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THE EFFECT OF TEMPERATURE ON ACTOMYOSIN

D. J. HARTSHORNE, E. M. BARNS, L. PARKER AND F. FUCHS*
Chemistry Department, Carnegie-Mellon University, Pittsburgh, Pa. 15213 (U.S.A.)
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SUMMARY

1. The effect of temperature on the ATPase activity of actomyosin systems was studied. The Arrhenius plot for natural actomyosin was curved between 0 °C and 40 °C for the Mg^{2+}-activated ATPase activity in the presence of calcium. A similar plot in the absence of calcium was linear over the same temperature range. The Arrhenius plot of the Mg^{2+}-activated activity of desensitized actomyosin in both the presence and absence of calcium was linear up to 40 °C. The addition of troponin B and tropomyosin to desensitized actomyosin did not alter the linearity of the plots. However, when troponin A was added (to complete the regulatory system) a non-linear plot resulted. It is proposed that the characteristic curvature of the Arrhenius plots for the Mg^{2+}-activated ATPase activity of natural actomyosin and myofibrils is a consequence of the binding of calcium to troponin A.

2. At temperatures above 40 °C all of the systems studied demonstrated a non-linearity, which was not influenced to any great extent by the regulatory proteins.

3. As the temperature was increased above 20 °C calcium sensitivity became less and at 50 °C was lost. It is unlikely that this effect was due to either the oxidation of critical sulfhydryl groups or to an alteration of the calcium binding characteristics of troponin. A possible explanation is suggested.

INTRODUCTION

One of the earliest studies of the effect of temperature on muscular activity was made by Shapley[1] in the 1920's. He showed that the walking rate of ants exhibited a non-linear relationship with temperature. It was later shown that the Arrhenius plots for the Mg^{2+}-activated ATPase activity of actomyosin[2] and myofibrils[3] were also non-linear with a break occurring at approx. 16 °C. Similar results prompted Levy *et al.*[4] to conclude that the rate limiting step in the walking rate of ants is probably the hydrolysis of ATP by actomyosin. Other workers have subsequently confirmed the deviation from linearity in Arrhenius plots for actomyosin[5] and myofibrils[6]. In contrast, it is known that the Arrhenius plot for myosin and the Ca^{2+}-activated ATPase activity of actomyosin is linear to 40 °C (refs 4, 5–11). Since the

Abbreviation: EGTA, ethyleneglycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

* Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pa. 15213, U.S.A.

Mg^{2+}-activated ATPase activity of actomyosin is generally accepted to be of physiological significance, there has been some speculation regarding the biphasic Arrhenius plot. Levy *et al.*[8] suggested that actin modifies myosin in such a way that temperature dependent conformational changes are allowed. It has also been proposed[5,6] that the high activation energy found below 16 °C, could be a consequence of increased dissociation of actin and myosin. This latter assertion has been challenged by Barouch and Moos[12], who found no evidence to indicate increased dissociation at lower temperatures, using the acto–heavy meromyosin system. They in turn speculated that the non-linear temperature dependence of actomyosin ATPase could be a consequence of some structural constraint, such as myosin aggregation. It has also been suggested[13] that at higher temperatures reversible denaturation becomes rate limiting and this causes non-linearity.

We became interested in the effect of temperature when we observed that in contrast to natural actomyosin, the Arrhenius plot for desensitized actomyosin was linear up to 40 °C. Thus, it became apparent that the regulatory proteins, which are absent from desensitized actomyosin, were influencing the temperature dependence in some way, and were in fact responsible for the biphasic nature of the Arrhenius plot. It appeared, therefore, that a systematic study of the influence of the regulatory proteins on the temperature dependence of actomyosin may help to gain an understanding of the mechanism of action of the regulatory proteins, as well as offering further information on the effect of temperature on the kinetics of actomyosin ATPase activity.

Most of the studies cited above have been confined to temperatures below 40 °C, presumably because of denaturation at higher temperatures. However, it has been shown with myofibrils that the Ca^{2+} sensitivity decreases with increasing temperature[14], and at temperatures above 40 °C the Ca^{2+} sensitivity of actomyosin is reduced[15–17]. Thus, the region above 40 °C is pertinent to the study of the regulatory proteins and for this reason our investigations have been extended to cover a wider temperature range (5–55 °C) than has previously been presented.

MATERIALS AND METHODS

Protein preparations

Troponin was isolated by the methods of Hartshorne and Mueller[19] and Yasui *et al.*[20], and tropomyosin was prepared by the former method. Troponin was separated into the A and B components by fractionation at acid pH[21]. Troponin B was further purified by chromatography on sulfoethyl-Sephadex[22]. Natural and desensitized actomyosins were prepared from myofibrils[23] as described by Schaub *et al.*[24]. Heavy meromyosin subfragment I was prepared by the method of Lowey *et al.*[25], and F-actin was made following the procedure of Tsuboi[26].

Assay of ATPase activity

The assay media and procedures were as described earlier[19]. Samples were exposed at each temperature for exactly 10 min, *i.e.* 5 min warm-up and 5 min assay time. In the Mg^{2+}-assay system, ethyleneglycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) was added when Ca^{2+}-free conditions were required, and omitted when trace amounts of Ca^{2+} (of the order of 10^{-5} M) were necessary. Ca^{2+}-activated

ATPase activity refers to the use of 2.5 mM $CaCl_2$, *i.e.* using Ca^{2+} as the only activating cation. Temperature ($\pm$ 0.1 °C) was regulated using a Haake circulating water bath.

Calcium binding

Ca^{2+} binding to troponin was measured by ultrafiltration using Amicon Centriflo membrane cones[27]. Details of this method will be published later.

Viscosity

Oswald Fenske viscometers (with water flow times at 25 °C between 80 and 100 sec) were used. The temperature was controlled to $\pm$ 0.01 °C.

Protein concentration

These were estimated from micro-Kjeldahl nitrogen analyses assuming a nitrogen content of 16 %.

RESULTS

Temperature dependence of the ATPase of natural actomyosin and myofibrils

The effect of temperature on the Mg^{2+}-activated ATPase activity of natural actomyosin is shown as an Arrhenius plot in Fig. 1. The two curves represent assays

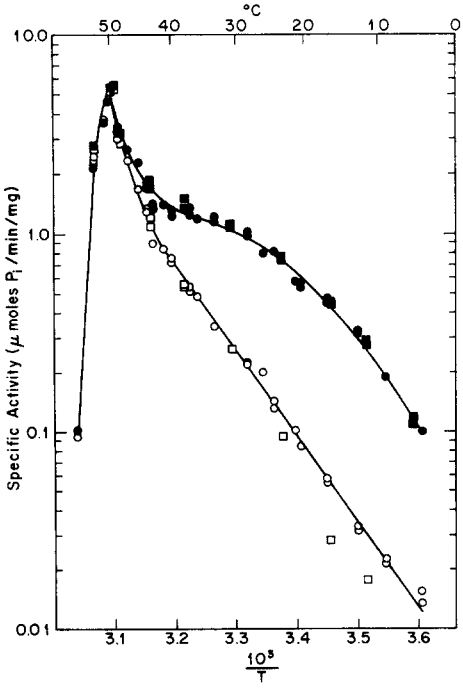


Fig. 1. Arrhenius plot for the ATPase activity of natural actomyosin. Assay conditions: 2.5 mM $MgCl_2$, 2.5 mM ATP, 25 mM Tris–HCl (pH 7.6). The natural actomyosin concentration was varied from approx. 0.4 mg/ml at 4 °C to 0.04 mg/ml at 50 °C. Results from two different preparations are shown. Solid symbols (●, ■) denote assays in the absence of EGTA, and open symbols (○, □) denote assays in the presence of 1 mM EGTA.

made in the absence and presence of trace amounts of Ca^{2+}. It is clear that the plot of the inhibited activity under the Ca^{2+}-free conditions is linear over the temperature range 0–40 °C. In contrast to this, a marked deviation from linearity is observed in the presence of Ca^{2+}. Below 16 °C the plot is linear and has a slope similar to the Ca^{2+}-free activity. Above 16 °C the plot becomes non-linear and exhibits a smooth curve up to about 40 °C. It should be emphasized that a critical temperature separating two linear portions of the plot is not apparent. Thus, any "event" which may be favored above 16 °C does not occur abruptly but rather over a range of temperature.

It is also of interest to note that at temperatures higher than 16 °C the two curves in Fig. 1 approach one another. This is shown more clearly in Fig. 2 and illustrates that the degree of Ca^{2+} sensitivity of natural actomyosin is dependent on the temperature of the assay system. At about 50 °C, there is no difference in ATPase activity between the Ca^{2+}-containing and Ca^{2+}-free conditions. The greatest loss of Ca^{2+} sensitivity occurs between 40 and 50 °C, and in this region the Arrhenius plot (Fig. 1) reflects a marked increase in activity. Above 50 °C, Ca^{2+} sensitivity is not evident and the ATPase activity declines rapidly due to denaturation.

The loss of Ca^{2+} sensitivity was not due to oxidation of critical sulfhydryl groups since identical results were obtained in the presence of dithiothreitol (final concentration in the assay media of 2.5 mM). Identical results were also found regardless

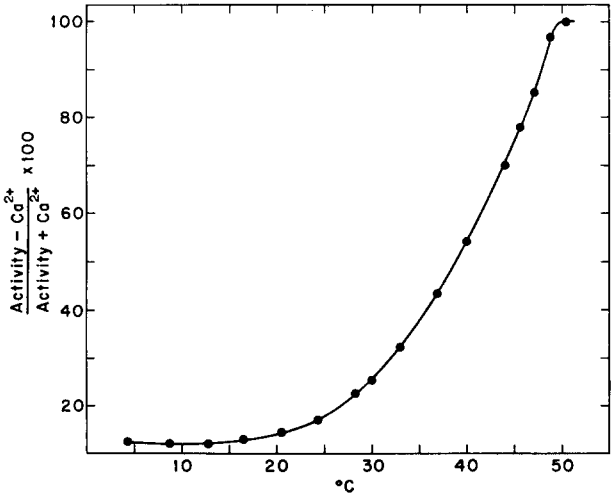


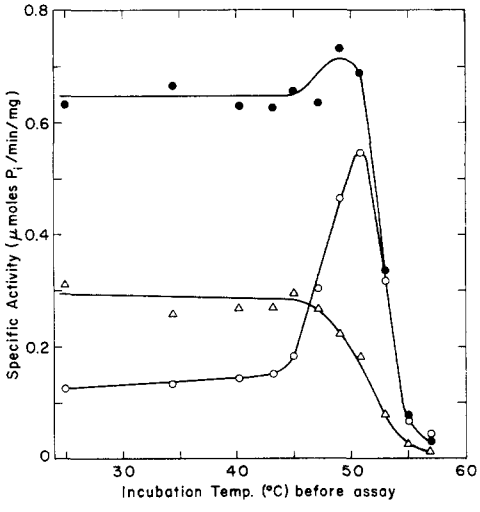
Fig. 2. The effect of temperature on the calcium sensitivity of natural actomyosin. ATPase activities were taken from Fig. 1, and the ATPase activity in the presence of EGTA ($-Ca^{2+}$) is expressed as a percentage of the ATPase activity in the absence of EGTA ($+Ca^{2+}$).

of whether the assay was started with actomyosin or ATP. Perhaps the most convincing demonstration that this affect was not due to oxidation can be found in Fig. 3, which illustrates the extent of reversibility of natural actomyosin ATPase activity exposed to different temperatures. Assays were set up, complete except for the ATP, and incubated for 10 min at the temperature indicated on the abscissa. The ATPase activity was then determined at 25 °C. The Mg^{2+}-activated ATPase activities under Ca^{2+}-containing and Ca^{2+}-free conditions, and also the Ca^{2+}-activated ATPase activity (2.5 mM $CaCl_2$) are shown in Fig. 3. Preincubation of actomyosin up to about 45 °C

does not markedly affect any of these activities, and the natural actomyosin was almost fully reversible. As the temperature of preincubation is increased the extent of reversibility decreases. This is shown by a drop of the Ca^{2+}-activated activity and a gradual reduction in the extent of Ca^{2+} sensitivity. Preincubation at about 53 °C is necessary to eliminate Ca^{2+} sensitivity. If the loss of Ca^{2+} sensitivity was due to oxidation then it would not be expected to be reversible. However, a comparison of Figs 2 and 3 shows clearly that over a wide range of temperatures some reversibility may be demonstrated, even at 50 °C.

It is unlikely that the effect of temperature on EGTA, either directly or *via* an alteration of the pH of the Tris–HCl buffer, could alter our results. Murphy and Hasselbach[14] eliminated one of these possibilities by showing that temperature did not markedly affect the binding of Ca^{2+} by EGTA. At 50 °C, the pH of our assay system dropped to 6.8; at this pH the level of EGTA which is used in our assays (1 mM) is still adequate to maintain a very low Ca^{2+} concentration. However, to demonstrate that pH changes did not cause spurious results, we repeated the Arrhenius plots using buffers adjusted to pH 7.6 at selected temperatures, and also at higher pH values (8.0 and 8.3 at 25 °C). In all cases, the results were essentially the same as those presented in Fig. 1.

The effect of temperature on the superprecipitation of natural actomyosin was also measured in both the Ca^{2+}-containing and Ca^{2+}-free assay media. After 2 min, the turbidity changes were complete under both sets of conditions at all temperatures and this value of $A_{550\ nm}$ was plotted *vs* temperature (Fig. 4). At 25 °C in the presence



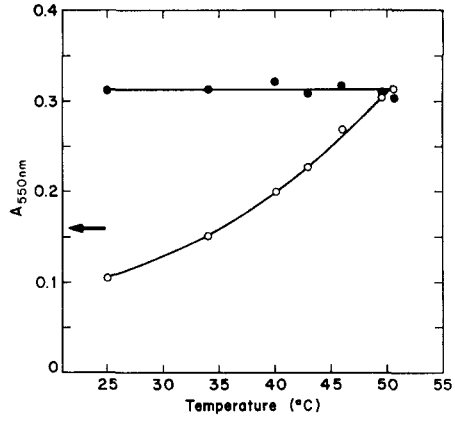


Fig. 3. The reversibility of the ATPase activity of natural actomyosin. Samples were incubated for 10 min at the indicated temperature and assayed at 25° C in the Mg^{2+} system (●) and the Mg^{2+} + EGTA system (○). Activity was also determined in 2.5 mM $CaCl_2$, 2.5 mM ATP, 25 mM Tris–HCl (pH 7.6) (△). Actomyosin 0.27 mg/ml.

Fig. 4. The effect of temperature on the super precipitation of natural actomyosin. Conditions were the same as in the enzymic assays. The reaction mixture was equilibrated at the indicated temperature for 5 min prior to the addition of ATP. 2 min after starting the reaction with ATP the absorbance at 550 nm was plotted for the EGTA containing assay mixture (○) and the assay mixture in the absence of EGTA (●). The arrow indicates the absorbance at 550 nm prior to the addition of ATP. Natural actomyosin concentration 0.22 mg/ml.

of Ca^{2+}, the natural actomyosin "contracted"; in the absence of Ca^{2+}, the natural actomyosin showed a clearing response. The difference between the two decreased with increasing temperature and at 50 °C no difference was observed. Thus, although the extent of superprecipitation was constant the control by Ca^{2+} was lost on increasing the temperature. This is consistent with the data shown in Fig. 1.

Before continuing with further studies on temperature dependence of actomyosin, we decided to establish that the Arrhenius plots were indeed a reflection of actin activated myosin ATPase. Our concern was that at the higher temperatures either myosin or actin alone could catalyze significant hydrolysis of ATP. This was tested in the Mg^{2+} assay system and found not to be the case. The extent of hydrolysis catalyzed by actin alone was very low at any temperature and the hydrolysis by myosin alone was maximum at about 40 °C but its specific activity was only of the order of 0.02 μmole P_i liberated per min per mg protein. Thus, over the entire temperature range, we felt justified in regarding the ATPase activity as due to actomyosin.

Bárány[5] found that with freshly prepared myofibrils from frog leg muscle the Arrhenius plot of the Mg^{2+}-activated ATPase activity was linear at low ionic strength (0.014). To examine the possibility that the non-linear plots were a consequence of

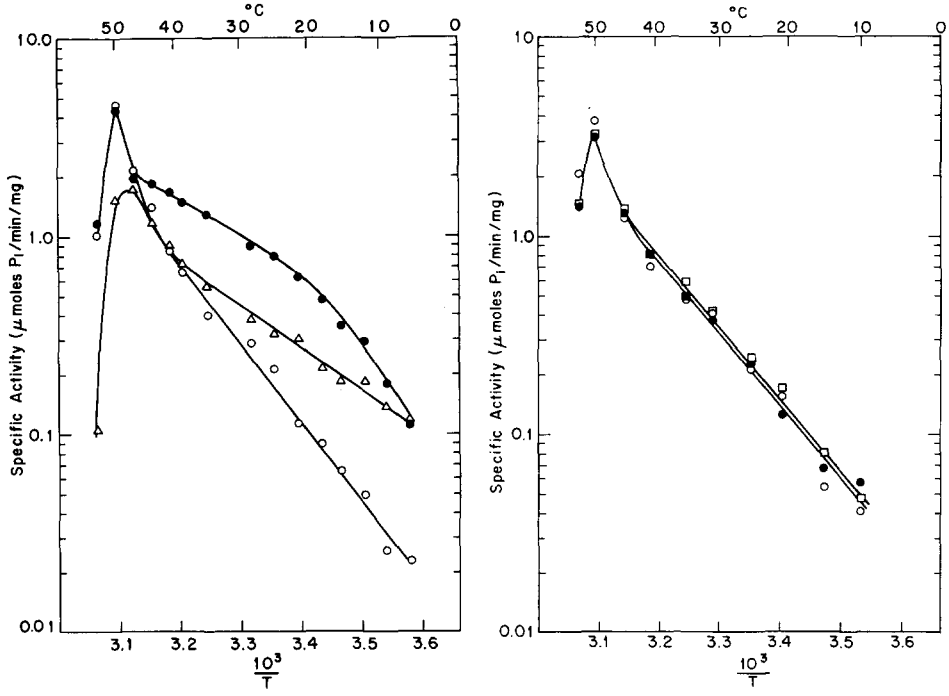


Fig. 5. Arrhenius plot for the ATPase activity of myofibrils. Activities were determined in the Mg^{2+} assay medium in the presence of EGTA (○) and in the absence of EGTA (●), under conditions similar to those given in Fig. 1. The ATPase activity was also determined in the Ca^{2+} assay medium (△).

Fig. 6. The effect of troponin A and tropomyosin on the Arrhenius plot of desensitized actomyosin. Activities were determined in the Mg^{2+} assay medium in the absence of EGTA. Conditions as in Fig. 1. Control desensitized actomyosin (□), *plus* troponin A, approx. 600 μg/mg actomyosin (●), *plus* troponin A and tropomyosin, approx. 600 μg of each per mg actomyosin (○).

aging, and also to compare myofibrils with natural actomyosin, we prepared myofibrils and began the ATPase assays within 2 h of the death of the rabbit. The results are shown in Fig. 5. The plot was essentially the same as in Fig. 1 and clearly exhibited a non-linearity in the Ca^{2+}-containing Mg^{2+} assay system. Myofibrils prepared as Bárány[5] described and assayed at ionic strengths ranging from 0.025 to 0.075 behaved similarly, and did not yield a linear Arrhenius plot. The Ca^{2+}-activated ATPase activity is also shown in Fig. 5. This was found to be linear although it exhibited a lower activation energy (see *Activation energies*).

Effects of the regulatory proteins on the temperature dependence of desensitized actomyosin

The observation which prompted this section of our studies is shown in Fig. 6; namely that the Arrhenius plot for desensitized actomyosin is linear over the temperature range 0–40 °C. Above 45 °C the shape of the curve is similar to that shown earlier. Thus, it was postulated that the regulatory proteins modified the Mg^{2+}-activated ATPase activity of actomyosin, in the presence of Ca^{2+}, resulting in curvature of the Arrhenius plot. The next point was to decide which of the regulatory proteins was responsible for this effect. It is shown in Fig. 6 that neither troponin A

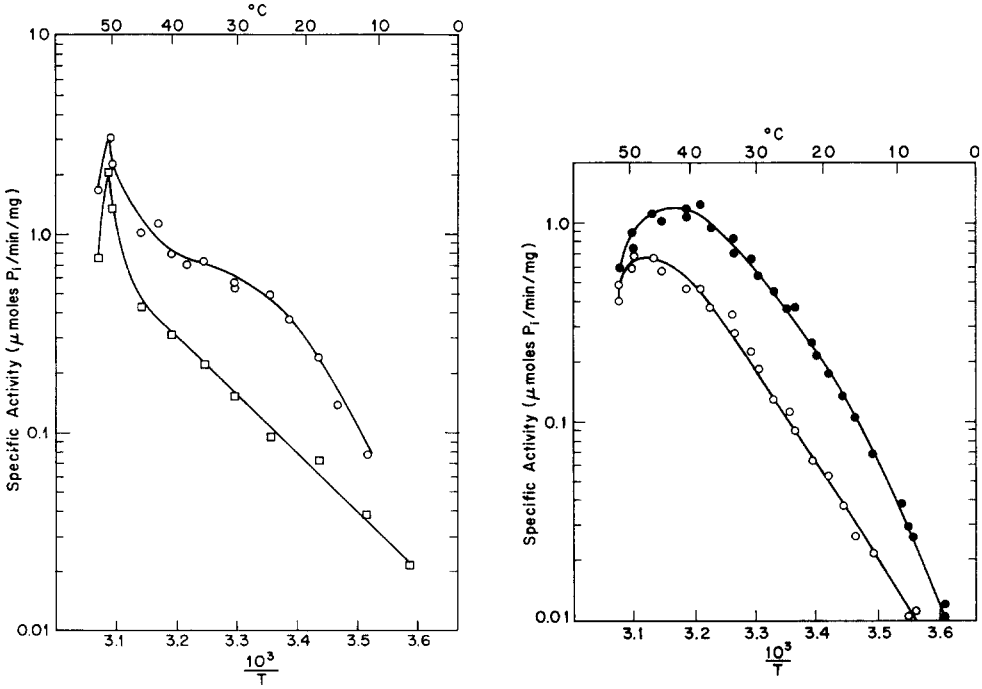


Fig. 7. The effect of troponin A, troponin B and tropomyosin on the Arrhenius plot of desensitized actomyosin. Activities were determined in the Mg^{2+} assay medium in the absence of EGTA. Conditions as in Fig. 1. Desensitized actomyosin *plus* troponin B and tropomyosin (□) at a weight ratio of 1:0.6:0.6, respectively. Desensitized actomyosin *plus* troponin B, tropomyosin and troponin A (○) at a weight ratio of 1:0.6:0.6:0.6, respectively.

Fig. 8. Arrhenius plot of the subfragment I–actin complex in the presence of troponin and tropomyosin. Activities were determined in the Mg^{2+} assay medium in the absence of EGTA (●) and in the presence of EGTA (○). Troponin and tropomyosin were added so that the final weight ratio of subfragment I:actin:troponin:tropomyosin was 2:1:0.25:0.25, respectively. Approximate concentration of subfragment I–actin complex ranged from 0.75 mg/ml at 4 °C to 0.1 mg/ml at 40 °C.

alone nor troponin A *plus* tropomyosin is adequate. These combinations of proteins were essentially identical to the control desensitized actomyosin plot.

Fig. 7 shows that the effect of troponin B *plus* tropomyosin again results in a linear plot over the 0–40 °C range. However, when troponin A is added to the system (Fig. 7) the characteristic curvature of the plot results. It would thus appear that the complete system is necessary even though troponin A seems to be the component which induces the non-linear behavior of the actomyosin ATPase activity. It should be emphasized that Ca^{2+} is also necessary, since in the absence of Ca^{2+}, the Mg^{2+}-activated ATPase activity of natural actomyosin was linear over the pertinent temperature range (see Fig. 1).

The effects of the regulatory proteins on the ATPase activity of desensitized actomyosin were quite stable since they were completely recovered after heating the troponin–tropomyosin complex for 10 min at 50 °C.

Temperature dependence of the ATPase activity of the heavy meromyosin subfragment I–actin complex

Barouch and Moos[12] have recently reported that the Arrhenius plot for the Mg^{2+}-activated ATPase activity of acto-heavy meromyosin was linear over the temperature range 5–35 °C. We decided to compare the characteristics of the heavy meromyosin subfragment I–actin complex to the findings of Barouch and Moos[12], and also to extend the temperature range. The results are shown in Fig. 8. The shape of the Arrhenius plot is different from those described earlier. Although the regulatory proteins were present, the results in the Ca^{2+}-containing assays did not deviate markedly from linearity. The slopes of the Ca^{2+}-containing and Ca^{2+}-free plots were in fact similar. Another difference can be seen at temperatures above 40 °C, as the subfragment I–actin complex did not exhibit the sudden increase of specific activity that was a characteristic of the actomyosin plots.

Activation energies

The activation energies were calculated from the Arrhenius plots and are shown in Table I. No attempt was made to analyze the data above 40 °C. In the Ca^{2+} assay system, the Arrhenius plot was linear over the 4–40 °C range and yielded activation energies between 7–9 kcal/mole. Inhibition of the Ca^{2+}-activated ATPase activity by tropomyosin[18] did not alter the shape of the Arrhenius plot nor the activation energy (see Table I). Assays performed in the Mg^{2+} system generally showed higher activation energies. The Arrhenius plot was linear between 4–40 °C for desensitized actomyosin both in the absence and presence of Ca^{2+}, and gave an activation energy of about 17 kcal/mole. A similar value was obtained for natural actomyosin and myofibrils below 16 °C in the presence of Ca^{2+} and between 4–40 °C in the absence of Ca^{2+}. The region between 20 and 40 °C for actomyosin in the presence of Ca^{2+} and the complete compliment of regulatory proteins (*i.e.* natural actomyosin, myofibrils, desensitized actomyosin *plus* troponin and tropomyosin) is interesting since here a marked drop in activation energy (6–8 kcal/mole) occurred. The Arrhenius plot showing the effect of troponin B *plus* tropomyosin on the Mg^{2+}-activated ATPase activity of actomyosin was linear over the range 4–40 °C, although the activation energy (14 kcal/mole) was lower than expected. This was a consistent observation

TABLE I

ACTIVATION ENERGIES CALCULATED FROM ARRHENIUS PLOTS

System	*Temperature range (°C)**	*Activation energy (kcal/mole)*
Natural actomyosin		
Mg^{2+} assay + Ca^{2+}	4–16	19
Mg^{2+} assay + Ca^{2+}	20–40	6
Mg^{2+} assay — Ca^{2+}	4–40	20
Desensitized actomyosin		
Mg^{2+} assay ± Ca^{2+}	4–40	17
Ca^{2+} assay**	4–40	7
Ca^{2+} assay + tropomyosin**	4–40	7
Mg^{2+} assay + tropomyosin + troponin B	4–40	14
Mg^{2+} assay + Ca^{2+} + tropomyosin + troponin B + troponin A	4–16	23
Mg^{2+} assay + Ca^{2+} + tropomyosin + troponin B + troponin A	20–40	6
Myofibrils		
Mg^{2+} assay + Ca^{2+}	4–16	19
Mg^{2+} assay + Ca^{2+}	20–40	8
Mg^{2+} assay — Ca^{2+}	4–40	19
Ca^{2+} assay	4–40	9
Heavy meromyosin subfragment I + actin		
Mg^{2+} assay + Ca^{2+} + tropomyosin + troponin	10–35	26
Mg^{2+} assay — Ca^{2+} + tropomyosin + troponin	10–35	23

* The data points over the indicated temperature range were assumed to fall on a straight line.
** This datum is not shown in the preceding figures.

and we have no explanation to offer for it. The heavy meromyosin subfragment I–actin complex did not show marked deviation from linearity in the Mg^{2+} system, and both the Ca^{2+}-containing and Ca^{2+}-free assays were approximated by a straight line with activation energies as shown in Table I.

Physical properties as a function of temperature

To check the possibility that at higher temperatures protein components were dissociated from actomyosin we incubated natural actomyosin and desensitized actomyosin under the conditions of assay at varying temperatures. The insoluble material was separated by centrifugation and after dialysis to remove ATP, the protein content of the supernatant was estimated. Even at 50 °C, there was an insignificant amount of protein released. Also, the heated actomyosin which was separated from the supernatant behaved identically to actomyosin samples which were not fractionated. Thus, it is unlikely that any of the features of the Arrhenius plot could be rationalized as a consequence of the release of protein components from the actomyosin complex.

The next point to check was the binding of Ca^{2+} to troponin at different temperatures. The results are shown in Table II, and clearly there is no marked temperature dependence of Ca^{2+} binding to troponin. Ca^{2+} binding studies to isolated myofibrils were carried out and also showed that the binding characteristics at 25 and 38 °C were similar.

TABLE II

TEMPERATURE DEPENDENCE OF Ca^{2+} BINDING TO TROPONIN

Parameters are given ± standard deviation. Figures in parentheses indicate the number of determinations made at each temperature. Solvent used was 50 mM KCl, 10 mM imidazole (pH 7.0). Troponin concentration was approx. 0.1 mg/ml. No attempt was made to define calcium binding to lower affinity sites.

Temperature (°C)	*μmoles Ca^{2+} bound per g troponin*	*Affinity constant* × 10^{-6} (M^{-1})
3 (3)	29.7 ± 4.1	2.1 ± 0.3
23 (7)	27.9 ± 4.8	1.6 ± 0.6
38 (8)	30.9 ± 8.1	2.2 ± 1.5

The effect of temperature on the viscosity of the troponin–tropomyosin complex is shown in Fig. 9. There is a marked reduction in viscosity as the temperature is increased, and the viscosity of the troponin–tropomyosin complex approaches that of tropomyosin alone. Thus, the effect of troponin is reduced as the temperature is increased.

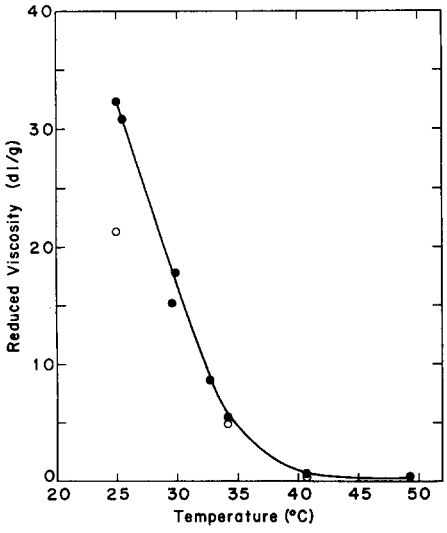


Fig. 9. The effect of temperature on the reduced viscosity of troponin *plus* tropomyosin (●) and tropomyosin alone (○). Troponin and tropomyosin were mixed in a 1:1 weight ratio. Both the troponin–tropomyosin complex and tropomyosin alone were used at a total protein concentration of 2 mg/ml. The solvent was 10 mM Tris–HCl (pH 7.6), 1 mM dithiothreitol.

DISCUSSION

The interesting features of the Arrhenius plots of the Mg^{2+}-activated ATPase activity of natural actomyosin or myofibrils are (a) the curvature in the presence of Ca^{2+} over the temperature range 4–40 °C, and (b) the rapid loss of Ca^{2+} sensitivity at temperatures above 40 °C. It is significant that the plot for desensitized actomyosin

is linear over the 4–40 °C range. It thus seems unlikely that the non-linearity of the natural actomyosin plots is due to aggregation[12] since it is to be expected that the tendency to aggregate would be similar in both preparations. Also the activation energies for natural actomyosin and desensitized actomyosin below 16 °C are similar and thus it is unlikely that an increased dissociation[5] of the actin and myosin is responsible for the biphasic nature of the natural actomyosin plot. In fact, we were unable to obtain linear plots using rabbit myofibrils such as Bárány[5] found with frog myofibrils. The loss of Ca^{2+} sensitivity was partly reversible and was not, therefore, due to oxidation of critical sulfhydryl groups on myosin as found by Levy and co-workers[15,17]. Murphy and Hasselbach[14] suggested that the loss of Ca^{2+} sensitivity which they observed with myofibrils was due to an increased proportion of the total ATPase activity being catalyzed by myosin alone. This is unlikely since the highest activity observed in the Mg^{2+} assay system for myosin alone was approx. 0.02 μmole P_i liberated per min per mg myosin at a temperature of 40 °C. (It should be emphasized that most myosin preparations contain actin, and specific activities this low are only obtained after the removal of actin.) F-actin alone catalyzed very little hydrolysis of ATP. Thus, we feel justified in regarding the rather complex Arrhenius plot as reflecting the actomyosin type of ATPase activity.

The region of the plots above 40 °C could be accounted for by assuming that the conformation of myosin at higher temperatures no longer "recognizes" troponin. Thus, although the ATPase activity is still actin-moderated, it is not regulated by the troponin–tropomyosin complex. At temperatures above 50 °C, the conformation may be further altered and the activation by actin lost. The similarity of the curves for natural actomyosin and desensitized actomyosin in this region support the idea that the regulatory proteins are not modifying the myosin. The reduction in viscosity with increasing temperature indicates that gross changes occur in the troponin–tropomyosin complex. This is consistent with earlier results on tropomyosin[28,29]. Thus, the loss of Ca^{2+} sensitivity could be explained by assuming that temperature dependent conformational changes occur in the actomyosin complex. It is not possible to localize any specific change and it seems likely that at this relatively high temperature, all the proteins will be altered in some way, although apparently none of these proteins are dissociated from the actomyosin complex.

It is of interest to note that the heavy meromyosin subfragment I–actin complex (Fig. 8) did not show the marked increase of activity between 40 and 50 °C although Ca^{2+} sensitivity was lost at about the same temperature as with natural actomyosin. The difference in the shape of the Arrhenius plots could be partly due to the possibility that subfragment I is less stable than myosin since it is cleaved from myosin following proteolytic digestion with papain. Also the extent of dissociation of the subfragment I–actin complex is greater than with actomyosin, and although the dissociation of the subfragment I–actin complex is probably not increased by decreasing the temperature, as Barouch and Moos showed for acto-heavy meromyosin, the concentration of free subfragment I at any given temperature will be higher than the concentration of free myosin. Thus, the Arrhenius plot may to some extent indicate the denaturation of subfragment I alone, unprotected by actin. The lack of a marked curvature between 4 and 40 °C for the subfragment I–actin complex in the presence of the regulatory proteins and Ca^{2+} may also be a reflection of a higher dissociation constant for this complex as compared to natural actomyosin. Our results shown in Fig. 8 were ob-

tained with a constant weight ratio of subfragment I to actin (2:1, respectively) and are not extrapolated to infinite actin concentration. However, a similar plot using extrapolated V values would not be expected to be very different and if anything would probably show a lower activation energy since it is likely that, as in the case of acto-heavy meromyosin[12], the apparent dissociation constant decreases with decreasing temperature. The effect of this would be to reduce the slope of the Arrhenius plot.

Let us now consider the region below 40 °C. Curvature or breaks in Arrhenius plots are not uncommon, and several explanations for this behavior have been proposed (see refs 30 and 31). In our case, we cannot define the physical or thermodynamic changes which may be occurring, but we wish to point out that these do in fact reflect the influence of the regulatory proteins. Unfortunately, the mechanism of action of troponin and tropomyosin on a molecular level is not understood, and thus any discussion on the control of ATPase activity must be at best speculative in nature. It should, however, be emphasized that it is unlikely that these effects were due to changes in the Ca^{2+} binding characteristics of troponin, since there was very little temperature dependence of Ca^{2+} binding to troponin and myofibrils up to 38 °C. In the absence of Ca^{2+} or in the absence of troponin A, the Arrhenius plot is linear below 40 °C. Thus, Ca^{2+} binding by troponin A appears to result in the curvature of the Arrhenius plot either by altering the activator (actin) or through an interaction with myosin. Which of these two possibilities is the most likely is difficult to assess. However, it is interesting to recall some of the results of Levy and co-workers[7,8,32]. They found that curvature of the Arrhenius plot for myosin alone could be induced using ITP as substrate, or by chemically modifying the myosin. In one of these papers, Levy *et al.*[8] concluded that the active site of myosin was somewhat flexible and could be modified by such agents as dinitrophenol, *p*-chloromercuribenzoate and actin, and that such changes were reflected in the thermodynamic and kinetic characteristics of the catalyzed reaction. From our results, it is clear that actin alone is not the modifier, but rather the actin–troponin–tropomyosin complex, and this only in the presence of Ca^{2+}. It is possible, extending Levy's hypothesis, that the Ca^{2+}-loaded troponin A interacts directly with myosin and thus modifies the ATPase characteristics as illustrated by the Arrhenius plot. If this were so, however, one might expect an effect of troponin A alone which was not found. On the other hand, it is also possible that the Ca^{2+}-loaded troponin A changes the activating properties of actin and that this is the effect reflected in the temperature studies. This to us seems to be the most likely explanation. In either mechanism, however, it is apparent that the role of troponin A is not entirely the passive process of turning on and off the inhibition by troponin B. If this were the case, then in the presence of Ca^{2+} the troponin–tropomyosin–actin complex would behave identically to actin alone. Since this was not found it is, therefore, suggested that troponin A in addition to regulating troponin B activity modifies in the presence of Ca^{2+} the ATPase activity of actomyosin.

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