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Interaction of Adipic Acid Dihydrazide Analogue of ATP with Myosin. Involvement of the Essential Sulfhydryl Groups†

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ABSTRACT: The hydrolysis by myosin of a soluble ATP analogue, adipic acid dihydrazide-ATP, is shown to proceed in a fashion similar to the hydrolysis of ATP by myosin modified at either of the two essential sulfhydryl groups. In both systems, the Mg^{2+}-activated hydrolysis of the nucleotide is increased, whereas the EDTA-stimulated activity is inhibited. Blocking of one of the two essential sulfhydryl groups of myosin, SH_1 or SH_2, leads to a complete inhibition of the analogue hydrolysis. The analogue is unable to expose the essential sulfhydryl group SH_2 for modification by thiol reagents. Evidence is presented to show that the nucleotide derivative does not label irreversibly the protein. It is concluded that adipic acid dihydrazide-ATP (ADH-ATP) need interact with only one of the two essential thiol sites of myosin. The hydrolysis of MgADH-ATP by myosin is inhibited by large excess of actin and does not result in contraction of actomyosin threads. MgADH-ATP is also a rather weak dissociating agent of the acto-heavy meromyosin complex. These properties of the ATP analogue are discussed in conjunction with the previous modification studies of myosin and the mechanism of ATP hydrolysis derived from them (Burke, M., Reisler, E., and Harrington, W. F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3793; Reisler, E., Burke, M., and Harrington, W. F. (1974a), *Biochemistry 13,* 2014).

Recently it was suggested that the low rate of ATP cleavage in the resting state of muscle results from formation of a stable complex structure involving the two essential thiol sites (SH_1 and SH_2) on each myosin head and MgATP (Burke et al., 1973; Reisler et al., 1974a). Activation was thought to occur by interaction of actin with myosin in the vicinity of one of the two essential thiol sites, thus breaking the stable complex and leading to a rapid dissociation of split products. In a way the action of actin was viewed as analogous to the chemical blocking of SH_1 sites of myosin; it was noted, in earlier studies, that such modification resulted in significant activation of myosin ATPase in the presence of Mg^{2+} ions (Sekine and Kielley, 1964) and the concomitant loss of the ability of actin to activate this hydrolysis reaction (Silverman et al., 1972). In terms of the proposed model (Burke et al., 1973; Reisler et al., 1974a), these two observations are corollary of the inability of the chemically modified SH_1 site to participate in the formation of the stable complex structure with MgATP. It is assumed implicitly, in this context, that the above two effects of modification of SH_1 sites of myosin are linked and stem from the same mechanistic reason.

If indeed true, such explanation would imply that the cyclic formation and opening of the inhibitory MgATP-myosin complex is an essential requirement of the contractile process. The last conclusion is supported within the qualifications set above, by the recent finding that the modification of SH_1 sites of myosin precludes contraction of actomyosin threads (Harrington et al., 1975).

The above suggestions rely, however, upon a reasonable but unproved thesis, that chemical modification does not introduce structural changes, which may produce the observed alterations in the enzymatic properties of myosin. Thus, it would be desirable to find experimental conditions which lead to, or support, the same conclusions, without involving a chemical assault on the protein. In principle, such a goal could be achieved by employing an appropriate ATP analogue, which could not form the assumed cyclic complex with the two thiol sites. Such an analogue should display hydrolytic behavior (with native myosin) comparable to that of ATP with myosin modified at SH_1 or SH_2 (i.e., activated MgATPase, inhibited EDTA(K^+) ATPase, very small, or no actin activation, etc.). We require, thus, a limited structural modification of ATP, which would preclude the precise interaction with the two thiol sites, but would not impair the hydrolysis reaction.

To our knowledge, none of the numerous ATP analogues previously tested with myosin meets the rather restrictive and specific requirements listed above (for a comprehensive review on ATP analogues and their interaction with myosin, see Yount, 1975).

The comprehensive studies of Tonomura et al. (1967) have shown a tolerance of modification of the ribose ring of ATP for cleavage of phosphate and much less for contraction. In view of this, our attention focused on ribose modified ATP derivatives prepared by Lamed et al. (1973) and successfully employed in affinity chromatography of myosin fragments (Lamed and Oplatka, 1974; Oplatka et al., 1975; Muhlrad et al., 1975). We have chosen to study the interaction and hydrolysis by myosin of adipic acid dihydrazide-ATP (ADH-

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ATP),[1] since it was observed to be cleaved at a rapid rate in the presence of Mg^{2+} cations, displayed no activation by EDTA, and failed to cause shortening of myofibrils (Lamed et al., 1973).

The results to be presented below suggest that ADH-ATP interacts with only one of the two essential thiol sites of myosin and that the ADH-ATP-myosin system has many characteristics in common with thiol modified myosin and ATP. We also show that the hydrolysis of ADH-ATP is inhibited by actin and does not result in contractions.

Materials and Methods

Glass-distilled water was used throughout and inorganic salts and reagents were of analytical grade. FDNB and MalNEt were the products of Eastman Kodak Co. (Rochester, N.Y.) and ATP of highest available purity was purchased from Sigma Chemical Co. (St. Louis, Mo.). The preparation of myosin and actin has been described elsewhere (Azuma and Watanabe, 1965; Lehrer and Kerwar, 1972). HMM and HMM S-I were obtained and purified using the previously described chromatographic procedure (Lamed and Oplatka, 1974). The preparation of Sepharose sebacic dihydrazide-ATP columns employed in purification of the proteins was somewhat modified. Sebacic dihydrazide was dissolved in 50% acetic acid (20 g/L) at room temperature and adjusted to pH 2.0 with HCl to ensure complete dissolution. Cyanogen bromide activated Sepharose 4B (0.5 volume) was added, and the mixture was stirred overnight at room temperature. Periodate oxidized ATP was then coupled to the resin as described earlier (Lamed and Oplatka, 1974).

The ATP derivative, adipic acid dihydrazide-ATP, was prepared as described by Lamed et al. (1973).

Selective modification of SH_1 and SH_2 groups of myosin with MalNEt or FDNB was carried out as reported by Reisler et al. (1974a). Reduction of the dinitrophenylated myosin with sodium dithionite and removal of the dinitrophenyl group by thiolysis with β-mercaptoethanol was performed as described before (Reisler et al., 1974a).

The reactions of myosin with MalNEt were carried out at 1% protein concentration in 0.5 M KCl-50 mM Tris-HCl, pH 7.9 at 5 °C. The reaction was terminated by a 20-fold dilution of the protein in 0.5 M KCl-Tris-HCl solvent containing a 50-fold excess of dithiothreitol.

The Ca^{2+}- and EDTA-stimulated ATPases and ADH-ATPases, as well as MgADH-ATPase activities of myosin were measured at 37 °C employing the procedures of Kielley et al. (1956) and Kielley and Bradley (1956).Temperature and pH dependence of myosin activities was monitored by a pH-stat titration method in 50 mM KCl, 1 mM ATP or ADH-ATP, 1 mM Mg^{2+} or Ca^{2+} cations. In pH studies, the temperature was maintained at 25 °C; in temperature dependence measurements, the pH was kept at 7.6.

Actin activation of the ATPase activity of myosin was measured on protein samples incubated for 5 min, at 37 °C, in a solution containing 50 mM KCl, 10 mM Tris-HCl, 5 mM Mg^{2+}, and 2 mM ATP or ADH-ATP, at pH 7.6. Following the incubation, the protein was precipitated by 10% trichloroacetic acid and the supernatant obtained on centrifugation was assayed for P_i by the method of Kielley et al. (1956).

The dissociation of acto-HMM or acto-HMM S-I complexes by ATP or its analogue was followed by light scattering. The measurements (at an angle of 90°) were conducted at 25 °C in a Fica (Paris, France) light scattering photometer employing octagonal cells as described by Eisenberg and Tomkins (1968). Protein concentrations in controls (uncomplexed HMM or actin) and in acto-HMM or acto-HMM S-I, were 0.07 mg/mL for HMM and HMM S-I, and 0.12-0.15 mg/mL for actin. To monitor the effect of ATP or ATP analogue on acto-HMM solutions, small volumes of the concentrated nucleotide solutions were added directly to the scattering cells containing the protein system. Prior to measurements, all solutions were filtered through 0.45-μm Millipore filters. In the presence of 1 mM MgATP, the acto-HMM complex is fully dissociated and the scattering of acto-HMM solution ($I_{\text{acto-HMM}}$) reduces to the sum of scatterings of its components, HMM and actin ($I_{\text{HMM}} + I_{\text{actin}}$). The observed drop in scattering ($\Delta I = I_{\text{acto-HMM}} - I_{\text{acto-HMM+nucleotide}}$) is a measure of the extent of dissociation of acto-HMM solutions relative to the dissociation in the presence of 1 mM MgATP.

Results

The ability of myosin and myosin fragments to hydrolyze soluble ATP derivatives modified on the ribose moiety was demonstrated by Lamed et al. (1973). These authors noted that the derivatives, and particularly adipic acid dihydrazide-ATP, were cleaved at a rapid rate in the presence of Mg^{2+} and Ca^{2+} cations but could not be hydrolyzed in the absence of divalent cations, i.e., in the presence of EDTA. Such hydrolytic behavior is rather similar to that of ATP with SH_1-MalNEt or SH_2-MalNEt modified myosin. Two possible explanations come to mind: either the derivative covalently binds to myosin and modifies its properties in a manner characteristic of a thiol reagent, or, because of altered geometry, it interacts reversibly with the protein without involving all the residues and structural transitions that ATP does. To examine these possibilities and their implications, we have studied the hydrolysis of ADH-ATP by myosin in greater detail.

The various ADH-ATPase activities of myosin are summarized in Table I. The first row discloses that contrary to ATP, Mg^{2+} is a potent activator of ADH-ATP cleavage. In fact, MgADH-ATPase is comparable with MgATPase of SH_1-MalNEt modified myosin and is manyfold higher than MgATPase of the native protein. Chelation of Mg^{2+} cations by EDTA activates the "normal" ATPase but almost completely inhibits the ADH-ATPase reaction in analogy to the effect of EDTA on cleavage of ATP by SH_1-MalNEt (or SH_2-MalNEt) myosin (row 2). In the presence of Ca^{2+} cations, the rates of hydrolysis of ATP and its analogue are not significantly different (row 1). The divalent metal requirement for ADH-ATPase activity of myosin is shown in Figure 1. The requirement for Mg^{2+} is rather low since even at 10^{-6} M level of this metal and 1 mM ADH-ATP most of the activity is preserved. In contrast to this, relatively high Ca^{2+} concentrations are required for Ca^{2+}-activated hydrolysis of the analogue and high Mg^{2+} levels are necessary for ATP hydrolysis by SH_1-MalNEt modified protein (dashed curve, from Burke et al., 1973). The shallow profile of ADH-ATPase dependence on Mg^{2+} level probably reflects rather tight binding of this metal to myosin. In the ATP-myosin system, such binding is necessary for Mg^{2+}-induced inhibition of the hydrolysis reaction.

An interesting situation arises when we examine the hydrolysis of ADH-ATP by SH_1-MalNEt modified protein (row

[1] Abbreviations used: ADH-ATP, adipic acid dihydrazide-ATP; AMP-PCP, α,β-methylene-ATP; AMP-PNP, β,γ-imino-ATP; ATP(γS), adenosine 5′-*O*-(3-thiotriphosphate); FDNB, fluorodinitrobenzene; MalNEt, *N*-ethylmaleimide; HMM, heavy meromyosin; HMM S-I, heavy meromyosin subfragment 1.

TABLE I: Specific Activities[a] of Native and Modified Myosins (μmol mg^{-1} min^{-1}).

	ATP			ADH-ATP		
	EDTA(K^+)	Ca^{2+}	Mg^{2+}	EDTA(K^+)	Ca^{2+}	Mg^{2+}
Myosin	2.6 (100)[b]	0.95 (100)[b]	0.015[c]	0.05	1.2 (100)[b]	0.45 (100)[b]
SH_1-MalNEt myosin	0.15	2.3	0.12[c]	0.01	0.08	~0
SH_1-DNP myosin	0.42 (16)	3.40 (350)			0.23 (19)	0.09 (20)
SH_1-reduced DNP myosin	1.95 (75)	3.30 (350)			0.66 (55)	0.38 (85)
SH_1-DNP myosin + β-mercaptoethanol	1.82 (70)	1.05 (110)			0.84 (70)	0.29 (65)

[a] Activity measurements were carried out at 37 °C, pH 7.6, in 0.05 M KCl for Ca-ATPase and in 0.8 M KCl for EDTA-ATPase (Kielley et al., 1956; Kielley and Bradley, 1956), unless otherwise specified. [b] The numbers in parentheses refer to relative activities. [c] Measurements carried out at 25 °C, pH 7.6, in 0.05 M KCl employing the pH-stat titration method.

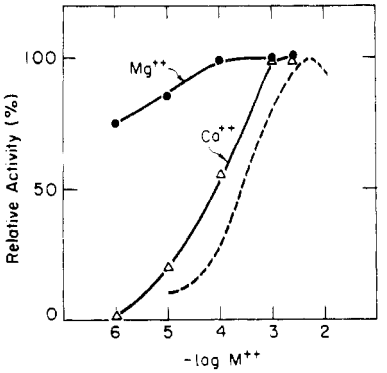

FIGURE 1: Relative ADH-ATPase activity of myosin as a function of the concentration of added divalent cations: (△) Ca^{2+}; (●) Mg^{2+}. The dashed line shows the relative ATPase activity of SH_1-MalNEt modified myosin as a function of Mg^{2+} ion concentration (from Burke et al., 1973). In the absence of M^{2+} cations, i.e., in the presence of EDTA, ADH-ATPase activity is inhibited.

TABLE II: Specific Activities of Myosin Incubated with MgADH-ATP (μmol mg^{-1} min^{-1}).[a]

Step	Present in the incubation mixture	Mg^{2+}	Ca^{2+}	EDTA
Step I	ADH-ATP, 1 mM	0.45	1.2	0.05
Step II	ADH-ATP (1 mM): ATP (5 mM)	<0.05	0.9	2.5

[a] The incubations of myosin assay system (Kielley et al., 1956) were carried out at 37 °C on solutions containing 0.025 mg/mL of protein and the tabulated ligands. One set of test tubes, incubated in the presence of ADH-ATP, was assayed for P_i liberation after 5 min; the second set was made up after 5 min to 5 mM ATP and Ca, Mg or EDTA, incubated for additional 5 min and assayed for P_i. ΔP_i between the two sets yields the values listed for step II of this table.

2); practically all of the analogue activities are lost. Clearly, retention of only one of the two essential thiol groups of myosin is not sufficient for expression of ADH-ATPase activities, although it is adequate for the cleavage of ATP itself. Involvement of the thiol sites in the reaction of myosin with ADH-ATP can also be tested through dinitrophenylation of SH_1 groups and subsequent manipulation of the attached label (Reisler et al., 1974a). As shown in Table I, modification of SH_1 groups with FDNB induces a similar, although less dramatic, effect as labeling with MalNEt (row 3). Reduction of the DNP label with sodium dithionite, i.e., formation of a nucleophilic center at the SH_1 site leads to a partial regeneration of Mg and Ca ADH-ATPases (row 4). Similar recovery of analogue activities is achieved on reversal of DNP binding by incubation of the modified protein with β-mercaptoethanol (row 5). Thus, in analogy with the ATP-myosin system (Reisler et al., 1974a), SH_1 sites are seemingly implicated in the interaction with the metal-analogue complexes. It should be mentioned, however, that also SH_2-MalNEt modified myosin, which could cleave CaATP and MgATP, did not hydrolyze ADH-ATP. It could be that, because of the proximity of the essential thiol groups spaced at about 10 Å apart (Reisler et al., 1974b; Haugland, 1975), blocking of one of them sterically prevents the interaction with the other site.

To check whether the cleavage pattern of ADH-ATP does not derive from an irreversible attachment of the analogue to the vicinity of SH_1 or SH_2 site, we have examined a composite ADH-ATP and ATP system. In the first step, myosin assay system was incubated in the presence of 1 mM ADH-ATP and 1 mM Mg, Ca, or EDTA. Following this incubation half of the solutions were assayed for P_i liberation and the other half was made up to 5 mM in ATP and Mg, Ca, or EDTA, to check for "normal" ATPase. As shown in Table II, when ATP is introduced into the ADH-ATP myosin system, it completely overrides the hydrolytically expressed interaction of analogue with the protein. Thus, it appears that no irreversible change has been conferred on myosin by incubation with ADH-ATP under conditions employed in this work.

The effective displacement of the analogue by ATP suggests that the affinity of the former for myosin is lower than that of ATP. This conclusion is verified by direct inhibition measurements presented in Figure 2b. ADH-ATP inhibits the EDTA (K^+) ATPase of myosin; yet considerable concentrations of the analogue are required to achieve full inhibition (Figure 2a). In the inverse case, inhibition of MgADH-ATP by ATP, low concentrations of the latter are sufficient to reach the maximum suppression of the analogue cleavage (Figure 2b). When compared, the data in Figures 2a and 2b indicate that Mg^{2+} induces a large difference in the relative affinities of ATP and ADH-ATP for myosin. Following modification of SH_1 sites by MalNEt, this difference is significantly diminished; about 2.5-fold excess of ADH-ATP is now adequate to inhibit the MgATPase activity. Interestingly, although SH_1-MalNEt practically does not hydrolyze MgADH-ATP, the analogue is still capable of interacting with the modified protein and inhibiting its normal ATPase.

Since the data presented so far suggest that neither an irreversible binding of the analogue to myosin nor modification of the thiol sites occurred, it seemed logical to seek an explanation for the properties of the myosin-ADH-ATP system in the altered mode of interaction of the analogue with the protein

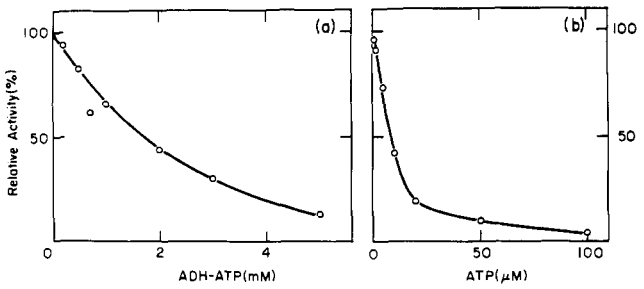

FIGURE 2: (a) Relative EDTA-ATPase of myosin as a function of ADH-ATP concentration; (b) relative MgADH-ATPase as a function of ATP concentration. The activities were measured at 1 mM concentrations of EDTA-ATP and MgADH-ATP.

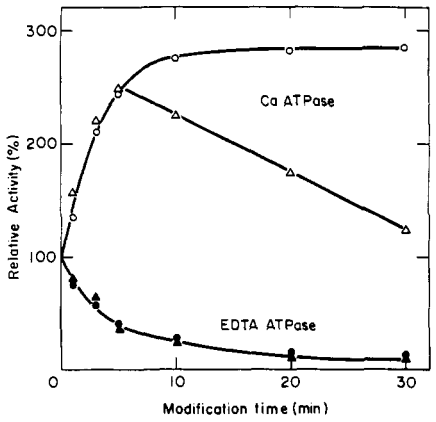

FIGURE 3: Relative Ca^{2+}- (○, △) and EDTA- (●, ▲) ATPase activities of myosin reacted at pH 7.9 with a fourfold molar excess of MalNEt and plotted as a function of time of modification. The modification was carried out in the presence of 1 mM EDTA-ATP (△, ▲) or 1 mM EDTA + 1 mM ADH-ATP (○, ●).

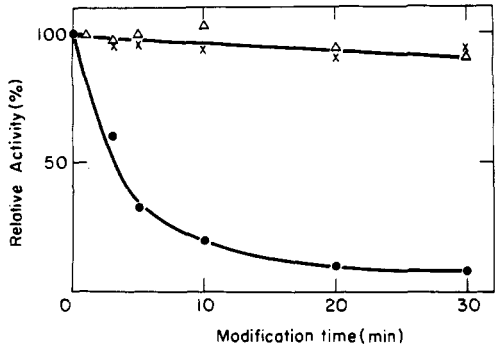

FIGURE 4: Relative CaATPase activity of SH_1-MalNEt myosin reacted at pH 7.9 with a fourfold molar excess of MalNEt and plotted as a function of time of modification. The reaction was carried out in the absence of ligands (X), in the presence of MgADH-ATP (△), or in the presence of MgATP (●).

and particularly with its essential thiol sites. One way to detect such differences is by monitoring the course of modification of the thiol groups by sulfhydryl reagents. Figure 3 shows the influence of ATP or ADH-ATP (in the presence of EDTA) on the reaction of myosin with MalNEt at pH 7.9. In the presence of ATP the modification by MalNEt is initially directed to SH_1 groups, as attested by elevation of CaATPase and loss of EDTA-ATPase activities and then proceeds also at the SH_2 groups, which leads to decrease of the CaATPase activity. Such pattern of modification reflecting exposure of SH_2 groups by ATP or by other nucleotides has been established before (Sekine and Yamaguchi, 1963) and can be viewed as indicative of the nucleotide-SH_2 site interaction. In the modification reaction conducted in the presence of ADH-ATP no loss in CaATPase was detected (Figure 3); in fact the reaction course was similar to that of a ligand free system where only SH_1 groups were labeled (Yamaguchi and Sekine, 1966; Reisler et al., 1974a). The failure of ADH-ATP to "unmask" the SH_2 groups can be even better demonstrated on the SH_1-MalNEt myosin system. Figure 4 shows that modification by MalNEt of SH_2 groups of SH_1-MalNEt protein, if attempted in a ligand free system, leads to only a minor decrease in activity. MgATP, which exposes the SH_2 groups for reaction (Yamaguchi and Sekine, 1966), affects dramatically the time profile of the modification whereas MgADH-ATP has no detectable effect (Figure 4). Therefore, it appears that the ATP analogue, although capable, when coupled to Sepharose, of strongly retaining the SH_1-MalNEt myosin (Lamed et al., 1976), does not perturb the SH_2 site. This, in turn, suggests that ADH-ATP interacts with only one of the two essential thiol sites of myosin at a time.

The difference between MgATP and MgADH-ATP complexes with myosin is also reflected in their fluorescence properties. MgATP enhances the tryptophan fluorescence of HMM by about 17% compared with about 5% increase by MgADP (Werber et al., 1972). The effect of MgATP was attributed by Werber et al. (1972) to formation of the rate-limiting complex M**ADP·P_i. No enhancement of fluorescence is observed with MgADH-ATP; the analogue even quenches somewhat the protein signal (Werber, 1976). This observation supports the kinetic evidence that ATP and ADH-ATP complexes with myosin are structurally different. Consequently, the analogue does not favor formation of the unique conformational state of myosin associated with the rate-limiting step in the hydrolysis of ATP (Burke et al., 1973).

Enzymatic and spectral data on ADH-ATP myosin suggest, in parallel to the above conclusions, that the interaction between the analogue and the protein resembles the interaction of SH_1-MalNEt (or SH_2-MalNEt) myosin with ATP. The common ground in both cases is the availability of only one thiol site for nucleotide binding and the resulting inability to form a transient conformational state ascribed to the M**ADP·P_i complex. To explore this similarity we have examined the temperature (Figure 5) and pH dependence (Figure 6) of the ADH-ATPase activity of myosin. The Arrhenius plots of the enzymatic activities are presented in Figure 5. For Ca- and Mg-stimulated ADH-ATPases as well as CaATPase and MgATPase (not shown) of SH_1-MalNEt modified protein, the plots are linear over the temperature range 5 to 37 °C. The appropriate activation energies are 80-90 kJ/mol for the ADH-ATPases and 90-100 kJ/mol for Ca- and MgATPases of SH_1-MalNEt myosin. These values contrast with much lower activation energies of Ca and MgATPases of unmodified protein (25-30 kJ/mol). Moreover, the last two activities yield biphasic Arrhenius plots which reflect temperature dependent transitions in the conformation of the active site of the protein (Watterson et al., 1975). Notable in this context is the fact that, in the low temperature range, where the activation energy of CaATPase (and MgATPase) of myosin is much increased, thiol modification studies (Watterson et al., 1975; Harrington et al., 1975) indicate in line with the kinetic evidence (Bagshaw and Trentham, 1974) that the M**ADP·P_i → M*ADP·P_i transition is no longer the rate-limiting step of the hydrolytic reaction.

The same general picture of similarities and differences between the experimental systems discussed above extends to

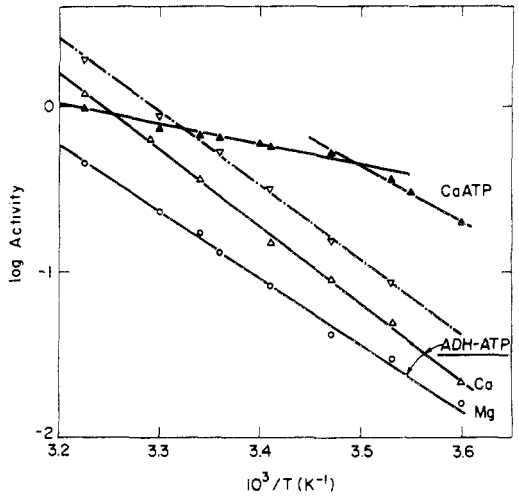

FIGURE 5: Arrhenius plots of log of specific activities of myosin against reciprocal temperatures. ▲, △, and ○ represent respectively CaATPase, CaADH-ATPase, and MgADH-ATPases of native myosin. The dashed line (▽) shows the CaATPase of SH_1-MalNEt modified myosin.

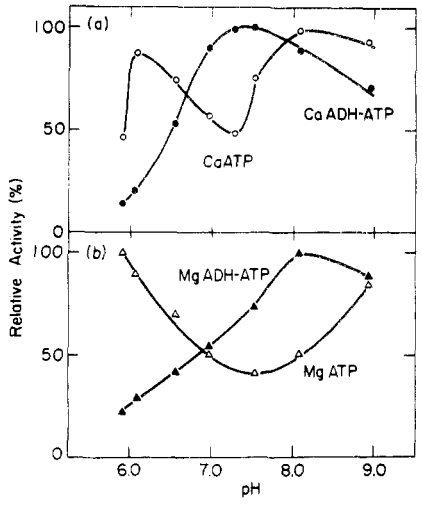

FIGURE 6: Effect of pH on relative ATPase (○, △) and ADH-ATPase activities (●, ▲) of myosin at 25 °C. (a) Ca^{2+}-stimulated and (b) Mg^{2+}-stimulated activities.

their behavior as a function of pH. Figure 6 demonstrates the difference between ADH-ATP and ATP activities of myosin as a function of hydrogen ion concentration. The ADH-ATP pattern in this figure is closely similar to that observed with ATP and SH_1-MalNEt modified myosin (Sekine and Kielley, 1964), and that of myosin-ATP system at low temperatures (Watterson et al., 1975). It is tempting to relate again the changed activity-pH profile of the last three cases with the absence of the rate-limiting complex, M**ADP·P_i, in these systems.

The results discussed so far raise the question of the interaction of the analogue with the actomyosin system. The observation of Lamed et al. (1973), that ATP derivatives of this type did not cause contraction of myofibrils, suggested that their hydrolysis is abortive in the mechanistic sense. We have verified this result by following the shortening of actomyosin threads employing previously described procedures (Harrington et al., 1975). MgADH-ATP was unable to cause shortening of threads, whereas MgATP brought about their rapid shrinkage. Activity assays carried out on actomyosin systems used for preparation of such threads disclosed a high

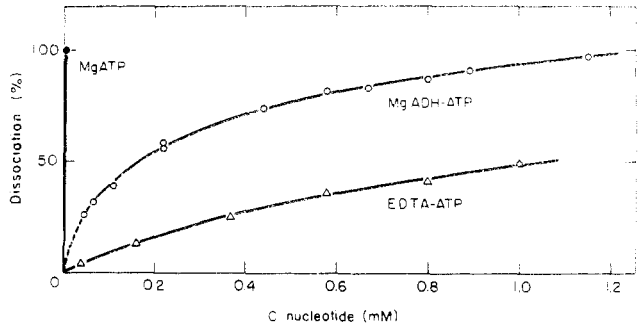

FIGURE 7: Dissociation of acto-HMM complex induced by MgATP (●), MgADH-ATP (○), and EDTA-ATP (△) plotted against their respective concentrations. The dissociation was followed by light scattering as described in Materials and Methods. One hundred percent dissociation refers to full dissociation of acto-HMM into actin and HMM in the presence of 1 mM MgATP.

rate of ADH-ATP hydrolysis, only slightly lower than that of ATP in the same system.

Increasing the ratio of actin to myosin has an inhibitory effect on the rate of cleavage of ADH-ATP. At 1:1 actin/myosin ratios (w/w) the inhibition of ADH-ATPase reaches about 75% of the initial activity value and exceeds 90% at higher actin/myosin ratios. The same behavior was also observed when myosin was replaced by HMM or HMM S-1. To rule out the possibility that actin promotes an irreversible inhibition of myosin by ADH-ATP, we have performed tests similar to those presented in Table II. Maximally inhibited MgADH-ATP actomyosin was supplemented with an excess of MgATP, whereupon rapid liberation of P_i ensued, thus confirming that myosin was not covalently modified. Similar inhibition of myosin ATPase by actin was noted before to occur in the presence of EDTA (Baron et al., 1966) and to a smaller extent in the presence of Ca^{2+} cations (Nanninga, 1964). It seems that in all these cases the inhibition by actin reflects a decreased affinity of the nucleotide for myosin and its consequent displacement by high concentrations of actin. A competition between actin and ATP also prevails in the presence of Mg^{2+} cations; yet in the latter case the binding of nucleotide to myosin is strong enough to dissociate the actomyosin complex, even at high actin/myosin ratios and thus allow for the cyclic interaction of MgATP and actin with myosin and stimulation of the hydrolysis rate. Figure 7 demonstrates that indeed MgADH-ATP and EDTA-ATP have apparently lower affinity for myosin than MgATP. Very low concentrations of MgATP (10 μM) are sufficient to induce full dissociation of acto-HMM, or acto-SH_1-MalNEt-HMM,[2] as detected by a drop in the light scattering of the solution to a value of combined scatterings of the isolated components, HMM and actin. Many-fold higher levels of MgADH-ATP (about 1 mM) or EDTA-ATP are required to achieve the same effect (Figure 7). It appears from the data of Figure 7, which were also reproduced for the acto-subfragment-I system, that at high actin/myosin ratios mM levels of MgADH-ATP or EDTA-ATP may be too small to displace actin. The net result of this is the suppression of hydrolysis of the appropriate nucleotide. It should be stressed, however, that the failure of

[2] At such low ATP concentrations, dissociation of acto-HMM or acto-SH_1-MalNEt-HMM is succeeded by the immediate recombination of actin and HMM following the hydrolysis of ATP. Note that the hydrolysis of MgATP by SH_1-MalNEt myosin is not inhibited by actin. Thus, both with respect to the dissociation of acto-HMM and inhibition of hydrolysis by actin the myosin-ATP analogue system differs from the thiol modified myosin-ATP system.

MgADH-ATP to elicit contraction of actomyosin threads is not due to an unfavorable competition for myosin between actin and the analogue since at equimolar ratios of actin to myosin their complex is almost completely dissociated by 1 mM MgADH-ATP and the hydrolysis of the latter is practically uninhibited. Also, increasing the nucleotide concentration about tenfold does not lead to contraction of the threads.

Discussion

The mechanism of ATP hydrolysis by myosin may be indirectly studied by monitoring the alterations in the enzymatic reaction of myosin following specific modifications of functional residues. In fact, such an approach has led to formulation of a model, which attributes the low rate of ATP cleavage in a resting muscle to the formation of a stable complex structure with the participation of MgATP and the two essential thiol sites (Burke et al., 1973; Reisler et al., 1974a). This model is reminiscent of the "product lock-in" explanation suggested by Werber et al. (1972). Wider use of the modification approach is limited not only for practical and technical reasons but also because of the realization that modifications change the protein and the interpretation of results accordingly requires caution. The conclusions derived from such investigations can be, however, reinforced if supported by experiments in which a modified substrate (analogue) rather than a modified protein has been used. The most frequently used ATP analogues, ATP(γS), AMP-PCP, and AMP-PNP, display either a changed hydrolysis reaction or their cleavage by myosin is totally inhibited. Clearly, the analogues generate different steady-state complexes from that obtained with ATP, a feature of great advantage for structural analysis of intermediate states of actively contracting muscle (Goody et al., 1975). However, because the analogues are different from ATP, they do not provide the final clue to the understanding of the mechanism of ATP hydrolysis by myosin. Obviously, they also cannot probe the chemical events which underlie the mechanochemical cycle.

In the particular case of myosin, the model derived from thiol modification studies suggests that an ATP analogue capable of interacting with but one of the two essential sulfhydryl groups of the protein should be an attractive one to test. From previous work of Lamed et al. (1973), it appeared that ADH-ATP might serve this purpose. As documented in Table I the hydrolysis of ADH-ATP by myosin resembles that of ATP by SH_1-MalNEt or SH_2-MalNEt myosin; Mg^{2+} cations activate the hydrolysis of ADH-ATP, whereas EDTA inhibits it. Blocking of either SH_1 or SH_2 groups of myosin leads to almost complete inhibition of ADH-ATPase (Table I) and, what perhaps is more significant, the analogue does not expose the SH_2 group for modification by thiol reagents (Figures 3 and 4) as does ATP. Since we have verified that, under the experimental conditions employed in this study, the analogue does not covalently label the protein, the above results lead to the conclusion that ADH-ATP interacts with only one essential thiol site at a time. Similar results were also obtained with pimelic and sebacic acid dihydrazide derivatives of ATP.

The proximity of SH_1 and SH_2 groups, of the order of 10 Å (Reisler et al., 1974b), precludes definitive resolution of the identity of the participating thiol site since the interaction of ADH-ATP with SH_1 or SH_2 may be sterically hindered by the label on the other group. That similar hydrolytic behavior could be observed for the ATP-thiol modified myosin system and the analogue-unmodified myosin system lends support to the utility of the modification studies and the conclusions derived from them. The results with ADH-ATP confirm that inhibition of phosphate liberation by Mg^{2+} cations requires interaction of the phosphate nucleotide with the two essential thiol sites of myosin of striated muscle.

The fact that the hydrolysis of MgADH-ATP by myosin is not activated by actin nor does it result in contraction in spite of a relatively high turnover rate is instructive. We correlate it with the inability of the analogue to interact simultaneously with the two essential sulfhydryl sites of myosin. Seemingly, such an interaction is necessary to produce a cyclic conformational change on the myosin head without which contraction cannot occur. Modification studies suggested this might be the case (Harrington et al., 1975), though they could not obviate the possibility that the loss of the contractile function and the activation of MgATPase of myosin through blocking of SH_1 groups may be only casually related. In this sense the results of ADH-ATP add credence to our previous proposal that contraction of striated muscle requires a cyclic opening and closing of a stable complex formed with the participation of MgATP and the two sulfhydryl sites, SH_1 and SH_2.

The stable complex, which we equate with the rate-limiting complex in the hydrolytic cycle of myosin, M**ADP·P_i, may be detected by a number of methods, most conveniently by fluorescence measurements (Werber et al., 1972). Whenever M**ADP·P_i cannot be detected, contraction or activation of myosin MgATPase by actin will probably not follow. Thus, for example, by fluorescence criterion, MalNEt modified HMM does not display the existence of M**ADP·P_i (Werber et al., 1972) and its capacity to hydrolyze ATP is little affected by actin (Silverman et al., 1972). The ATP analogue examined in this work neither supports contraction nor does it enhance the fluorescence of myosin. The lack of the fluorescence effect with MgADH-ATP suggests, in line with the kinetic evidence, that M**ADP·P_i is not formed, or is not predominant. One has to note, however, that detection of the optical properties ascribed to the M**ADP·P_i state should not be equated with the contractile function, though their absence does imply the loss of this function. Optical (Werber et al., 1972) and chemical (Watterson et al., 1975) probes attest that M**ADP·P_i is formed with CaATP as well; yet no contraction follows. Evidently, the CaATP and MgATP complexes with myosin differ structurally in spite of giving rise to similar perturbation of spectral properties of the protein (see also Reisler et al., 1974a).

The characteristics of the M**ADP·P_i state may be extended to explain the pH and the temperature dependence of myosin ATPase and to correlate the transition in their profiles with changes in population of the intermediates of the hydrolytic cycle. Both Mg^{2+} and Ca^{2+} cations induce at room temperature a double maximum pH profile of ATPase activity. Moreover, CaATPase and MgATPase display a biphasic Arrhenius plot with activation energies about 20–30 kJ/mol. When conditions of the enzymatic assay are changed, in a way which depletes the system from the M**ADP·P_i state, the pH dependence of ATPase is transformed into a single maximum profile and the activation energies are significantly increased. Interestingly, such change can be achieved simply by lowering the temperature of the assay to 0 °C (Watterson et al., 1975), which, as known from other studies, decreases the concentration of M**ADP·P_i in the system (Bagshaw and Trentham, 1974; Seidel, 1975; Harrington et al., 1975). A similar shift of the pH profile of ATPases and increase in the activation energy of the reaction (to about 90–100 kJ/mol) can be observed as a result of modification of the SH groups of myosin (Sekine and Kielley, 1964). The Arrhenius plots of the ac-

tivities of the thiol modified myosin also become linear. Hydrolysis of ADH-ATP, which by kinetic, spectral, and chemical evidence does not yield the M**ADP·P_i complex with myosin, falls into the same pattern of temperature and pH dependence (Figures 5 and 6). The high activation energy of the analogue activities of native myosin and ATPase activities of modified myosin can be compared with the large temperature dependence of the product dissociation step (MADP $\rightleftarrows$ M + ADP). Bagshaw (1975) reports for this step an activation energy of about 130 kJ/mol, whereas the rate-limiting isomerization (M**ADP·P_i $\rightleftarrows$ M*ADP·P_i) is according to them practically temperature independent. Thus, the results presented in Figure 5 are consistent with other lines of evidence that M**ADP·P_i is not formed (or is not predominant) with ADH-ATP.

In view of the analogies drawn above, it would be interesting to extend the conformational, kinetic, and contractile studies on the ATP-myosin system into the range of low temperatures, below 0 °C. The attractive possibility in such a study is that one can reach conditions where M**ADP·P_i will be almost completely depleted from the system. The recent work of Seidel (1975) suggests that, though at 0 °C the rate-limiting complex still exists, lowering the temperature should bring us to the desired goal. Under such conditions, of no M**ADP·P_i, one may be able to characterize the other intermediates of the hydrolytic cycle and hopefully to establish, by a different approach, that contraction requires the cyclic formation and disruption of the Mg^{2+} induced M**ADP·P_i conformation.

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