EFFECT OF TEMPERATURE ON THE RATE OF HYDROLYSIS OF ADENOSINE TRIPHOSPHATE AND INOSINE TRIPHOSPHATE BY MYOSIN WITH AND WITHOUT MODIFIERS. EVIDENCE FOR A CHANGE IN PROTEIN CONFORMATION*

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SUMMARY

The temperature dependence of the myosin-catalyzed hydrolysis of inosine triphosphate gives a biphasic Arrhenius plot curving sharply near 16° C. In contrast, the Arrhenius plot with adenosine triphosphate as substrate is linear. When enzyme modifiers such as 2,4-dinitrophenol or *p*-chloromercuribenzoate are added to the system, the temperature dependence of ATP hydrolysis becomes very similar to that for ITP with native enzyme.

Using ^{32}P-labeled ATP, it has been shown that ATP and ITP compete for the same site and that even in the presence of dinitrophenol, ATP is bound to the enzyme far more tightly than ITP.

These data can be explained by assuming a temperature-induced change in the conformation of the enzyme–substrate complex. A consistent framework to explain the complex relationship between temperature, substrate, metal ion, and modifier can be obtained using this concept of a flexible active site.

INTRODUCTION

Many different compounds modify the nucleotide triphosphatase activity of myosin. These include 2,4-dinitrophenol studied by GREVILLE AND NEEDHAM[2] and CHAPPEL AND PERRY[3], *p*-chloromercuribenzoate studied by KIELLY AND BRADLEY[4], aminoethyl thiuronium studied by MORALES *et al.*[5], ethylenediamine tetraacetic acid studied by FRIESS[6] and pyrophosphate studied by GALLOP *et al.*[7]. The interesting studies of these workers have implicated the protein sulfhydryl groups in the action of these modifiers and also indicated that they are involved with protein-bound metal ions. Moreover, these studies have given insight into the myosin substrate complex. For

Abbreviation: PCMB, *p*-chloromercuribenzoate.

* A preliminary account of some of this work has been published[1].

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example, BLUM[8] has postulated an interaction with the 6-amino group in the action of some of these agents.

GILMOUR AND GRIFFITH have emphasized that there is a provocative similarity in the effect of many of these modifiers[9]. For example, dinitrophenol and *p*-chloromercuribenzoate activate ATPase activity at low concentrations and inhibit at high levels. Moreover, both agents activate the hydrolysis of 6-amino nucleotides but only inhibit the hydrolysis of 6-hydroxynucleotides when calcium ions moderate the reaction[8,10]. Under these conditions activation occurs only at the higher temperatures, for example, 25°[3,9]. It has since been shown that the response to these modifiers is not only substrate-dependent but also metal-dependent and that the above generalizations do not hold when magnesium is used instead of calcium[11]. Furthermore, DNP and PCMB appear to react with different groups on the enzyme[11]. Nevertheless, all the work taken together indicates that the SH-groups of the protein, the group at the six position at the purine or pyrimidine ring on the substrate, the metal ion, and the temperature all play key roles in determining the effects of a modifier.

In earlier studies on the role of temperature in the myosin system, correlation of some of these phenomena could be obtained by assuming a temperature-induced conformation change in the myosin molecule[1]. This change was found to depend not only on the nucleotide but also on the metal ion and the modifying agent. Such a conclusion has further been supported by the work of GILMOUR[12] who studied the pH-dependence at various temperatures. In this work the previous studies have been extended and additional support for such an induced conformation change has been obtained.

MATERIALS AND METHODS

Myosin was prepared by the method of KESSLER AND SPICER[13] and reprecipitated three times. After dissolving the precipitate in 0.6 *M* KCl, the solution was clarified by centrifugation for 30 min at 25000 rev./min in the No. 30 head of a Spinco preparative ultracentrifuge. The enzyme solutions were stored at 0° and used no later than 10 days after preparation. ATP and ITP were obtained from the Sigma Chemical Company and Nutritional Biochemicals as the disodium salts. [^{32}P]ATP, labeled in the two terminal phosphates, was prepared by an exchange reaction between ATP and $^{32}P^{32}P$ using the tryptophane-activating enzyme[14]. The $^{32}P^{32}P$ was prepared by pyrolysis of $K_2H^{32}PO_4$ obtained from Oak Ridge.

The reactions were carried out in solutions having the following composition: 0.1 *M* KCl, 0.125 *M* Tris buffer, *ca.* 0.2 mg of L-myosin/ml and 5 m*M* nucleotide triphosphate with or without the following additions: 0.01 *M* MgCl, 0.01 *M* $CaCl_2$, 0.01–0.03 *M* DNP, 4 μmoles PCMB/100 g protein. The final pH was 7.3–7.4.

The reaction was started by the addition of substrate and quenched by precipitation with trichloroacetic acid. The rate of the reaction was followed by assaying reaction mixtures at four or more different times (1–5 min with Ca^{2+}; 5–20 min with Mg^{2+}). The protein was separated by centrifugation and the supernatant was analyzed for inorganic phosphate by the isobutanol extraction method[15]. Reactions were carried out in a water bath controlled to within $\pm 0.5°$, or in an ice–water mixture.

In the experiment which involved the release of ^{32}P the isobutanol extract obtained in the analysis for inorganic phosphate was also assayed for radioactivity.

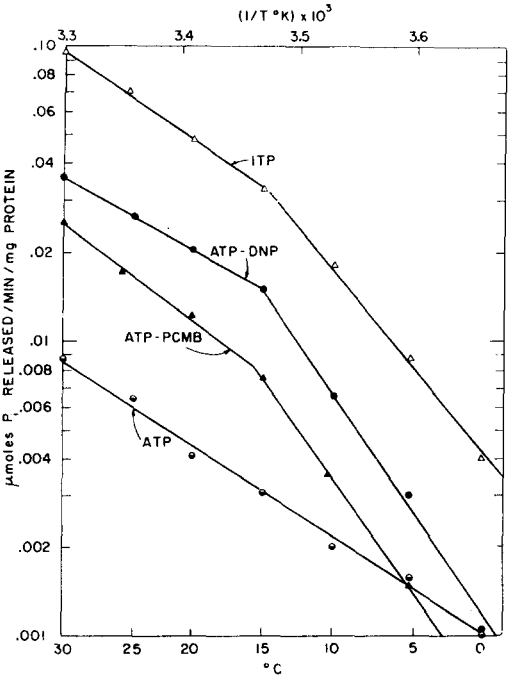

Fig. 1

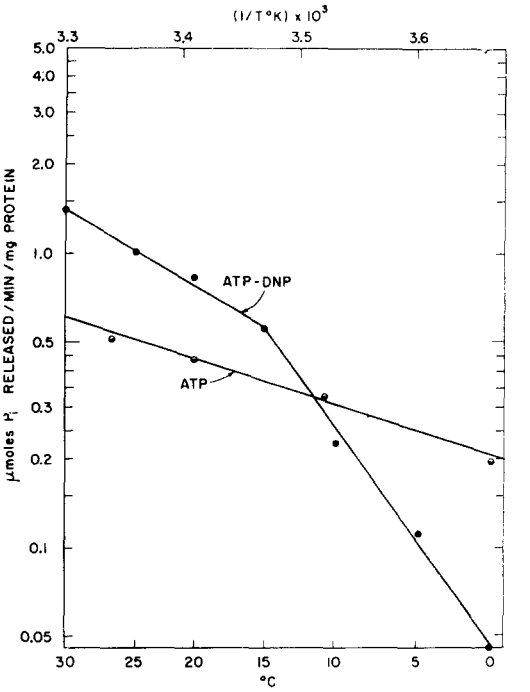

Fig. 2

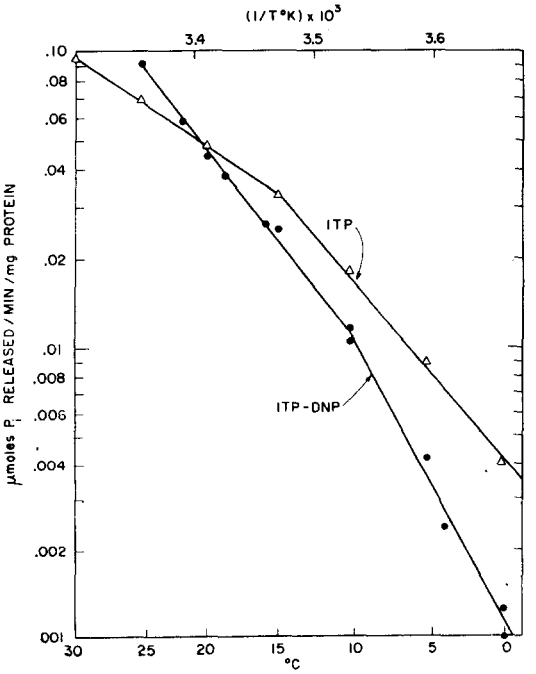

Fig. 3

Fig. 1. Temperature dependence of the magnesium-moderated reactions catalyzed by myosin. Conditions: 0.01 *M* $MgCl_2$; 0.1 *M* KCl; 0.125 *M* Tris (pH 7.4), 5 m*M* ATP or ITP; myosin concentration depended on temperature, *ca.* 0.2–0.4 mg/ml; when present PCMB concentration was 0.4 μmole/100 mg of protein and dinitrophenol was 0.03 *M*.

Fig. 2. Temperature dependence of calcium-moderated reactions catalyzed by myosin. Conditions: same as Fig. 1 but with 0.01 *M* $CaCl_2$ instead of $MgCl_2$, and 0.01 *M* DNP in place of 0.03 *M* DNP. The shape of this curve with 0.01 *M* DNP is virtually the same as others obtained with 0.03 *M* DNP. The higher concentration of DNP only displaces the curve upward (greater activation).

Fig. 3. Temperature dependence of magnesium-moderated ITPase of myosin with and without 0.03 *M* DNP. Conditions: same as for Fig. 1 except ITP for ATP.

This was done by drying an aliquot of the extract on an aluminum planchet and counting with a gas flow counter.

Rates of reaction were calculated using the straight-line portions of the rate curves (usually between 5 and 50 % hydrolysis). The very early portions of the curves were neglected in view of the fact that this starting rate with myosin is sometimes anomalously high[16].

RESULTS

When the kinetic data are put in the form of an Arrhenius plot, the curves in Figs. 1–3 are obtained. Each point represents the slope of a line derived from five phosphate analyses at appropriate times intervals. Typical rate curves are shown in Fig. 4. Each curve in Figs. 1–3 represents a series of measurements made on the same day with the same myosin preparation; for each set of conditions the results were confirmed with at least one other preparation. The specific activities (in μmoles of phosphate released/mg of protein/min) depended somewhat on the individual preparation and its age. However, all the enzyme preparations used in this study gave values of 0.4–0.6 μmole ATP split/min/mg under the standard conditions with Ca^{2+} at 25° and these agree well with values obtained by other workers. Regardless of the specific activity of an individual preparation, the ratios at different temperatures were constant.

In Fig. 1, comparison between ATP, ITP, ATP–DNP, and ATP–PCMB is made with Mg^{2+} as the moderating metal ion. Here there is a qualitative as well as a quantitative difference between the ATP and ITP curves, or between ATP in the absence or presence of modifier. Moreover, the ITP, ATP–DNP and ATP–PCMB curves are similar. These latter curves are drawn as two intersecting lines, but, of course, a more or less sharp curve is the true representation of the actual phenomena. The intersecting lines are drawn, however, to indicate the transition temperature and to show that a single straight line gives a poor fit.

One of the important features of Fig. 1 is the qualitative difference between the shape of the ATP and ITP curves. A biphasic temperature dependence has been observed with other enzymes, but in this work one substrate shows a biphasic Arrhenius

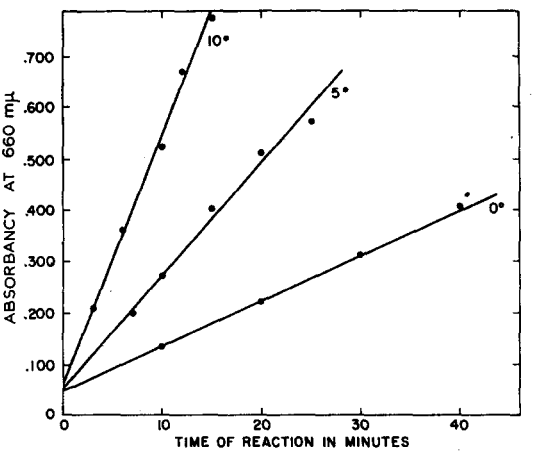

Fig. 4. Typical rate curves for hydrolysis of ATP by myosin. Conditions: 0.01 *M* Ca^{2+}, 0.1 *M* KCl, 0.125 *M* Tris (pH 7.5), 0.005 *M* ATP, *ca.* 0.2 mg myosin/ml.

plot and the other does not. Moreover, the two substrates in this study are very similar, and the same kind of bond is broken during the reaction.

The different temperature dependence of these substrates could mean that the ITP and ATP are bound to different and separate active sites on the enzyme. To test this the kinetic experiments shown in Figs. 5 and 6 were performed. The two bottom

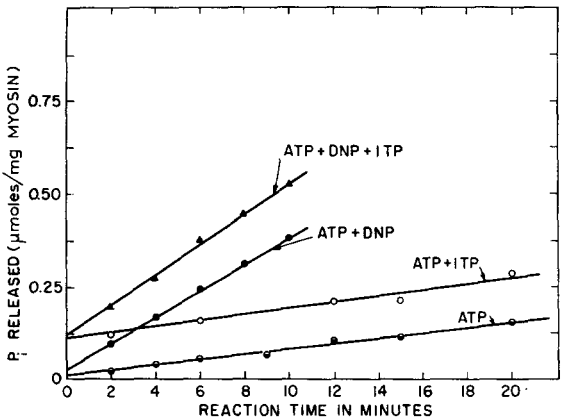

Fig. 5. Rate of total inorganic phosphate-release by myosin in mixtures of ATP and ITP. Conditions: 0.01 *M* Mg^{2+}, 0.05 *M* Tris (pH 7.5), 25°, *ca.* 0.2 mg myosin/ml, 0.1 *M* KCl, 5 m*M* ATP. DNP when present was 0.01 *M*. ITP when present was 5 m*M*. Intercepts differ because of greater P_i contamination in ITP.

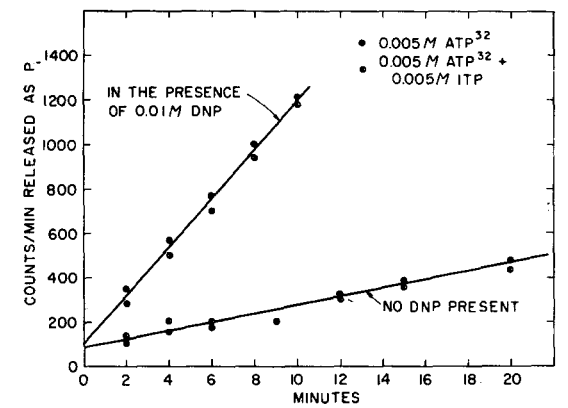

Fig. 6. Competition of [^{32}P]ATP and ITP for active site of myosin in presence and absence of dinitrophenol. Conditions: 0.01 *M* Mg^{2+}, 0.05 *M* Tris (pH 7.5), 25°, *ca.* 0.2 mg/ml myosin, 0.1 *M* KCl, 5 m*M* [^{32}P]ATP. ITP when present was 5 m*M*. DNP when present was 0.01 *M*.

lines in Fig. 5 show the rates of hydrolysis of ATP alone and in the presence of ITP. It is seen that the addition of ITP fails to increase the rate of P_i liberation despite the fact that ITP alone is hydrolyzed at about ten times the rate of ATP. Since the Michaelis constant of ATP is known to be less than that of ITP[17,18], the probable explanation is that ATP is so firmly bound to the myosin that none of the faster reacting ITP can gain access to the active site. To add direct support to this conclusion, the hydrolysis of ^{32}P-labeled ATP in the presence and absence of unlabeled ITP was followed as shown in Fig. 6 (bottom line). Here it is seen that the presence of ITP has no effect on the rate of ^{32}P release and hence on the rate of ATP hydrolysis. As would be predicted from these two results, the specific activity of the $^{32}P_i$ showed that within experimental error all of P_i released came from ATP. Thus, ATP prevents the access of ITP to the active site and hence the possibility that the results can be explained by two different sites is eliminated. Incidentally, the much tighter binding of the less

readily hydrolyzed substrate shows that correlation of tightness of binding and velocity of hydrolysis does not occur.

The same experiment was performed with 0.01 *M* DNP modifying the reaction. The results are essentially the same as in the absence of DNP as far as competition for the active site is concerned. Both the rate study (Fig. 5, top) and the isotopic data

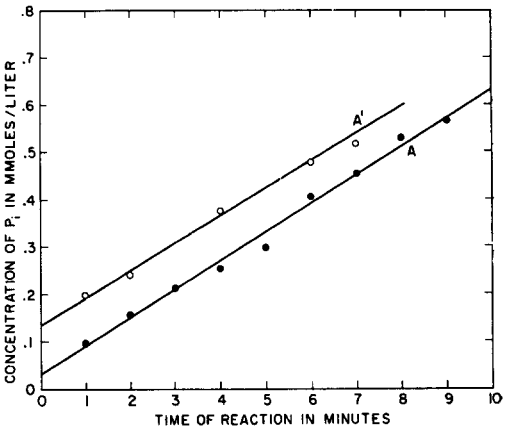

Fig. 7. Effect of reaction at 30° on rate of subsequent reaction at 0°. Conditions: reagent concentrations same as for Fig. 4. A′: after incubating enzyme solution in absence of ATP and DNP at 30° for 1.5 min, ATP and DNP were added and allowed to react for 1 min. The solution was then cooled to 0° and [P_i] was measured as a function of time. A: same enzyme solution as A′ measured at 0° without prior reaction at 30°.

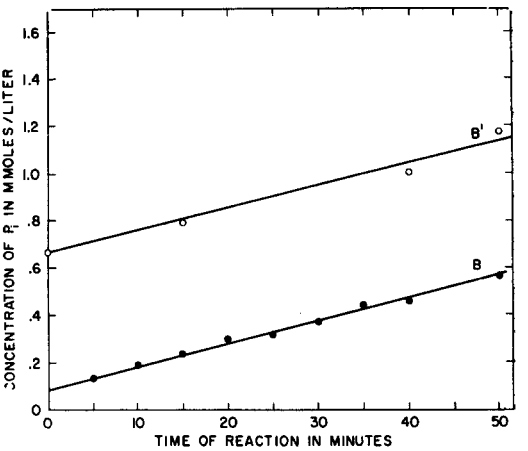

Fig. 8. Effect of reaction at 0° on rate of subsequent reaction at 30°. Conditions: same concentrations as Fig. 4. B′: after incubating enzyme solution for 1.5 min at 0° in absence of ATP and DNP, ATP and DNP were added and allowed to react for 20 min at 0°. The solution was then brought to 30° and the [P_i] measured as a function of time. B; same enzyme solution as B′ measured at 30° without prior reaction at 0°.

(Fig. 6, top) establish that even if binding of ATP is weakened by the action of DNP it is still strong enough to prevent access of ITP to the active site.

With Ca^{2+}, dinitrophenol activates at high temperature and inactivates at low. With Mg^{2+} there is some activation even at 0°. The confusing state of affairs seen here and in other work, *i.e.* that dinitrophenol activates sometimes and inactivates at others, has been discussed by GILMOUR AND GRIFFITH[9]. The complete temperature data, however, help clarify the situation. The effect of dinitrophenol is seen to be qualitatively similar for the two metals. A biphasic curve is noted in each instance with the transition occurring in the region of 16°. But since the calcium–DNP curve crosses the Ca^{2+} curve, DNP activates at some temperatures and inhibits at others, whereas with Mg^{2+} it activates throughout the temperature range. If the Mg^{2+} curves

could be extended to lower temperatures, however, they most probably would intersect. It is the effect of DNP on the shape of the curve rather than the absolute rate at any given temperature which seems to be of key significance.

The change in slope of the ATP–DNP curve might be the result of some irreversible denaturation at the high or low temperatures. To check this possibility, experiments were performed as shown in Figs. 7 and 8. In Fig. 7 the enzyme was first allowed to react at 30°. Then the temperature was lowered to 0° where the rate of reaction was measured. In Fig. 8 the enzyme was first allowed to react at 0°. Then the temperature was raised to 30° where the rate of reaction was measured. In each run the measured rate was the same as a control allowed to react at only one temperature. The biphasic nature of the Arrhenius plot, therefore, is not caused by irreversible denaturation at any temperature.

DISCUSSION

The results lead to the following specific conclusions: (*i*) ATP and ITP react at the same active site on myosin; (*ii*) in the presence or in the absence of 0.01 *M* DNP, ATP is more firmly bound to enzyme than is ITP; (*iii*) the Arrhenius plot for ATP without modifier is a straight line whereas for ITP it is biphasic; (*iv*) with DNP or PCMB, ATP hydrolysis is more like ITP hydrolysis in both magnitude and shape of the Arrhenius plot, (*v*) the biphasic nature of the ATP–DNP plot is observed for both Mg^{2+} and Ca^{2+} despite the fact that the velocities of hydrolysis differ by a factor of 20 for these two metals, (*vi*) DNP with ITP shows a different temperature dependence than any of the other systems.

These results must be considered within the framework of other work in this field which is cited in the introduction. This work had led to general agreement on the following points:

1. There are certain SH and other functional groups on the myosin molecule which, directly or indirectly, inhibit the enzymic activity; other chemically similar groups contribute in a positive way to the enzyme's function.

2. The dual action of modifiers such as DNP and PCMB which increase ATPase at lower concentrations and then inhibit at higher levels is due to preferential reaction with rate-retarding groups on the protein.

3. The enzyme interacts in a similar way with nucleotides having a 6-amino group. These substrates, more tightly bound than the 6-hydroxy nucleotides, appear to be more strongly inhibited by rate-retarding groups on the enzyme, *i.e.* more markedly activated by reagents such as DNP and PCMB.

4. A number of modifiers alter the interaction between enzyme and 6-amino nucleotide, making it more similar to that between enzyme and 6-hydroxy nucleotide.

The work presented here adds support to these general conclusions. Moreover, it has led us to a mechanism for explaining the relationship between substrate, modifier, and temperature. This may be described as follows:

(a) Portions of the myosin molecule including the active site are flexible and can change conformation as a function of temperature.

(b) The shape, flexibility, and temperature sensitivity of the active site are modified through interactions with bound metal and substrate, and with agents such as DNP, PCMB, and actin.

(c) Changes in the conformation of the active site may be reflected in the thermodynamic and kinetic characteristics of the catalyzed reaction.

It is well known that changes in temperature can cause major conformational changes in proteins, some reversible and some irreversible. Such a reversible change appears to occur with myosin in the region of 16°. We believe this involves a change in shape of the enzyme–ITP complex and that this is reflected in the biphasic Arrhenius plot. With ATP as substrate the change is not apparent because the 6-amino group of the purine ring contributes to a tighter fit. Thus, ATP is able to stabilize one conformation of the active site (supporting a slow rate of hydrolysis) over the entire temperature range studied. This effect is considered analogous to the well known phenomenon of substrate protection against irreversible heat denaturation. It is emphasized that the ATP–protein interaction need not prevent changes in shape at regions removed from the active site.

A conformation change at the active site becomes apparent even with ATP when the stabilizing action of this substrate is weakened by a modifier. Thus, DNP or PCMB with ATP promotes a situation more similar to ITP, *i.e.*, increases the rate of hydrolysis and shows a biphasic Arrhenius plot with activation energies of 12 and 25 kcal instead of the 12-kcal plot of ATP alone. These agents do this, presumably, by altering the interaction between the enzyme and the 6-amino group of ATP.

However, we cannot conclude that ITP, with no 6-amino group, is indifferent to the actions of these modifiers. It has been pointed out before that at 25° DNP actually increases the rate of hydrolysis of ITP with Mg^{2+} (ref. 11); this effect is seen in Fig. 3. And even with calcium the hydrolysis of 6-hydroxy nucleotides is not *unchanged* but rather *inhibited* by DNP and SH-binding agents[10]. Therefore, when the Mg–ITP–enzyme complex is modified by DNP, the conformation of the active site changes in still another way. And under these conditions the apparent heat of activation is near 25 kcal even above 16°, giving an almost linear Arrhenius plot (Fig. 3).

Non-linear temperature plots have been observed before with other enzymes and have been explained in various ways[19]. The main alternatives to the mechanism proposed here, *i.e.* a reversible flexibility of the active site, are: (*i*) solvent changes at a critical transition temperature; (*ii*) irreversible denaturation of the enzyme, and (*iii*) complex temperature effects on a reaction sequence, *i.e.* two or more consecutive reactions one of which is "mastering" above 16° and another one below. While each of these alternatives might explain some of the observations presented here, their correctness in the myosin system is considered less likely for these reasons: (a) the fact that two very similar substrates undergoing the same kind of chemical reaction give qualitatively different curves makes a general solvent effect unlikely, (b) the experiments with ATP–DNP (Figs. 7 and 8) demonstrate complete reversibility of the temperature effects under these conditions; (c) although a shift from one to another rate-limiting step cannot be rigorously excluded, it seems improbable that complex kinetic factors would balance out so often under such varied conditions to give a transition at or near 16°, despite large changes in over-all rate. This conclusion is supported further by the finding that actomyosin (here actin is the modifier) with ATP alone gives the same transition temperature[20]. This example of the similar effect of actin and the other modifiers on myosin ATPase is of special interest in view of the observations by CHAPPEL AND PERRY which indicate that actin and DNP compete for binding to myosin[3].

The above considerations offer a general framework which allows a correlation of a large number of the properties of myosin, but there are many points which still need clarification. Thus, not all modifiers under all conditions induce an enzyme–ATP conformation with a temperature dependence similar to enzyme–ITP. For example, MORALES AND HOTTA[21] have observed that AET-activated ATPase gives a linear Arrhenius plot. Also it appears from the work of BLUM[22] that not all substrates lacking a 6-amino group show a non-linear temperature dependence. These other studies were done with myosin b under somewhat different conditions and hence do not conflict with the data reported here. However, they suggest that further work will be needed to understand all of the interactions of these reagents in the myosin system. A conformation change at the active site which can be influenced by nucleotide specificity, temperature, and metal ion appears at first to introduce added complexity. It is seen, however, that it provides a basis for explaining seemingly conflicting experiments and may in the future provide a basis for further understanding of the nucleotide–protein interactions of the myosin system.

ACKNOWLEDGEMENTS

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