rise in pH at a characteristic temperature (the "melting temperature," T_m) which is due to structural changes within the protein. Since the pH shift turns out to be insensitive to the degree of protein aggregation, we have obtained acceptable melting curves even under conditions where the protein coagulates during melting. The melting profiles and T_m values of myosin, myosin rod, and light meromyosin have been found to be remarkably similar (T_m = 40 ± 1°, 0.5 M KCl, pH 5.9). Proton binding which occurs during melting coincides with the unfolding of a section of myosin rod. Taken in the context of other studies, the proton binding is thought to occur near the "hinge region."

Myosin (EC 3.6.1.3, ATP phosphohydrolase) is the major protein of the thick filaments of muscle fibers. It can be visualized as a hybrid molecule which contains two globular ATPase moieties attached to one end of a rod-like, α-helical tail (Lowey et al., 1969). Helix ⇌ coil transitions in its rod-like portion are thought to be central to the process of muscle contraction (Flory, 1956; Davies, 1963; Pepe, 1967; Huxley, 1969; Harrington, 1971). Such transitions can be produced experimentally by heating the purified protein to temperatures a few degrees above physiological temperature (Burke et al., 1973; Jacobson and Henderson, 1973). These transitions may be viewed as the melting of regions of "crystalline structure" within the molecule (Flory, 1956; Harrington, 1971); and they may be characterized by a "melting temperature" (T_m), which gives a useful estimate of the conformational stability of the molecule. Solvent conditions ordinarily exert a large influence on the thermal stability of proteins (Joly, 1965). For this reason, we have sought to use T_m values to evaluate the influence of solvent conditions on the conformational stability of myosin.

The solvent conditions of immediate interest were pH and ionic strength. Unfortunately, myosin is highly prone to heat coagulation (Lowey and Holtzer, 1959; Yasui et al., 1968), and this tendency is exaggerated at pH values below neutrality (Takahashi et al., 1962). We have found that heat coagulation is also favored at ionic strengths below

about 0.3. Thus, ordinary physical methods (e.g., ultracentrifugation, viscometry, and spectral methods) were inappropriate for our studies. A method recently introduced by Bull and Breese (1973a) appeared to hold potential for circumventing this problem. The method relies on the principle that thermal denaturation of a protein is ordinarily accompanied by a net uptake or release of protons caused by changes in the environment of ionizable residues. In application, the pH of an unbuffered protein solution is precisely monitored as a function of temperature while the temperature is raised through the T_m of the protein. Melting of the protein is manifested by a relatively abrupt shift in pH centered at a temperature corresponding to T_m. No discontinuity in the melting curve of pH vs. temperare is observed when the protein aggregates or coagulates during melting (Bull and Breese, 1973a). Melting observed by this method cannot be attributed simply to protein aggregation since a number of proteins aggregate only at temperatures considerably above their observed melting temperatures. We have, therefore, sought to use the method of Bull and Breese to study the melting behavior of myosin and its fragments. This paper, then, describes the first study of the proton-binding process which accompanies the thermally induced unfolding of the myosin molecule.

Methods

Purification and Analysis of Proteins

Protein Preparation. Myosin and its helical fragments were prepared according to standard methods with the minor modifications given below. All procedures except ethanol precipitations of the fragments were performed at tem-

peratures below 4°. Glass-distilled water was used through-out, and all chemicals were of reagent grade or better.

MYOFIBRILS served as a convenient starting material for protein isolations. A preparative method similar to that of Perry and Grey (1956) was used, with a major modification consisting of the use of the following buffer suggested by Goll et al.:[1] 0.1 M KCl, 2 mM MgCl₂, 1 mM NaN₃, and 20 mM potassium phosphate (pH 6.8). New Zealand rabbits were sacrificed, and the back- and hind-leg muscles were dissected out. The tissue (about 300 g) was subjected to five steps (5–10 sec each) of low-speed homogenization with cold buffer (3.5–5.0 volumes) in a 1-gal Waring Blendor. After each of the first three homogenizations, the slurry was centrifuged 10 min at 1000g in a Sorvall RC 2-B centrifuge. Pelleted material was retained for the next step. After the fourth step, the homogenate was strained through cheesecloth and centrifuged 10 min at 1000g. Pelleted material was subjected to the fifth homogenization and then centrifuged for 1 hr at 1450g. The resulting pellet was suspended in 3.5 volumes (v/v) of 0.1 M KCl, centrifuged 10 min at 1000g, and resuspended. Aliquots of the suspension were taken for protein estimation by a biuret method.

MYOSIN. A method suggested by Huxley and Hanson (1954) and detailed by Goll et al.[1] was used for myosin extraction. A suspension of myofibrils containing about 7 g of protein was centrifuged 10 min at 5000g. The pellet was suspended in about 600 ml of a buffer composed of 0.1 M sodium pyrophosphate and 10 mM MgCl₂ (pH 7.0). After 2 hr of stirring, the suspension was centrifuged 10 min at 14,000g to remove the residue. Myosin was salted out of the supernatant solution with ammonium sulfate between 35 and 45% saturation. The precipitate was collected by centrifugation at 8000g for 15 min and redissolved in 0.5 M KCl, with the pH adjusted to 7.0 by dropwise addition of saturated KHCO₃ solution. The myosin solution was then dialyzed against several 100-volume changes of 0.5 M KCl, and "recrystallized" at least once by dialysis against 0.05 M KCl, centrifugation, and redissolution of the precipitated myosin in 0.5 M KCl. The final protein solution was stored in stoppered vials at 5°. Preparations which could not be used within a week were glycerinated and stored at −20° by the method of Harris and Suelter (1967).

MYOSIN ROD. Precipitated myosin, purified as previously described, was used for preparation of myosin rod, following essentially the method of Harrison et al. (1971). The procedure was modified by the use of a final iodoacetic acid concentration of 5 mM to assure the quenching of proteolysis. The ethanol fractionation procedure of Szent-Györgyi et al. (1960) was used for purification of the rod from the papain digest. Rod preparations were stored at −20° by a modification of the method of Riddiford and Scheraga (1962).

LIGHT MEROMYOSIN was prepared from precipitated myosin by the method of Lowey and Cohen (1962), which was modified by the addition of a fourfold excess of trypsin inhibitor to quench proteolysis. The protein was stored under the same conditions used for myosin rod.

Characterization of Protein Preparations. ELECTROPHORESIS. Proteins were electrophoresed by the method of Weber and Osborn (1969) on acrylamide gels containing sodium dodecyl sulfate. An acrylamide concentration of 10% was used, with half the usual amount of cross-linking.

The gels were calibrated with appropriate protein standards for molecular weight determinations.

PROTEIN CONCENTRATIONS. Solution concentrations of myosin and myosin rod were calculated from A_{280} using E_{280} (1%) measured for our protein preparations.

ATPASE ASSAYS. Ca²⁺ ATPase assays were performed under the conditions of Bárány et al. (1964). P_i was assayed by the method of Clark (1964).

Methods for Studies on Protein Phase Transitions

General Features of the pH Method. ASSEMBLY OF THE MELTING APPARATUS. An apparatus similar to that described by Bull and Breese (1973a) was assembled, with the refinements of automatic temperature programming and continuous recording of temperature and pH. A linear temperature rise (r = 0.999) was programmed by driving the rotor of a contact thermometer in a circulating bath with a gear train connected to a synchronous motor.

The pH meter output was fed through a 100-mV zero-suppression circuit to a chart recorder which was calibrated to full-scale deflection of 0.1 pH unit. A thermistor probe in the sample cell was connected to a resistance bridge, and the unbalanced voltage of the bridge was fed through a voltage divider to another recorder.

CALIBRATION OF THE APPARATUS. The voltage vs. temperature output of the bridge circuit was calibrated to an accuracy of ±0.05° with a set of Brooklyn Thermometer Company thermometers which had been calibrated by the National Bureau of Standards. The pH meter was adjusted for meter zero and for coincidence of the extremes of the expanded scales. Double-buffer adjustments were carried out at 25°, using standard 0.05 M phthalate (pH 4.008) and 0.05 M sodium/potassium phosphate (pH 6.865) buffers described by Bates (1964). Adjustments were not considered satisfactory unless both buffers gave readings within ±0.005 pH unit of their accepted values. The meter was checked for drift with 0.05 M phthalate buffer and found to drift less than 0.002 pH/hr.

GENERAL PROTOCOL FOR A MELTING EXPERIMENT. All experiments were begun at 25°. The pH meter was calibrated as previously described. Calibration of the temperature bridge was checked by the use of decade resistance box to dial in resistance values corresponding to known temperatures. Electrodes and sample compartment were thoroughly rinsed with distilled water and blotted dry. Residual buffers were removed from protein solutions by exhaustive dialysis, and particulate matter was removed by centrifugation prior to use. The sample solution (5.0 ml), containing 1–2 mg/ml of protein, was pipetted into the cell, and the electrodes were immersed; 1 ml of purified cetane (Humphrey-Wilkinson) was layered on the protein solution through a polyethylene tube in the top of the cell. Stirring was begun, and the solution was adjusted to the desired initial pH with either 0.1 N HCl or 0.1 N NaOH delivered from a syringe microburet. The temperature and pH recorders were started (both running at the same chart speed) and indexed, and the linear temperature programmer was started. In the studies reported here, the rate of temperature rise was 0.44°/min and data were usually collected up to at least 60°. After the cell was cooled to room temperature, the sample compartment was washed, rinsed, and dried. The electrodes were rinsed with mild detergent (or immersed in 6 M guanidine hydrochloride for removal of protein precipitates), then rinsed with H₂O, and blotted dry. Calibration of the meter was rechecked with the stan-

---

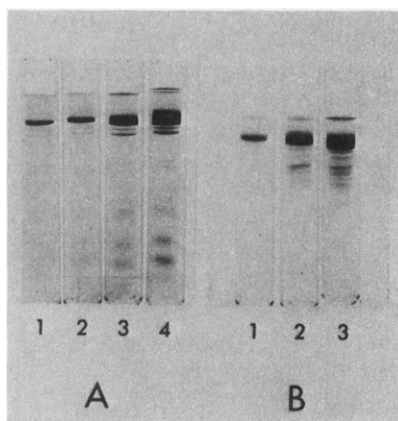[1] D. E. Goll, R. Robson, and M. Stromer, personal communication.

FIGURE 1: Electrophoresis of myosin and myosin rod on SDS polyacrylamide gels. (A) Myosin, 1–4: 10, 20, 50, and 100 μg of protein/gel; (B) myosin rod, 1–3: 10, 25, and 50 μg of protein/gel.

Table I: Spectral Data for Myosin and Myosin Rod.

Protein	E_{280} (1%)	A_{280}/A_{260}	E_{280} (1%)[b]	A_{280}/A_{260}[a] (Lit.)
Myosin	5.55 ± 0.2	1.68	5.50	1.74
Myosin rod	1.99 ± 0.08	1.74		

[a] Harris and Suelter (1967). [b] Godfrey and Harrington (1970).

dard phosphate buffer (pH 6.865) at 25°. If a deviation of more than 0.02 pH unit from the proper value was observed, the results of the experiment were discarded.

COMPENSATION OF PH FOR TEMPERATURE. For convenience, the temperature compensator on the pH meter was kept at its 25° setting throughout the melting experiment and the measured pH values were mathematically corrected for the temperature effect on hydrogen ion activity by the empirical function used by Bull.[2] A derivation of the correction function from general principles will be presented elsewhere.

DATA ANALYSIS. Melting curves were constructed by plotting the corrected pH values against their corresponding temperatures. The derivative was calculated at each temperature, T, and a plot of $(\Delta pH/\Delta T)$ vs. T constructed. The melting point (T_m) was assigned to the temperature corresponding to the center of the major band in the derivative curve. The pH at the melting point (pH$_m$) was assigned to the pH value corresponding to T_m.

Results

Homogeneity of Protein Preparations. Electrophoresis of myosin heavy chains in the presence of sodium dodecyl sulfate (SDS)[3] yielded a single major band with a molecular weight of about 206,000 (Figure 1). Electrophoresis of myosin rod under the same conditions showed a single major band of 80–90% purity with a molecular weight of 129,000 (Figure 1). The molecular weights obtained for myosin and myosin rod are similar to literature values, though the molecular weight of the rod is slightly higher than most com-
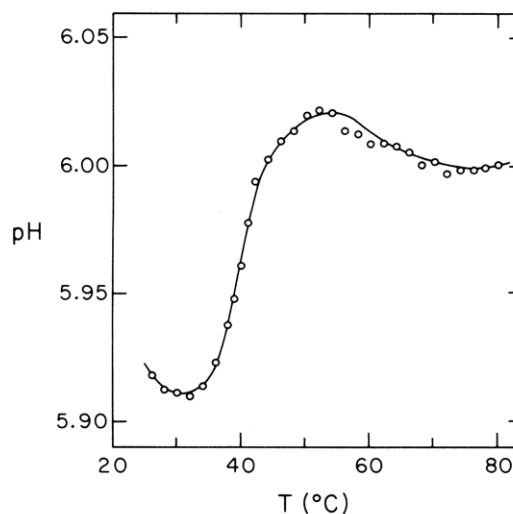
[2] H. B. Bull, personal communication.
[3] Abbreviation used is: SDS, sodium dodecyl sulfate.

FIGURE 2: Melting of myosin (1.85 mg/ml) in 0.5 M KCl. The values of pH$_i$, pH$_m$, and T_m were, in order: 5.92, 5.97, and 41.0°.
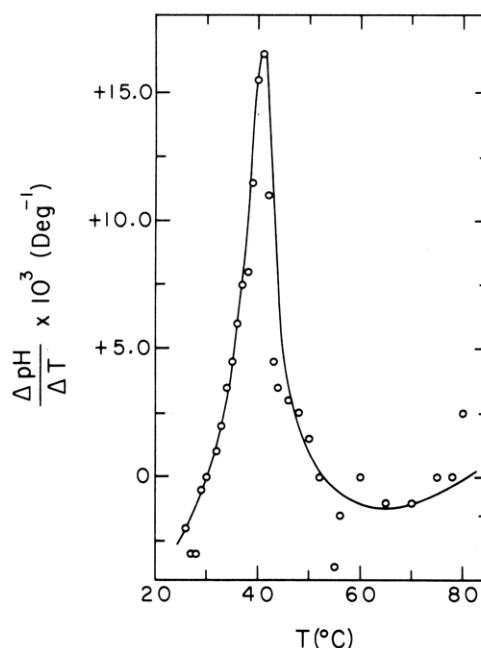

FIGURE 3: Derivative of the myosin melting curve. The curve was calculated from the melting curve in Figure 2. Note that T_m corresponds to the peak of the curve (41.0°).

monly reported values. Spectral data for myosin show good agreement with literature values (Table I). We are unaware of reported values of E_{280}(1%) for myosin rod; however, our value is small, consistent with the low aromatic amino acid content of this fragment. Ca^{2+} ATPase activities of our myosin preparations were comparable to those reported by Bárány et al. (1964).

Melting Studies. Initial melting studies were performed on myosin near its isoelectric pH (no acid or alkali added) in 0.5 M KCl. A relatively smooth melting curve of pH vs. temperature was obtained (Figure 2), which yielded a sharply peaked derivative curve (Figure 3). T_m was easily assigned to the center of the peak (41.0°). In general, the derivative points described a smooth curve on the low-temperature side of the peak, but exhibited some scatter on the high-temperature side. Initial studies on myosin rod near its isoelectric pH in 0.5 M KCl produced a remarkably similar
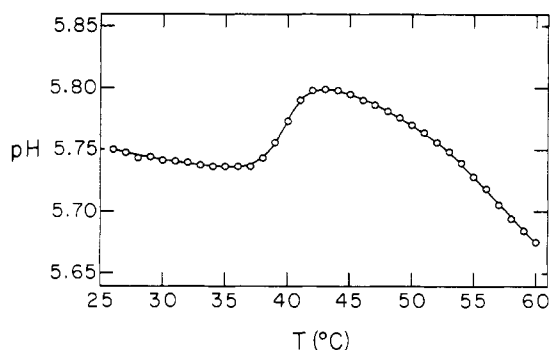
FIGURE 4: Melting of myosin rod (0.45 mg/ml) in 0.5 M KCl. The values of pH_i, pH_m, and T_m were, in order: 5.75, 5.77, and 39.7°.

FIGURE 5: Derivative of the myosin rod melting curve. The curve was calculated for the melting curve in Figure 4.

melting curve (Figure 4) and an even smoother derivative curve (Figure 5), which allowed assignment of a T_m of 39.7°. A few experiments on light meromyosin (not shown) produced very similar melting curves, yielding T_m values within ±0.5° of those of the rod. Preliminary studies were not done on subfragment-1 because only limited quantities of it could be prepared. Later studies have strongly suggested that it makes little contribution to the myosin melting curve between 20 and 60°.

During the course of melting, myosin coagulated into a fibrous mass, and myosin rod and light meromyosin solutions often became turbid. These obvious changes in the degree of aggregation produced no discontinuities in the melting curve, except in cases where they interfered with the stirring of the solution. The results of experiments in which coagulation interfered with stirring were immediately discarded, and the experiments were repeated. No systematic attempt was made to determine the precise temperature of coagulation, but it generally seemed to occur a few degrees above the melting temperature.

The presence of residual buffer in the protein solution was easily detected as an apparent failure of the protein to melt. Whenever this occurred, the protein was further dialyzed against 0.5 M KCl, and melting was repeated. Dialysis against five to six changes of 100 volumes each was often required before the melting curve became independent of the amount of dialysis.

The melting curves of myosin, light meromyosin, and myosin rod were remarkably similar. All appeared to consist of a cooperative increase in pH which was superimposed upon a smooth, overall decrease in pH. Light meromyosin and rod gave smoother curves than myosin, possibly reflecting the absence of small contributions from the globular portion of the myosin molecule. By virtue of the near identity of the T_m for all three species, one must conclude that the globular portion of the molecule either has a T_m almost identical with that of the fibrous portion of the molecule or that the globular portion makes almost no contribution to the melting curve generated by this method.

Control Studies. Since the advancement of the pH method for monitoring the melting of proteins by Bull and Breese (1973a), we have developed an automated technique and a new form of data handling based on their original method. The validity of these methods is demonstrated by the control experiments discussed in the following sections.

"Melting" of Solvent. It is sensible to question whether the solvent makes any contribution to the melting curve. Thus, we have performed "melting" experiments on 0.5 M KCl (our standard solvent) in the absence of protein. Melt-
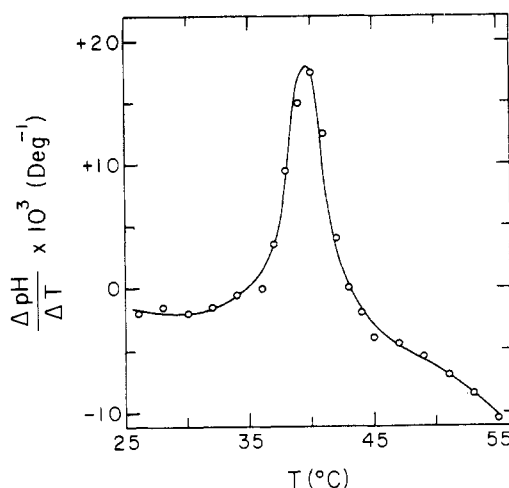
ing curves and their derivatives were obtained at several pH values, and typical results are displayed in Figure 6. While the shapes of the curves vary considerably with pH, it is apparent that heating of the solvent produces no pH change which might be construed as a melting phenomenon.

Melting of Deaerated Samples. The observed "melting curves" for 0.5 M KCl are undoubtedly produced by small amounts of dissolved CO_2, rather than by the neutral salt. Since the solubility coefficient of CO_2 is strongly temperature-dependent, the gas is undoubtedly driven from solution during heating. Although there is no compelling evidence that myosin binds CO_2, we were concerned that should this be the case, the release of bound CO_2 might produce phenomena resembling melting. Accordingly, several myosin rod samples were exhaustively deaerated and melted under an atmosphere of N_2. Compared with the nondeaerated controls, the resulting melting curves showed a smaller pH change across the melting region but showed no alteration of T_m.

Rate of Heating and T_m. In most types of melting studies (*e.g.,* scanning microcalorimetry), one finds a marked variation in T_m with the rate of heating. This variation in T_m was investigated for myosin by melting it at three different heating rates. As the rate of heating was lowered from 1.86 to 0.44°/min, T_m fell by about 1.5°. Extrapolation of the curve of T_m *vs.* heating rate to a heating rate of zero (equilibrium) showed that the equilibrium value of T_m was only 0.4° lower than the value obtained at our standard heating rate of 0.44°/min (Figure 7). Since this deviation falls within the usual experimental error, we may conclude that our T_m values are a good approximation to equilibrium values.

Discussion

Applications of Method. The present study is the first reported examination of the conformation of myosin in which pH has been used as the observable. Because of the novelty of the method used, a closer look is worthwhile. In its simplest application, the method consists of monitoring the pH of an unbuffered protein solution as the temperature is raised at a uniform rate. At lower temperatures, a monotonic shift in pH occurs, but as thermal denaturation (melting) sets in, an abrupt increase or decrease occurs (see Figure 2). Such behavior has been noted in titration curves when proteins denature (Steinhardt and Zaiser, 1955; Tanford,
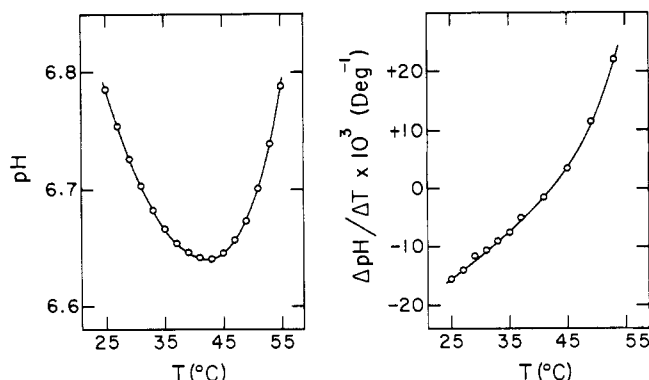
FIGURE 6: Control: "melting" of 0.5 M KCl initiated at pH 6.78. (A) Melting curve; (B) derivative curve.

FIGURE 7: Influence of heating rate on T_m value for myosin. Conditions: 1.85 mg/ml of protein in 0.5 M KCl; pH_m was 6.0 ± 0.2.

1962). The temperature at the center of this abrupt shift is given the term "melting temperature" and denoted by T_m (Flory, 1956). T_m varies from protein to protein and is characteristic for the protein in a particular solution environment (Bull and Breese, 1973a,b).

As in the use of other observables to monitor denaturation, one makes certain assumptions in using pH. We assume that changes in pH reflect changes in protein structure. Thermally induced ionizations of protein residues could cause such an effect; however, they produce only monotonic shifts in pH and are incapable of explaining the abrupt shift which we attribute to melting (see Goodno and Swenson, 1975). Thus, we can reasonably presume that our observed pH shift is indeed caused by a structural change. The abrupt change in pH is assumed to be *synchronized* with the abrupt change in structure which accompanies melting. The best justification for this assumption is the empirical fact that the pH method yields essentially the same T_m values as do other methods (Bull and Breese, 1973a,b). The assumptions behind the pH method, then, are no more sweeping than those of other methods for observing protein denaturation. Thus, we regard pH as an observable which complements other methods for the study of protein conformational transitions. Its availability as a conformational probe is of particular importance in the study of myosin as most other methods fail because of the aggregation that occurs in the system. Criteria for its validity as a probe of myosin conformation changes are prescribed in the following sections.

Validity of pH as a Conformational Probe for Myosin. It is apparent that the pH shifts we have observed are due to changes in the protein rather than changes in the solvent or the apparatus. One might logically wonder how such small pH shifts can be reproducibly measured. Routine measurements of pH values are generally limited to an accuracy of about ±0.01 pH unit, primarily because of the buffers used in the standardization process. Nevertheless we have measured pH changes which are sometimes much smaller than 0.01 unit. As pointed out by Bull and Breese (1973a), the success of these measurements relies only on pH differences rather than absolute values. With our apparatus, small pH differences can be detected quite accurately, and these differences yield a precision of about ±0.5° for duplicate T_m determinations.

It is critical to know whether the melting phenomenon is caused by changes within the myosin molecule or simply by the process of coagulation. This question may be approached from the standpoint of the equilibrium and the ki-
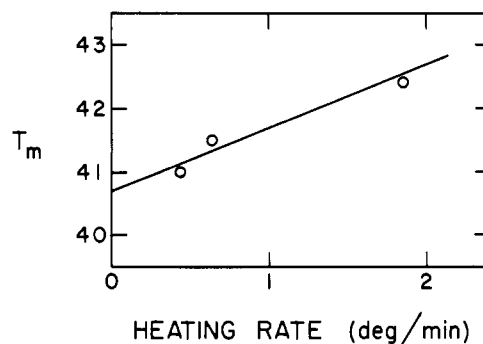
netics of coagulation. Since coagulation is a form of polymerization, it should be sensitive to concentration. If coagulation is the source of the pH range, then, T_m should be sensitive to protein concentration. We have measured T_m for myosin over a 50-fold change in concentration with no effect (see following paper for details). This result is similar to the observations of Bull and Breese (1973a) on ovalbumin. From the kinetic standpoint, we can compare the rate of thermal aggregation of myosin with the dependence of T_m upon the heating rate (Figure 1). Thermal aggregation of myosin proceeds rapidly at elevated temperatures (Lowey and Holtzer, 1959; Takahashi *et al.*, 1962). If coagulation were the source of the pH changes, T_m should be quite dependent upon heating rate. We find that, within the precision of our measurements (±0.5°) T_m is independent of heating rate. Thus, we may conclude that our studies have observed structural changes *within* the protein molecule. (Myosin, myosin rod, and light meromyosin are considered jointly in this context.)

Two features of our melting curves deserve special note. First, they show a single melting transition with very similar melting temperatures for myosin and its helical fragments. The viscosity results of Jacobson and Henderson (1973) (myosin) and Burke *et al.* (1973) (rod and smaller fragments) support this observation. The ORD results of Burke *et al.*, however, show an additional transition at higher temperature. Apparently the pH method is sensitive to the same structural change which produces the marked decrease in intrinsic viscosity. Burke *et al.* have speculated that the viscosity change is due to the "flexing" of the rod portion of myosin. We might, therefore, speculate that the observed pH changes occur as a result of this flexing. Second, melting occurs with an *uptake* of protons. In this regard, we might speculate that the binding of protons might be able to trigger an isothermal melting of a section of the helical portion of myosin.

In summary, we have demonstrated that the pH method can probe the conformational stability of myosin and its helical fragments even under conditions where aggregation occurs. Other methods in general use fail under such conditions. Thus the method is of particular importance for allowing the examination of myosin under a wider range of solution conditions. Flexing of the rod portion of the myosin molecule, which is apparently reflected by our observed conformational transitions, has been suggested as a possible mode of tension generation in muscle contraction (Flory, 1956; Davies, 1963; Pepe, 1967; Huxley, 1969; Harrington, 1971). Further, our results suggest the existence of a proton-binding process which is coupled with myosin conformational changes. This coupling may be of importance as

an energy-transduction mechanism (Harrington, 1971). The following paper further explores this and other implications of the process.

Acknowledgments

References

Bárány, M., Gaetjens, E., Bárány, K., and Karp, E. (1964), *Arch. Biochem. Biophys. 106*, 280.

Bates, R. G. (1964), Determination of pH, New York, N.Y., Wiley.

Bull, H. B., and Breese, K. (1973a), *Arch. Biochem. Biophys. 156*, 604.

Bull, H. B., and Breese, K. (1973b), *Arch. Biochem. Biophys. 158*, 681.

Burke, M., Himmelfarb, S., and Harrington, W. F. (1973), *Biochemistry 12*, 701.

Clark, J. M., Jr. (1964), Experimental Biochemistry, San Francisco, Calif., W. H. Freeman.

Davies, R. E. (1963), *Nature (London) 199*, 1068.

Flory, P. J. (1956), *Science 124*, 53.

Godfrey, J. E., and Harrington, W. F. (1970), *Biochemistry 9*, 886.

Goodno, C. C., and Swenson, C. A. (1975), *Biochemistry*, following paper.

Harrington, W. F. (1971), *Proc. Nat. Acad. Sci. U.S. 68*, 685.

Harris, M., and Suelter, C. H. (1967) *Biochim. Biophys. Acta 133*, 393.

Harrison, R. G., Lowey, S., and Cohen, C. (1971), *J. Mol. Biol. 59*, 531.

Huxley, H., and Hanson, J. (1954), *Nature (London) 173*, 973.

Huxley, H. E. (1969), *Science 164*, 1356.

Jacobson, A. L., and Henderson, J. (1973), *Can. J. Biochem. 51*, 71.

Joly, M. (1965), A Physico-chemical Approach to the Denaturation of Proteins, London, Academic Press.

Lowey, S., and Cohen, C. (1962), *J. Mol. Biol. 4*, 293.

Lowey, S., and Holtzer, A. (1959), *J. Amer. Chem. Soc. 81*, 1378.

Lowey, S., Slayter, H. S., Weeds, A. G., and Baker, H. (1969), *J. Mol. Biol. 42*, 1.

Pepe, F. (1967), *J. Mol. Biol. 27*, 203.

Perry, S. V., and Grey, T. C. (1956), *Biochem. J. 64*, 184.

Riddiford, L., and Scheraga, H. (1962), *Biochemistry 1*, 95.

Steinhardt, J., and Zaiser, E. M. (1955), *Advan. Protein Chem. 10*, 151.

Szent-Györgyi, A. G., Cohen, C., and Philpott, D. E. (1960), *J. Mol. Biol. 2*, 133.

Takahashi, K., Yasui, T., Hashimoto, Y., and Tonomura, Y. (1962), *Arch. Biochem. Biophys. 99*, 45.

Tanford, C. (1962), *Advan. Protein Chem. 17*, 70.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Yasui, T., Kawakami, H., and Morita, F. (1968), *Agr. Biol. Chem. 32*, 225.