Mechanism of Inhibition of Relaxation by *N*-Ethylmaleimide Treatment of Myosin†

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ABSTRACT: It has remained unexplained why *N*-ethylmaleimide (NEM) treatment of myosin can inhibit relaxation in actomyosin systems from rabbit skeletal muscle which appear to be regulated solely through tropomyosin and troponin. Since rigor complexes between (nucleotide-free) myosin and actin affect the troponin-tropomyosin system, the possibility was explored that, as a result of NEM treatment, some of the myosin maintains rigor complexes with actin in the presence of ATP which might be responsible for inhibition of relaxation. Evidence is presented indicating that such a mechanism might account for the effects of NEM treatment. First, after exhaustive NEM treatment of heavy meromyosin (HMM), acto-HMM complexes were no longer dissociated by ATP. Second, admixture of such NEM-treated, enzymatically inactive HMM or myosin to native regulated actomyosin or acto-HMM inhibited relaxation.

It has been known for a number of years that regulated rabbit actomyosin may lose its ability to relax on removal of calcium if certain free sulfhydryl groups of myosin are blocked, e.g., by *p*-chloromercuribenzoate or by *N*-ethylmaleimide (NEM[1]). This was first suggested by Tonomura's experiments (Tonomura and Yoshimura, 1960) and later demonstrated by Bremel (Weber and Bremel, 1971; Bremel, 1972) and Daniel and Hartshorne (1972). This observation has been puzzling since in vertebrate skeletal muscle regulation by calcium seems to depend on troponin and tropomyosin (Ebashi and Ebashi, 1964; Ebashi and Endo, 1968) which are components of the regulated actin filament and do not combine with myosin (Endo et al., 1966; Spudich et al., 1972; Weber and Bremel, 1971). In the absence of calcium, troponin is thought to hold tropomyosin in a position peripheral to the central groove, in the domain of the myosin binding site, so that tropomyosin sterically interferes with the binding of ATP-activated myosin (Wakayabashi et al., 1975; Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973). On addition of calcium, troponin permits tropomyosin to return to the position near the central groove of the actin filament, away from the myosin binding site. There is no evidence for myosin regulation (cf. Szent Györgyi et al., 1973) in rabbit actomyosin (Bremel and Weber, 1975).

However, myosin can inhibit relaxation by the troponin-tropomyosin system when myosin is combined with actin in rigor complexes (between nucleotide-free myosin and actin) (Bremel and Weber, 1972) presumably because the rigor complexes cause a shift in the tropomyosin position toward the central groove (Huxley, 1972; Haselgrove, 1972). Conceivably NEM treatment may preferably damage the ATP site so that some of the myosin molecules complexed with actin are no longer dissociated by ATP. As a result rigor complexes exist in the presence of saturating ATP concentrations; i.e., ATP resistant rigor complexes occur. We verified the existence of ATP resistant rigor complexes with exhaustively NEM-treated HMM (the double-headed proteolytic myosin fragment) and showed that admixture of so treated, enzymatically inactive myosin or HMM to native, regulated actomyosin or acto-HMM inhibited relaxation of the latter.

Materials and Methods

N-Ethylmaleimide and ATP were purchased from Sigma (St. Louis, Mo.), ^{3}H-labeled NEM and ^{14}C-labeled iodoacetamide were from New England Nuclear, creatine phosphate was from Calbiochem (La Jolla, Calif.), and creatine phosphokinase was from Worthington (N.J.). Contractile and regulatory proteins were prepared as previously described: using modifications (Bremel and Weber, 1975) of the procedures of Portzehl et al. (1950) for myosin, of Straub (1942) as further modified by Tsuboi (1968) for actin, which was purified according to Spudich and Watts (1971), and of Ebashi and Ebashi (1964) for the troponin-tropomyosin complex. Polymerizable actin was removed from the latter by a 2-h centrifugation (100 000*g*, 10 mM imidazole (pH 7.0), 0.1 M KCl, and 2 mM $MgCl_2$). Regulated actin filaments were reconstituted from pure actin and the troponin-tropomyosin complex according to Bremel and Weber (1975), by combining actin with a large excess of redissolved lyophilized troponin-tropomyosin complex (2:1 weight ratio of complex-actin). Heavy meromyosin (HMM) was prepared by brief trypsin digestion of a myosin suspension followed by ammonium sulfate precipitation according to Lowey et al. (1969).

Myosin was treated with NEM by incubating a suspension of myosin (12 mg/ml), in 50 mM Tris buffer, pH 7.6, with 1-2 mM NEM for 30 min at room temperature. (The molarity of NEM was assessed by absorption measurements at 305 nm, using a molar extinction coefficient of 0.62×10^3 (Riordan and Vallee, 1967).) The reaction was terminated by the addition of dithiothreitol to a final concentration of 25 mM. The concentration of KCl was raised to 0.6 M and the pH adjusted to 7.0 with imidazole buffer. HMM (18 μM final concentration) was combined with regulated actin, equimolar to the concentration of heads (i.e., 36 μM), and the solution adjusted to 0.1

† From the Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19174. *Received May 17, 1976.* Supported by the Muscular Dystrophy Association of America and National Institutes of Health Grant HL 15692. S.P. had a fellowship from the Muscular Dystrophy Association of America.

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[1] Abbreviations used: NEM, *N*-ethylmaleimide; HMM, heavy meromyosin, trypsin fragment of myosin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; IAM, [^{14}C]iodoacetamide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

M KCl, 10 mM imidazole, pH 7.0, before incubation with 1.1 mM NEM for 30 min at room temperature. After termination of the reaction by addition of dithiothreitol to a final concentration of 8 mM, the protein was dialyzed overnight against 10 mM imidazole, pH 7.0, and 0.2 mM dithiothreitol. Identically and in parallel we treated one batch of acto-HMM with cold NEM (for ATPase experiments), another with [^{3}H]NEM (for sedimentation measurements) and, for control experiments, one batch of regulated actin (without HMM) with cold NEM and another with [^{3}H]NEM. These control experiments served to determine cofactor activity of actin in the NEM-treated acto-HMM complex and were necessary to estimate radioactivity of [^{3}H]NEM-HMM which remained combined with the protective actin throughout the experiment (see below).

Iodoacetamide-labeled HMM was prepared according to Marston and Weber (1975) by incubating overnight at 4 °C 7 mg/ml HMM with equimolar (per "head") [^{14}C]iodoacetamide in 10 mM imidazole, pH 7.0, followed by dialysis against 0.2 mM dithiothreitol, 10 mM imidazole, pH 7.0, overnight.

Protein concentrations were determined according to Lowry's method (1951), standardized against myosin and actin calibrated by Kjeldahl measurements. The concentration of the troponin–tropomyosin complex was arbitrarily read from the actin curves. The quality of the myosin preparations was evaluated by measurements of the K^+-activated ATP hydrolysis at 24 °C (0.6 M KCl, 15 mM Tris buffer, pH 8.0, 2 mM EDTA, and 5 mM ATP) (cf. Seidel, 1969), using the method of Taussky and Shorr (1952) for the determination of inorganic phosphate. Myosin preparations with a rate of 40–50 s^{-1} (mol of ATP hydrolyzed (mol of myosin)$^{-1}$ s^{-1}) are considered good quality.

For measurement of actin-activated ATPase activity, the various species of myosin, singly or in the desired mixtures, were first combined with actin in 0.6 M KCl before they were diluted into the desired assay system. The concentration of actin was maintained at a constant value in any single experiment and only the concentrations of myosin were varied when so desired. The assay system (2 ml) contained 1 mM $MgCl_2$, 2.5 mM MgATP, 10 mM imidazole, pH 7.0, had a final ionic strength of 0.055 M, and contained in addition, either 1 mM EGTA or CaEGTA. The assays were started by the addition of ATP, incubated for 5 to 10 min at 5 °C, and analyzed for inorganic phosphate after protein precipitation with trichloroacetic acid. ATPase measurements using HMM were modified in the following manner: 1.0 mM MgATP, 1 ml assay volume, 1 min incubation, and native actin and native HMM were added separately to the assay system. When NEM-treated acto-HMM was present in an assay system together with native regulated actin, the two were preincubated together for 1 to 2 h at 4 °C before mixing them with the assay solution. For measurements of ATPase activity as a function of increasing ATP concentrations, creatine phosphate (5 mM) and creatine phosphokinase were added (2 mg/ml), and creatine analysis was performed according to Eggleton et al. (1943), after termination of ATP hydrolysis by *p*-mercuribenzoic acid, and protein precipitation by $ZnSO_4$ (subsequently removed by $Ba(OH)_2$) according to Somogyi (1945). Relaxation was calculated according to the following expression:

$$\% \text{ relaxation} = 100 \times [1 - (\text{ATP hydrolyzed in the absence of Ca})/(\text{ATP hydrolyzed in the presence of Ca})]$$

The fraction of HMM bound to actin was defined as the fraction sedimenting with polymerized actin (during 15 min of centrifugation at 100 000*g* + 10 min of acceleration time, in a swinging bucket rotor over a distance of 8 mm, conditions which permit the sedimentation of all actin filaments) minus the fraction of HMM which sedimented under these conditions in the absence of actin. [^{3}H]NEM-treated HMM was liberated from its complex with actin by incubating overnight with a 50-fold excess of native (unlabeled) HMM. Since the sedimentation experiments in the presence of ATP were meant to measure the extent of dissociation of the HMM–actin complex by ATP, the solutions were kept at 0 °C to preserve ATP. The medium contained 1 mM $MgCl_2$, 10 mM imidazole, pH 7.0, 1 mM EGTA, 0.05 M ionic strength, and 1.2 mM MgATP (when present). ATP was added immediately before centrifugation; 30 min elapsed between ATP addition and the end of sedimentation.

In order to determine how much [^{3}H]NEM-labeled HMM remained in the supernatant, supernatant radioactivity was corrected for that associated with actin depolymerized as a result of NEM treatment. The latter was determined by treating actin alone with [^{3}H]NEM (see above) which was found to destroy the ability of one-third of the actin to sediment at 100 000*g*. The specific radioactivity of [^{3}H]NEM-treated HMM was estimated by subtracting, from the radioactivity in the original [^{3}H]NEM-treated acto-HMM preparation, the actin radioactivity determined in the control experiment without HMM (see above). The accuracy of either subtraction is not crucial to the general outcome of the experiment, because HMM had a sixfold greater specific activity (per mole of protein) than actin.

Results

In the presence of NEM-treated myosin, the actin-activated ATPase activity of native myosin can be identified most easily if the ATPase activity of the NEM-myosin is insignificant. We, therefore, rather extensively treated the myosin with NEM: 1–2 mM concentrations were added for 30 min at room temperature, thereby virtually abolishing actin-activated ATPase activity (Table I, column 6) or reducing it to very low values (Figure 1). Nevertheless, such enzymatically inactive myosin, when added to a mixture of native myosin and regulated actin, considerably inhibited relaxation after removal of calcium. Figure 1 compares the effect of increasing ATP concentrations in the absence of calcium on the actin-activated ATPase activity of *native* myosin before and after the addition of NEM-treated myosin. Regulated actin with *native* myosin *alone* relaxed 61% at 1.0 mM MgATP; on decreasing ATP below 0.1 mM, ATPase activity progressively increased in the absence of calcium and relaxation became less (Figure 1A). Relaxation completely disappeared at 0.01 mM MgATP, at which concentration ATPase activity in the absence of calcium reached a maximum. This suppression of relaxation at low ATP levels is caused by increasing saturation of the actin filaments with rigor complexes (Bremel and Weber, 1975; Bremel et al., 1972). After substituting one-half of the native with NEM-treated myosin, the rate of ATP hydrolysis at 1 mM MgATP was considerably increased in the absence of calcium and relaxation was reduced to 31% (Figure 1B). It can be seen from Figure 1B that the addition of NEM-treated myosin increased the rate of ATP hydrolysis not only in the absence of calcium but also in its presence, as in potentiation described by Bremel and his colleagues (1972).

Increasing the ratio of NEM-treated myosin to native myosin tended to intensify suppression of relaxation and to in-

TABLE I: ATPase Activity as a Function of Increasing Ratios of NEM-Treated Myosin to Native Myosin.[a]

	Native Myosin Concn		Native + NEM-Myosin[b] Ratio (NEM-Myosin/Native)			NEM-Myosin Concn
	0.4 μM	0.2 μM	0.33	1.0	3.0	0.8 μM[c]
+ Ca	1.2	1.3	2.1	2.4	3.5	0
− Ca	0.05	0.04	0.9	1.5	2.8	0
% relaxation	96	97	56	37	20	
+ Ca	1.7	1.6	3.6	4.1	3.6	0
− Ca	0.09	0.14	1.0	1.9	2.5	0
% relaxation	95	91	72	54	30	
+ Ca	1.8	2.2	2.7	6	8.6	0
− Ca	0.14	0.1	1.4	2.9	4.2	0
% relaxation	92	95	48	52	51	

[a] The table shows the effects of NEM treatment on three different batches of myosin. Units are mol of ATP s^{-1} (mol of myosin heads)$^{-1}$. Actin concentration held constant at 0.8 μM. [b] The sum of native + NEM-myosin (NEM-myosin is NEM-treated myosin) was held constant at 0.4 μM. [c] Columns 2-6, ATPase activity is expressed in terms of native myosin "heads", column 7, in terms of NEM-treated myosin "heads".

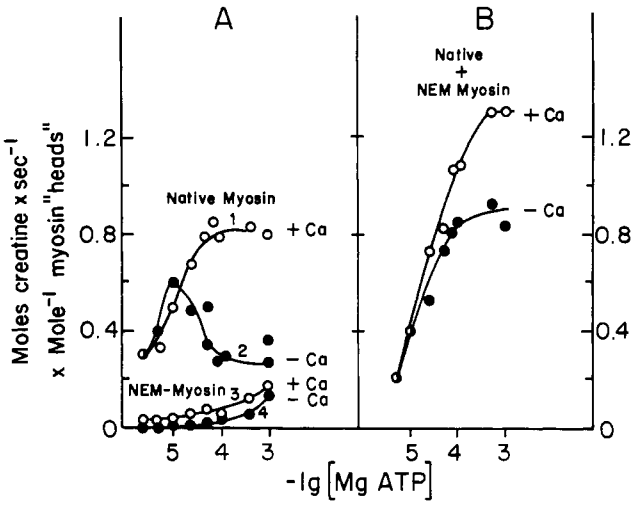

FIGURE 1: The effect of increasing ATP concentrations on actin-activated ATP hydrolysis of native, NEM-treated and a mixture of native and NEM-treated myosin. (A) Curves 1 and 2, native myosin (0.14 μM); curves 3 and 4, NEM-treated myosin (0.07 μM). (B) NEM-treated myosin/native myosin ratio = 1 (0.07 μM each). Actin constant, 0.3 μM (A and B); ionic strength, 0.06 M (for other conditions, see Materials and Methods).

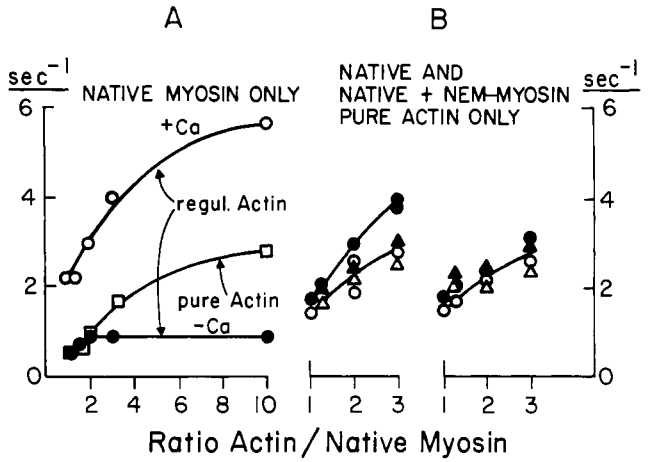

FIGURE 2: The effect of increasing ratios of actin to native myosin on ATP hydrolysis. (Open Symbols) Calcium present; (closed symbols) calcium absent. (A) Only native myosin present, in decreasing concentrations (0.6 to 0.06 μM); pure and regulated actin constant, 1.4 μM. (B) Two different experiments comparing ATP hydrolysis activated by pure actin with and without addition of NEM-treated myosin; native myosin decreasing from 0.6 to 0.15 μM; NEM myosin increasing from 0.15 to 0.45 μM; actin, 1.2 μM. With unregulated actin, either pure or containing inactive troponin, removal of calcium always produces a small stimulation of ATPase activity: (circles) native myosin; (triangles) native and NEM-myosin.

crease ATPase activity in the presence of calcium (Table I, two out of three experiments). Although the same general effect always occurred, quantitative variations between experiments were considerable.

Since in our experiments NEM-treated myosin was added at the expense of native myosin, the ratio of actin to native myosin was increased. Figure 2 and Table I show that such change in ratio by itself, i.e., without the addition of NEM-treated myosin, did not decrease the extent of relaxation. However, often, although not always (compare columns 1 and 2 in Table I with Figure 2; cf. Beinfeld and Martonosi, 1975), an increase in this ratio caused an increase in the rate of ATP hydrolysis during activation by pure actin, and by regulated actin in the presence of calcium. Nevertheless, the experiments of Table I with 50% substitution of native by NEM-treated myosin indicate true potentiation: in the presence of calcium the ATPase activity is significantly higher on substituting NEM-treated myosin than after increasing the ratio of actin to native myosin by withdrawing native myosin. It has not yet been shown why sometimes an increase in the actin concentration beyond equivalence with myosin "heads" leads to an increase in the rate of ATP hydrolysis. We suspect that, in the large disordered aggregates of actin and myosin filaments, the chances for each myosin head to interact with an actin monomer often are improved with an excess of actin filaments. (For a look at such aggregates by electron microscopy, cf. Niederman and Pollard, 1975.)

Figure 2B also shows that in the absence of regulatory proteins the addition of NEM-treated myosin did not increase the actin-activated ATPase activity of native myosin.

After we had shown that NEM-treated myosin "turns on" the regulated actin filaments, we wanted to know whether NEM-treated myosin forms complexes with regulated actin that persist at high ATP concentrations (1.2 mM) even in the absence of calcium. Since it is difficult to demonstrate the existence of such complexes at low ionic strength with the insoluble myosin, we treated the soluble proteolytic fragment HMM with NEM, after protecting the actin binding site with equimolar amounts of regulated actin. HMM was extensively

TABLE II: Inhibition of Relaxation after Addition of NEM-Treated HMM.[a]

NEM-Treated HMM (μM)	Native HMM (μM)	Native Regulated Actin (μM)	ATPase[a] Activity + Ca (s^{-1})[b]	ATPase[a] Activity − Ca (s^{-1})[b]	Relaxation (%)
4.3			0.18	0.18	0
4.3		5.2	0.18	0.18	0
	5.0	5.2	3.3	1.2	64
4.3	5.0	5.2	3.0	2.5	17

[a] Conditions as described in Materials and Methods; assays with NEM-treated HMM contained 3.9 μM NEM-treated actin. NEM-treated actin did not activate ATP hydrolysis by native HMM. [b] Mol of ATP split s^{-1} (mol HMM "heads")$^{-1}$.

modified with 10–12 mol of NEM bound per mol of HMM, and it had lost most of its enzymatic activity (Table II). Control experiments with NEM-treated regulated actin and native HMM suggested that the NEM treatment completely destroyed all enzymatic cofactor activity of the regulated actin. Nevertheless, the addition of this enzymatically inactive complex to a mixture of regulated actin and native HMM inhibited relaxation considerably; ATPase activity of the native HMM in the absence of calcium was doubled (Table II), and relaxation was reduced from 64 to 17%. To allow transfer of the NEM-treated HMM from the protecting NEM-treated actin to *native actin,* we preincubated the NEM-treated complex with native regulated actin for a prolonged period of time. (The half-time for dissociation of the native rigor complex was measured by J. Loscalzo to be about 30 min. We did not measure it for the rigor complex between NEM-treated HMM and actin, but assumed it to be similar.) The addition of NEM-treated HMM did not increase the rate of ATP hydrolysis by native HMM in the presence of calcium. As will be discussed later this difference between the effects of NEM-treated HMM and NEM-myosin may only be apparent, due to the difference in the kinetics between the soluble and the insoluble system.

We measured complex formation between HMM and actin by centrifugation, under conditions that caused complete sedimentation of actin filaments but left nonaggregated HMM in the supernatant (see Materials and Methods). For these experiments our "native" HMM was actually lightly labeled with ^{14}C-labeled iodoacetamide (IAM), a modification that does not interfere with relaxation. The fraction of aggregated "native" HMM could easily be determined by centrifugation without added actin (line 1, Table III). The aggregated [^{3}H]NEM-HMM had to be measured by a different procedure since it was prepared while complexed to actin and remained complexed by over 50% (line 4, Table III). We determined the fraction of [^{3}H]NEM-HMM that sedimented after being displaced from actin (NEM-actin and the same amount of native actin used in the assay for dissociation) by a 50-fold excess of native (*not* IAM labeled) HMM (line 5, Table III). Ninety-one percent of the IAM-labeled and 77% of the NEM-treated HMM were not aggregated (lines 1 and 5, Table III). NEM treatment had reduced the fraction of nonaggregated HMM capable of forming rigor complexes with actin to 75% (81-23/100-23) as compared with the 95% (95-9/100-9) found for IAM-labeled HMM. However, on addition of ATP 66% of these rigor complexes remained intact (61-23/81-23) as compared with only 22% (28-9/95-9) of the rigor complexes containing IAM-labeled HMM. Furthermore, in evaluating this difference one must take into account that the ATP level decreased more rapidly in the control experiment with IAM-labeled HMM because even in the absence of calcium the rate of ATP hydrolysis was much higher than that with NEM-treated HMM (Table II). Probably the rigor complexes with IAM-labeled HMM were formed in the lower part of the tube where the ATP may have been exhausted at the end of the centrifugation. It appears that NEM treatment created a species of HMM—and by implication of myosin—that forms ATP-resistant rigor complexes with actin.

Discussion

A large number of NEM molecules can easily be attached to myosin. Progressive modification with NEM affects ATPase activity in amazingly different ways, which depend upon salt concentration, ionic species, nucleotide content, temperature, etc., during incubation with NEM (Watterson et al., 1975), suggesting considerable variability of the myosin conformation. Inhibition of relaxation, as described by Weber and Bremel (1971) and Bremel (1972), results from a very restricted set of conditions during NEM treatment (Daniel and Hartshorne, 1972, 1973; Weber and Bremel, 1971) and is quite variable (Bremel, 1972).

We were able to reproduce the behavior of such NEM-treated myosin preparations by mixing myosin, which had been enzymatically inactivated by NEM treatment, with native myosin and regulated actin. We obtained similar results with HMM, showing that the fragment behaves similar to myosin also in this respect. In addition, we demonstrated that NEM treatment produced a species of HMM that formed complexes with actin which were not dissociated by ATP. One may describe them as ATP-resistant rigor complexes because they, just as native rigor complexes, seemed to be responsible for the "turning on" of the regulated actin filaments in the absence of calcium.

Potentiation which would also be expected in the presence of rigor complexes occurred with NEM-treated myosin (Table I) but apparently not with NEM-treated HMM (Table II). However, this difference may only have been apparent for the following reason. Under the conditions of the HMM experiment, actin-activated ATPase activity was linearly proportional to the free actin concentration. Since that may have been reduced by as much as 50% by the ATP-resistant rigor complexes, an increase in ATPase activity due to potentiation may have been compensated by the decrease resulting from the reduced concentration of free actin. In the insoluble actomyosin system, the rate of ATP hydrolysis is not dependent on the concentration of free actin but only on the molar ratio of actin to myosin. This ratio was never decreased by the addition of NEM-treated myosin since we always removed an amount of native myosin equal to the added NEM-treated myosin. Therefore, any degree of potentiation could manifest itself as an increase in the rate of ATP hydrolysis.

The species of HMM forming ATP-resistant resistant rigor complexes (or by inference that of myosin) probably had suffered such deformation of the ATP binding site that it either no longer bound ATP, or no longer responded to ATP binding by the appropriate conformational change. The actin binding site, however, must have remained sufficiently intact to permit complex formation at the protein concentrations used in these experiments, implying a differential effect of NEM inactivation on these two sites. Preferential inhibition of myosin-ATP interactions by blocking of free SH groups, permitting continued actomyosin complex formation, has been described

TABLE III: Binding of NEM-Treated HMM to Actin in the Presence of High ATP Concentrations.

Conditions of Assay Subjected to Centrifugation						
HMM			Regulated Actin			
"Native",[a] IAM Treated (μM)	Native (μM)	NEM Treated (μM)	NEM Treated (μM)	Native (μM)	MgATP (μM)	HMM Sedimented (%)
						IAM Treated
1.3					1.2	9
1.3				14	0	95
1.3				14	1.2	28
						NEM Treated
		0.46	0.42		0	57
	20	0.40	0.37	5.3	0	23
		0.46	0.42	6.7	0	81
		0.46	0.42	6.7	1.2	61

[a] IAM-treated means labeled with [^{14}C]iodoacetamide; conditions as described under Materials and Methods.

many years ago (Bárány and Bárány, 1959). The attacks on the different sites are not sharply separated as indicated by our experiment with HMM: while a fraction of the protein still retained its ability to be dissociated by ATP, another had lost all function of the actin binding site so that it no longer formed rigor complexes (Table III). This, together with the ease by which conformational interconversions of myosin occur (Watterson et al., 1975), may explain the variability in the results of NEM treatment.

It should be pointed out that myosin or myosin fragments capable of forming ATP-resistant rigor complexes most likely also may be generated spontaneously in any in vitro preparation. (Preliminary experiments by J. M. Murray (personal communication) suggest that S-1 preparations often contain a small contamination of such modified S-1.) The appearance of ATP-resistant rigor complexes could significantly affect the behavior of actomyosin with respect to tension development, potentiation, calcium sensitivity, etc., and therefore this possibility should be kept in mind during the interpretation of in vitro experiments.

The experiments described here offer a plausible explanation for the effects of restricted NEM treatment of myosin on its interaction with regulated actin as described by Daniel and Hartshorne (1972) and Weber and Bremel (1971). Relaxation on removal of calcium is inhibited, and in the presence of calcium ATP hydrolysis is activated above that of native actomyosin, as in potentiation. In other words, the effects are very similar to those obtained by us by mixing enzymatically inactive myosin capable of forming ATP resistant rigor complexes with native myosin. We suggest that during *restricted* NEM treatment only a fraction of the myosin becomes enzymatically inactive and capable of forming ATP-resistant rigor complexes whereas a large part retains its ability to interact normally with regulated actin. Relaxation of the latter is lost because actin is turned on in the absence of calcium by the ATP-resistant rigor complexes of the inactivated fraction. An admixture of rigor complexes to "native" myosin is also suggested by a shift in activating calcium levels to lower concentrations (Weber and Bremel, 1971; Bremel, 1972) since Bremel and Weber (1972; Weber, 1975) showed that rigor complexes increase the apparent affinity of calcium for troponin. While we cannot prove that this explanation applies, it seems to be plausible, and it shows that one need not invoke myosin regulation in rabbit myosin in order to understand inhibition of relaxation subsequent to modification of the myosin molecule.

Acknowledgment

We thank M. K. Knox for the excellent execution of the HMM experiment.

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