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Characterization of Modified Myosin at Low Ionic Strength. Enzymatic and Spin-Label Studies†

Deborah Bennett Stone* and Shirley Clift Prevost

ABSTRACT: In order to gain insight into the nature of the actin-induced acceleration of the myosin-catalyzed hydrolysis of ATP several chemical modifiers of myosin have been studied at the low ionic strength conditions where actin is most effective. From determinations of myosin catalytic activity under a variety of conditions it was found that actin and chemical modifiers (*e.g.*, sulfhydryl reagents, butanol, substitution of ITP for ATP) share common properties in a low ionic strength medium. Modification results in activation of the myosin catalytic activity if Mg^{2+} is present; below a certain critical Mg^{2+} concentration inhibition occurs. This Mg^{2+}-moderated activation varies with the pH of the assay medium; maximal activation by the modifiers occurs at pH 7 (*i.e.*, at the pH of minimal activity for the unmodified myosin). The effect of modification on the nucleotide-induced conformational change was investigated with a spin-labeled preparation of heavy meromyosin. The magnitude of the ADP-induced mobilization of spin labels attached to the SH_1 groups of heavy meromyosin was reduced by modification. For three vastly different modifiers (actin, butanol, substitution of inosine nucleotide for adenine nucleotide) the potency of activation of the myosin catalytic activity was inversely related to the concentration of Mg^{2+} required to support activation and the magnitude of the nucleoside diphosphate induced spectral change. These findings suggest that the modifiers impair the nucleoside diphosphate induced conformational change in myosin and that such impairment may be associated with acceleration of the catalytic activity.

A central question for the mechanism of muscle contraction is how the chemical energy of ATP is converted into the mechanical energy responsible for the relative translation of the thin and thick filaments. Hydrolysis of ATP is essential for contraction and this process is catalyzed by the thick filament protein, myosin. Under physiological conditions (*i.e.*, at low ionic strength and in the presence of Mg^{2+}) the catalytic activity of myosin alone is poor. However the thin filament protein, actin, enhances this activity to levels sufficient for contraction. Attempts to study the unique properties of the actin-modified myosin ATPase have been thwarted by the fact that myosin is insoluble in the ionic conditions which support actin activation. Attention has therefore been focused on a number of chemical reagents (*e.g.*, a variety of alcohols, sulfhydryl reagents, and substitution of ITP for ATP) which activate the Ca^{2+}-moderated ATPase of myosin at high ionic strength (*i.e.*, conditions where myosin can be studied in solution). Recent studies with molecular probes (Duke *et al.*, 1966; Cheung and Morales, 1969; Cheung, 1969;

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Quinlivan *et al.*, 1969; Mattocks *et al.*, 1967) have provided support for the earlier proposal (Rainford *et al.*, 1964; Levy *et al.*, 1962; Gilmour, 1960; Blum, 1960) that chemical modification induces small, localized changes in the myosin conformation.

It is tempting to suppose that modification by actin involves similar conformational changes and the experiments reported in this paper were designed to answer that question. Two lines of investigation were pursued. In the first of these enzymatic assays were carried out to determine the properties of chemically modified myosin in a low ionic strength Mg^{2+} medium (*i.e.*, under conditions where actin works best). Previous studies (see, for example, Levy and Ryan, 1961) have indicated that the chemical modifiers do activate the Mg-ATPase of myosin in a low ionic strength medium but knowledge of the properties of this modified activity (*e.g.*, pH dependence) which might implicate a change in protein conformation is lacking. The second line of inquiry employed the spin-labeling technique (Stone *et al.*, 1965; McConnell and McFarland, 1970) to directly probe the conformation of discrete areas of the myosin molecule before and after modification by actin and chemical reagents. For these studies we used HMM,[1] a tryptic fragment of myosin which retains the ATPase and actin-binding properties of myosin, but remains soluble at low ionic strengths. Previous studies with spin labels having an iodoacetamide reactivity have detected changes in myosin conformation accompanying modification with *p*-ClHgBzO (Quinlivan *et al.*, 1969) and binding of nucleotides (Seidel *et al.*, 1970; Stone, 1970; Seidel and Gergely, 1971). Recently Stone (1973) observed that the alterations in spin label mobility associated with ADP binding to HMM are reversed by actin. This effect of actin was attributed to a decrease in the affinity of HMM for ADP as well as a reduction in the magnitude of the conformational change associated with nucleotide binding. In the present study we have looked for effects of chemical modification on the conformational change associated with nucleotide binding.

Experimental Procedure

Materials. Myosin A was obtained (J. Botts, unpublished) by a 2-hr extraction of coarsely minced rabbit skeletal muscle in 3 volumes of KCl solution (0.6 ionic strength, pH 6.8) containing ATP at an initial concentration of 2.0 mM. The enzyme was then purified by alternately lowering (I = 0.04 M) and raising (I = 0.5 M) the ionic strength, in the presence of ATP, to precipitate and redissolve the myosin. Following the third dissolution step (from which ATP was omitted), the myosin was centrifuged at 150,000*g* for 1 hr and dialyzed against 0.5 M KCl lightly buffered with $KHCO_3$ at pH 7.2. HMM was isolated from a 5-min tryptic digest of myosin (1 mg of trypsin/300 mg of myosin) by the procedure of Lowey *et al.* (1969) and concentrated by ammonium sulfate precipitation (Young *et al.*, 1965). Actin was extracted from an acetone dried muscle powder with a cold ATP solution and purified by repeated polymerization–depolymerization as described previously (Stone *et al.*, 1970). The spin label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, was purchased from Synvar Associates. In order to reduce the level of trace metal contaminants KCl (analytical reagent grade) was recrystallized from glass-distilled water.

[1] Abbreviations used are: HMM, heavy meromyosin; *p*-ClHgBzO, *p*-chloromercuribenzoate; R_{21}, ratio of the second and first peak heights, respectively, of the electron paramagnetic resonance (epr) spectrum.

The velocity of the nucleoside triphosphatase reaction was determined by measuring the rate of H^+ production with a Radiometer recording pH-Stat at pH 8 and 25°. The control reaction mixtures contained 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, and 0.1–0.2 mg of myosin/ml in a final volume of 25.0 ml. The reaction mixtures were magnetically stirred under a purified nitrogen atmosphere and the rate of addition of 5 or 25 mM KOH necessary to maintain the initial pH was followed for approximately 10 min. In studies of the pH dependence of the myosin-catalyzed reaction, rate measurements were carried out in buffered media and the production of P_i was followed by the colorimetric technique of Fiske and Subba-Row (1925) as modified by Morales and Hotta (1960). The 25-ml reaction mixtures contained 25 mM Tris-chloride, 25 mM histidine chloride, 20 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, and 0.05–1.0 mg of myosin/ml. The two methods gave approximately the same activities both for unmodified and for chemically modified myosin. The higher activity of actin-modified myosin in the buffered medium is presumably due to differences in ionic strength or KCl concentration between the two media.

Protein Concentrations. The concentration of actin and myosin preparations was measured by the colorimetric procedure of Lowry *et al.* (1951) using bovine serum albumin as a standard. To avoid interference by K^+, myosin was diluted in 0.5 M NaCl and actin in distilled water. The concentration of HMM preparations was determined by ultraviolet absorption at 280 nm using an extinction coefficient of 647 cm^2/g (Young *et al.*, 1964).

Techniques of Spin Labeling. The fast reacting thiol groups (SH_1) of HMM were allowed to react with the iodoacetamide spin label as described previously (Stone, 1973). Epr spectra were recorded at room temperature with a Varian E-3 epr spectrometer (Stone *et al.*, 1970).

The concentration of Ca and Mg in stock solutions of proteins and reagents as well as in duplicate reaction mixtures was measured with a Perkin-Elmer Model 303 atomic absorption spectrophotometer equipped with a dual element (Ca–Mg) hollow cathode lamp.

Results

Effect of Modification on the Low Ionic Strength Mg^{2+}-Moderated Activity of Myosin. In the presence of trace levels of Ca^{2+} (~1 μM in the nonbuffered reaction mixtures) the low ionic strength Mg^{2+}-moderated ATPase activity of myosin is low, ~0.1–0.2 μmol of P_i g^{-1} sec^{-1}. It is well documented that actin strongly accelerates this catalytic activity of myosin. In preliminary studies it was ascertained that the following chemical modifiers also accelerate this activity: butanol, propanol, 2,4-dinitrophenol, dioxane, *p*-ClHgBzO, and substitution of ITP for ATP. The only chemical modifiers which were previously shown to activate the high ionic strength Ca^{2+}-moderated activity but failed to enhance the low ionic strength Mg^{2+}-moderated activity were the metal ions, Cd^{2+}, Cu^{2+}, and Zn^{2+}. Only inhibition was observed for these modifiers when present in concentrations of 1–100 μM.

When studied in a high ionic strength Ca^{2+} medium, the chemical modifiers typically have a biphasic effect: activation at low levels of reagent is followed by inhibition at higher levels of reagent. This property is also observed when activity measurements are carried out in a low ionic strength Mg^{2+} medium. Typical results with *p*-ClHgBzO are shown in Figure 1. Modification by actin or by substitution of ITP for ATP is only monophasic; however subsequent modification with

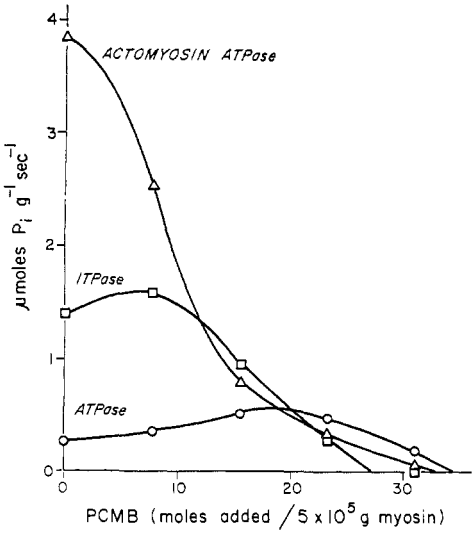

FIGURE 1: Effect of *p*-ClHgBzO on the ATPase, ITPase, and actin-modified activities of myosin. Myosin (10 mg/ml) was allowed to react with *p*-ClHgBzO on ice for 24 hr. Activity was measured by a pH-Stat (pH 8.0, 25°) in a medium containing 50 mM KCl, 1 mM $MgCl_2$, and the following additions: (○) 1 mM ATP, 0.2 mg of myosin/ml; (□) 1 mM ITP, 0.08 mg of myosin/ml; (△) 1 mM ATP, 0.04 mg of myosin/ml, 0.04 mg of F-actin/ml.

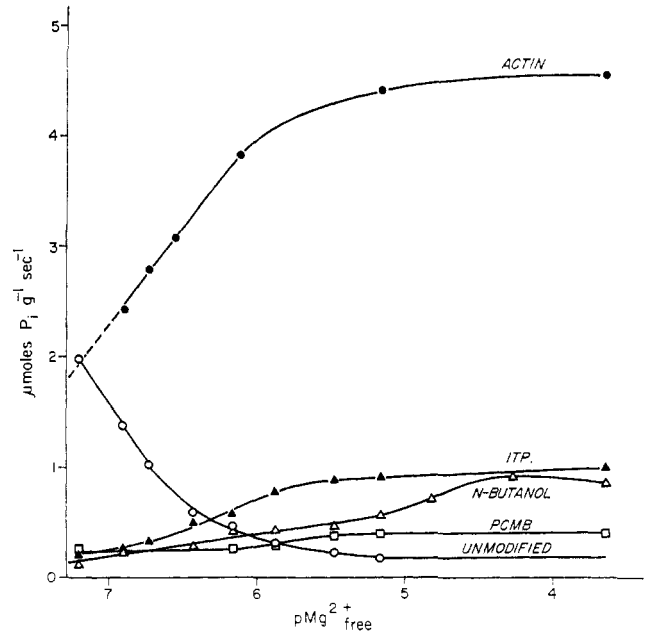

FIGURE 2: Magnesium dependence of the activity of unmodified and modified myosin. Activity was measured by a pH-Stat (pH 8.0, 25°) in a medium contaiing 50 mM KCl, 1 mM ATP, 0.001–1.0 mM total (=added plus contaminant) $MgCl_2$ and the following additions: (○) 0.1 mg of unmodified myosin/ml; (□) myosin reacted on ice for 24 hr with 20 mol of *p*-ClHgBzO/5 × 10^5 g, 0.2 mg/ml; (△) 0.44 M *n*-butyl alcohol and 0.2 mg of myosin/ml; (▲) ITP substituted for ATP, 0.1 mg of myosin/ml; (●) 0.1 mg of myosin/ml and 0.05 mg of F-actin/ml. The contaminant Mg^{2+} concentration was 1 μM for reaction mixtures containing unmodified and chemically modified myosin and 2 μM for those containing actin-modified myosin. The concentration of free Mg^{2+} was calculated by assuming only ATP binds Mg. The constants used in this calculation are: $pK_3 = 4.10$, $pK_4 = 6.80$; the affinity constant for binding of K^+ to ATP^{4-} is 8.0 M^{-1} (Botts *et al.*, 1965); affinity constants for Mg^{2+} binding to ATP^{3-} and ATP^{4-} at 0.05 ionic strength are 170 M^{-1} and 2.20 × 10^4 M^{-1}, respectively, and were obtained by extrapolation from plots of K *vs.* $(I)^{1/2}$ using the data of J. Botts, A. Chashin, and L. Schmidt (personal communication) for ionic strengths 0.1, 0.2, 0.3, and 0.6. The same constants were used for computing Mg^{2+} binding to ITP since it has been reported that the stability constants of the Mg^{2+} complexes of ATP and ITP are the same (Walaas, 1958).

low levels of a "biphasic" reagent (*e.g.*, *p*-ClHgBzO, see Figure 1) inhibits both of these activities.

In a low ionic strength medium the catalytic activity of unmodified myosin is strongly inhibited by Mg^{2+} while that of modified myosin is activated (Figure 2). Consequently, concentrations of modifier which maximally activate in the presence of high levels of Mg^{2+} produce inhibition when only trace levels of Mg^{2+} are present. The conversion from inhibition to activation occurs at a free Mg^{2+} concentration of approximately 10^{-6} M for the chemical modifiers and 10^{-7} M for actin. In the case of actin this conclusion is based on extrapolated data; however, Barron *et al.* (1966) have shown (through the use of chelators) that actin inhibits the ATPase activity of myosin when the free Mg^{2+} concentration drops below 10^{-7} M.

One of the more important effects of chemical modification is the conversion of the pH dependence of the high ionic strength, Ca^{2+}-moderated activity from the complex triphasic dependence typical of native myosin to a more normal monophasic titration curve. Since such a change in the pH dependence is strongly suggestive of a conformational change near the active site it seemed of interest to determine whether similar changes occurred in the low ionic strength, Mg^{2+}-moderated activity. The triphasic nature of the pH dependence of unmodified myosin is retained at these low ionic strength conditions (Figure 3). Modification by *p*-ClHgBzO or by substitution of ITP for ATP (Figure 3) did not significantly alter the triphasic nature of the activity–pH relationship, although the minimum of the curve near neutrality was shifted toslightly more alkaline pH values. Modification by actin, however, greatly reduced the abberations in the neutral range of the pH curve (Figure 4). The actomyosin activity reached a maximum at pH 8 and dropped sharply at higher pH values. From a plot of the ratio of activities for modified and unmodified myosin (Figure 5) it is clear that actin, *p*-ClHgBzO, and substitution of ITP for ATP all bring about marked activation at pH 7; the degree of activation is significantly reduced at lower or higher pH values.

Effect of Modification on the Epr Spectrum of Spin-Labeled HMM. A typical spectrum of spin-labeled HMM is shown in Figure 6A. The rotational motion of the spin label is very strongly restricted as indicated by the intensity of the outermost lines (labeled 1 and 5) of the spectrum. In the presence of ADP (Figure 6, spectrum B) there is an increase in peak 2 (representing weakly immobilized label) and a decrease in the intensity of the outermost lines of the spectrum as reported previously (Seidel *et al.*, 1970; Stone, 1970). Binding of IDP (Figure 6, spectrum C) also produces an increase in spin label mobility, although the magnitude of this change is less than observed with ADP. Figure 7 shows the dependence of the spectral change (measured from the ratio of the heights of the second and first peaks, respectively, of the spectrum) on the concentration of IDP. At saturating IDP the change in R_{21} was 0.150, compared with a value of 0.199 obtained in the presence of saturating ADP. From the data of Figure 7 an apparent dissociation constant was calculated by the method previously described by Stone (1973). A value of 155 μM was obtained for IDP compared with a previously reported value of 5 μM for ADP under similar ionic conditions.

Seidel and Gergely (1971) reported that the spectral change

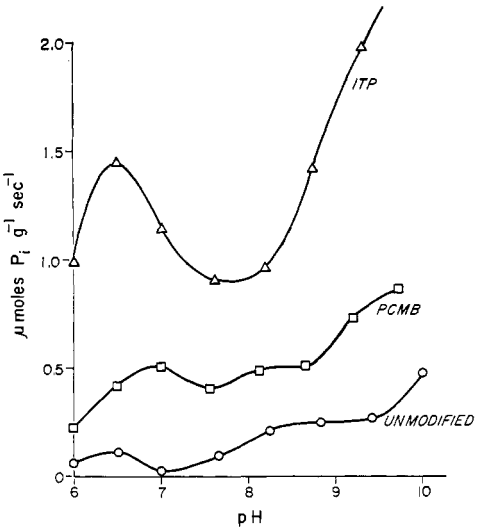

FIGURE 3: pH dependence of unmodified and chemically modified myosin. Activity was assayed by P_i production in a medium containing 25 mM Tris-chloride, 25 mM histidine chloride, 20 mM KCl, 1 mM ATP, 1 mM $MgCl_2$, and the following additions: (○) unmodified myosin, 1.0 mg/ml; (□) myosin treated on ice for 48 hr with 15 mol of *p*-ClHgBzO/5 × 10^5 g, 0.4 mg/ml; (△) ITP substituted for ATP, 0.2 mg of myosin/ml.

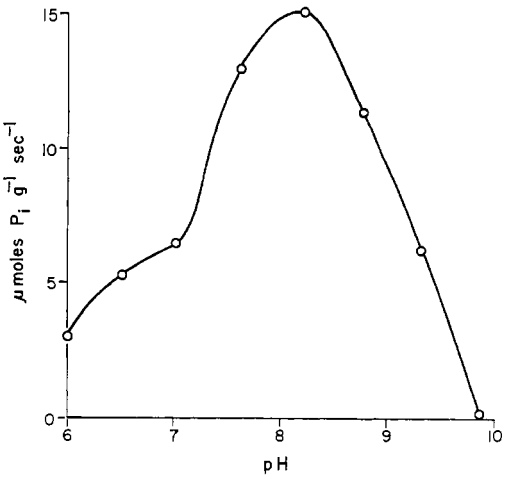

FIGURE 4: pH dependence of the ATPase activity of actin-modified myosin. The ATPase activity was measured as described in Figure 3 with 0.05 mg of myosin/ml and 0.05 mg of F-actin/ml.

observed during steady-state hydrolysis of ATP by spin-labeled myosin is very much greater than that produced either by the binding of ADP or by the binding of ATP in the absence of hydrolysis. We have observed essentially similar spectral differences for the adenine nucleotides with spin-labeled HMM (Stone, 1973; see also Table I). However, during hydrolysis of ITP we have observed only a slight increase in spin label mobility over that produced by the binding of IDP. The value of R_{21} observed during hydrolysis of ITP was very similar to that produced by the binding of ADP (Table I).

Figure 8 shows the effect of butanol on the spectral parameter, R_{21}, and the catalytic activity of spin-labeled HMM. The catalytic activity of unlabeled HMM is also included for comparison. The mobility of the spin labels attached to the HMM·ADP complex decreased with increasing butanol concentration; at 0.44 M butanol the value of R_{21} was 0.280 ± 0.005 compared with 0.325 ± 0.004 in the absence of butanol.

TABLE I: Effect of Adenine and Inosine Nucleotides on the Spectrum of Spin-Labeled HMM.[a]

Additions	R_{21}
4 mM $MgCl_2$	0.122
4 mM MgADP	0.256
10 mM MgATP	0.872[b]
4 mM MgIDP	0.205
10 mM MgITP	0.247[b]

[a] The samples contained 33 μM spin-labeled HMM in 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0), 5 mM KCl plus $MgCl_2$, and nucleotide as indicated. Under similar solvent conditions spin-labeled HMM had a catalytic activity of 2.41 μmol of P_i g^{-1} sec^{-1} in the presence of 1 mM MgATP and 1.08 μmol of P_i g^{-1} sec^{-1} in the presence of 2 mM MgITP. [b] Recorded during nucleotide hydrolysis. Following depletion of substrate the spectra were indistinguishable from those obtained by adding the corresponding nucleoside diphosphate.

At higher concentrations of butanol there was a dramatic increase in spin label mobility. This effect could be observed both in the presence and absence of nucleotide and is most likely due to protein denaturation. Since the spin label is a very effective modifier, further chemical modification with butanol only results in inhibition of the catalytic activity of HMM. However, the diphasic effect of butanol on the ATPase activity of unlabeled HMM coincides exactly with the changes in spin label mobility: the rise in catalytic activity occurs over the same concentration range as the decline in R_{21}; likewise the decline in enzymatic activity coincides with the marked increase in R_{21}.

Discussion

When studies are carried out in a low ionic strength medium actin and the chemical modifiers of myosin are found to share several distinctive properties, (1) modification results in activation of the myosin catalytic activity if Mg^{2+} is present; below a certain critical Mg^{2+} concentration inhibition occurs. (2)

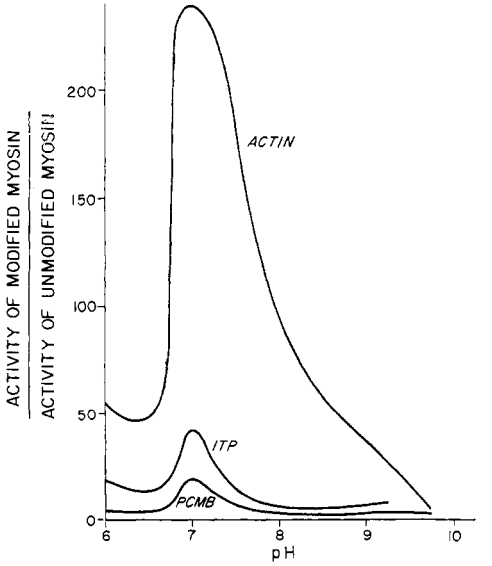

FIGURE 5: pH dependence of the catalytic activity of modified myosin. The ratio of activity of modified myosin to that of unmodified myosin was calculated from the data of Figures 3 and 4.

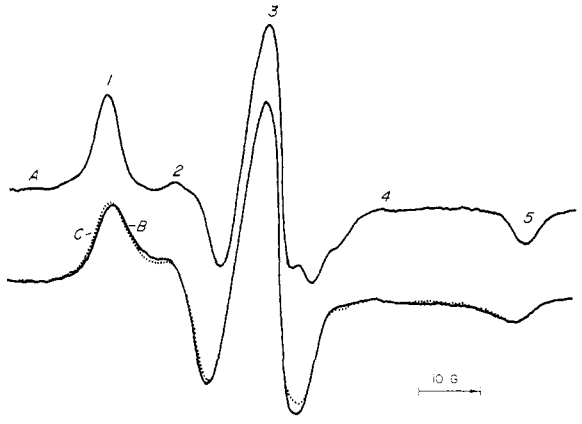

FIGURE 6: Effect of ADP and IDP on the epr spectrum of spin-labeled HMM. Each sample contained 45 μM spin-labeled HMM in 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0), 5 mM KCl, 4 mM $MgCl_2$, and the following additions: (A) no addition; (B) 7.2 mM ADP; and (C, broken line) 7.2 mM IDP.

This Mg^{2+}-moderated activation varies with the pH of the assay medium; maximal activation occurs at pH 7 (*i.e.*, at the pH of minimal activity for the unmodified myosin). (3) The magnitude of the ADP-induced mobilization of spin labels attached to the SH_1 groups of HMM is reduced by modification. Further evidence for a common mode of action for actin and the chemical modifiers comes from the finding (Figure 1) that following modification by actin or by substitution of ITP for ATP there is a shift in the *p*-ClHgBzO dependence of the catalytic activity. If the modifiers were acting independently the expected effect of double modification would be the sum of the effects of the modifiers acting alone. However, it is observed that prior modification by actin or by substitution of ITP for ATP shifts the inhibitory action of *p*-ClHgBzO modification to much lower concentrations of reagent.

Table II summarizes some of the properties of modified myosin. It is evident that potency as an activator is inversely related to the magnitude of the spectral change and the concentration of Mg^{2+} required to support activation. Elsewhere (Seidel *et al.*, 1970; Stone, 1970) it has been argued that the

TABLE II: Summary of Properties of Modified Myosin.

Modifier	Activation of Mg-ATPase[a]	[Mg^{2+}] Required for Activation[b]	ΔR_{21} (% of Control)[c]
Butanol	4.7	8.6×10^{-7} M	80
Adenine nucleotide replaced by inosine nucleotide	5.5	5.0×10^{-7} M	69
Actin	25.1	6.4×10^{-8} M	23

[a] The activity of modified myosin is expressed as a multiple of the activity of unmodified myosin. Data are taken from Figure 2 (total [Mg] = 1.0 mM). [b] The free [Mg^{2+}] at which the catalytic activity for modified myosin equals that for unmodified myosin is calculated from the data of Figure 2. [c] Calculated from ΔR_{21}(modified) $\times$ 100/ΔR_{21}(unmodified), where ΔR_{21} is the change in R_{21} occurring at saturating nucleoside diphosphate. Data are taken from Figures 7 and 8 of this paper and Figure 5 of Stone (1973).

nucleotide-induced spectral change results from a change in protein conformation. Taken together, these findings provide direct experimental support for the proposal of Gilmour (1960) that the modifiers act by preventing a nucleotide-induced conformational change.

Previous studies have provided ample evidence that chemical modification alters the conformational state of myosin in the absence of nucleotide (Duke *et al.*, 1966; Mattocks *et al.*, 1967; Cheung and Morales, 1969; Cheung, 1969; Quinlivan *et al.*, 1969). Modification by actin has also been shown to alter the mobility of spin labels attached to myosin (Stone *et al.*, 1968; Seidel *et al.*, 1971; Tokiwa, 1971). The present study provides the first indication that the nucleoside diphosphate induced conformational change is altered by modifiers such as actin, butanol, and substitution of IDP for ADP. In all probability the two findings are related. For instance, it is conceivable that the modifier-induced conformational

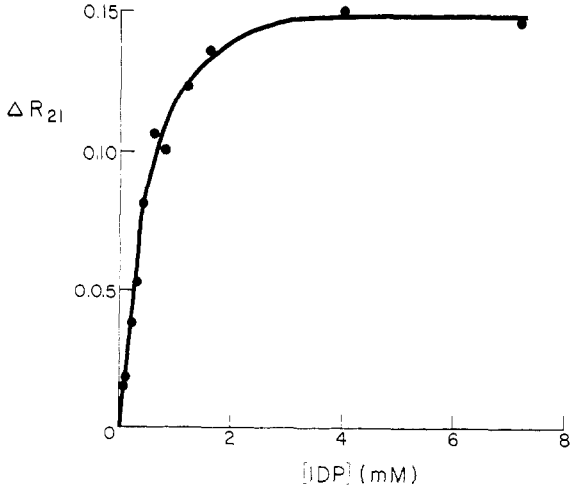

FIGURE 7: Spectral change as a function of IDP concentration. Conditions: 45 μM spin-labeled HMM in 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0), 5 mM KCl, 4 mM $MgCl_2$, and 0–7.2 mM IDP. The change in the ratio of the height of peak 2 to that of peak 1 is plotted *vs.* the total IDP concentration. R_{21} in the absence of IDP was 0.124.

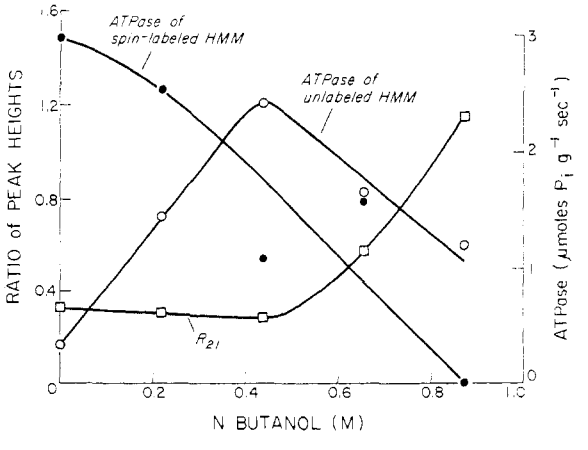

FIGURE 8: Effect of butanol on the ADP-induced spectral change and on the ATPase activity of HMM. Samples for spectral analysis (□) contained 40 μM spin-labeled HMM in 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0), 5 mM KCl, and 4 mM MgADP. Catalytic activity was assayed by P_i production in a medium containing 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0), 5 mM KCl, 1 mM MgATP and 0.26 mg/ml of unlabeled (○) or 0.12 mg/ml of spin-labeled (●) HMM.

change alters the myosin in such a way as to hinder the nucleotide-induced conformational changes. Since the spin label itself is a modifier it is probable that the nucleotide-induced conformational change is already reduced in the spin-labeled protein relative to unlabeled HMM.

Kiely and Martonosi (1969) have shown that the affinity of myosin for ADP is reduced by actin and 2,4-dinitrophenol. The data of Figure 7 indicate that the affinity of spin-labeled HMM for IDP is considerably weaker than for ADP. If the rate of nucleoside triphosphate hydrolysis is limited by product dissociation (Blum and Felauer, 1959; Taylor *et al.*, 1970) then these findings suggest that the modifiers accelerate this rate by promoting dissociation of the myosin–product complex. According to this line of reasoning the nucleoside diphosphate induced conformational change might strengthen the binding of the products of hydrolysis. However, one important group of modifiers, the sulfhydryl reagents, does not reduce ADP affinity (Malik and Martonosi, 1972; Stone, 1973). Moreover, recent experiments suggest that a step prior to product dissociation may be rate limiting for the hydrolysis of ATP (Trentham *et al.*, 1972). Attempts to demonstrate an effect of modifiers on the conformation of HMM*ADP·P (the species currently believed to predominate during steady-state hydrolysis (Viniegra-Gonzalez and Morales, 1972; Trentham *et al.*, 1972)) have produced mixed results. Actin had no effect on the mobility of spin labels attached to HMM*ADP·P (Seidel and Gergely, 1972; Stone, 1973). However studies of protein absorption (Yoshino *et al.*, 1972) and fluorescence (Werber *et al.*, 1972) as well as the spin label studies reported here indicate that the conformational changes detected during hydrolysis of ITP are less than those observed during ATP hydrolysis. In the absence of additional information it is impossible to decide whether this reduction is due to a reduction in the concentration of the intermediate species or results from an alteration in the conformation of the intermediate. If the latter is found to be the case, then this would be evidence for an effect of modifiers on the conformational change induced by this intermediate species. A change in conformation at this step in the reaction sequence would strongly suggest that the actual catalytic mechanism (rather than protein–nucleotide affinity) may be altered by modification.

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