N-ETHYLMALEIMIDE-MODIFIED HEAVY MEROMYOSIN

A Probe for Actomyosin Interactions

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ABSTRACT

Treatment of rabbit skeletal muscle heavy meromyosin (HMM) with the sulfhydryl reagent N-ethylmaleimide (NEM) produces a species of HMM which remains tightly bound to actin in the presence of MgATP. NEM-HMM forms characteristic “arrowhead” complexes with actin which persist despite rinses with MgATP. NEM-HMM inhibits the actin activation of native HMM-ATPase activity, the superprecipitation of actomyosin, the contraction of glycerinated muscle myofibrils, and the contraction of cytoplasmic strands of the soil amoeba Chaos carolinensis. However, NEM-HMM does not interfere with in vitro microtubule polymerization or beating of demembranated cilia.

KEY WORDS: actomyosin, N-ethylmaleimide, cell motility, heavy meromyosin

Microfilament systems are ubiquitous components of eukaryotic cells, serving as cytoskeletal elements and functioning in cellular processes such as cell movements, cytoplasmic streaming, and morphogenetic shape changes (4, 16, 25). The major molecular components of these systems are actin, which is usually organized in 5- to 7-nm filaments; myosin, an ATPase which can generate shear forces by interacting with actin; and control proteins capable of regulating the functioning of actin and myosin. The interactions of these components are best understood in the highly ordered structures found in muscle (11). Actomyosin systems of nonmuscle cells contain similar protein components, but the organization is less ordered, less stable, and varies greatly among cell types (4, 25). Moreover, it is unclear how the arrangement of these proteins contributes to cell motility.

Nonmuscle cells also contain other elements potentially capable of serving cytoskeletal and contractile functions. Microtubules have been shown to play a structural role in many cells, and can interact with ATPase crossbridges, i.e., dynein, to generate shear forces (31). Filamentous structures such as myonemes and spasmonemes found in ciliates can undergo calcium-induced contractions (1). Complex motile events such as nerve growth and axonal transport, cell shape changes, and chromosome movement during mitosis may involve the interaction of two or more of these systems.

The proteolytic subfragment of myosin, heavy meromyosin (HMM),1 has proven to be a useful probe in the study of microfilament-based systems (10, 12, 22). HMM binds tightly to myosin-binding sites of actin filaments and releases in the presence of MgATP. Formation of characteristic arrowhead complexes between skeletal muscle HMM and actins from cell types throughout the plant and animal kingdoms has allowed the study of actomyosin interactions in nonmuscle cells.

1 Abbreviations used in this paper: BSA, bovine serum albumin; DTT, dithiothreitol; HMM, heavy meromyosin; IAM, iodoacetamide; NEM, N-ethylmaleimide; NEM-HMM, N-ethylmaleimide-treated heavy meromyosin; PIPES, piperazine-N,N'-bis(2-ethane sulfonic acid).
animal kingdoms has provided unequivocal identification of actin filaments (10, 12, 22). HMM decoration has also yielded information about the organization of microfilament systems, specifically the periodicity of binding sites and polarity of the filaments.

HMM modified to prevent its dissociation from actin in the presence of MgATP would be expected to make myosin-binding sites unavailable to native myosin, and consequently to block force generation in actomyosin systems. Such a modified HMM would serve as a probe to determine the dependence of a given cellular process on actomyosin interactions. It would also be useful in studying the involvement of actomyosin in complex motility processes where more than one contractile system may be involved. We report here that chemical modification of HMM using the sulfhydryl reagent N-ethylmaleimide (NEM) produces a population of HMM which binds actin, does not release with MgATP, and can block actomyosin interactions in vitro and in cell models.

The differential sensitivity of myosin's actin-binding and ATPase activities to sulfhydryl reagents has been previously studied (2, 9, 13, 23, 26, 27). Ishiura et al. (13) have shown that p-chloromercuribenzoate-treated myosin fragment S, does block actin superprecipitation, but this modified protein also inhibits nonactin related processes such as in vitro microtubule polymerization. In this report we study some properties of NEM-treated HMM (NEM-HMM) and determine its suitability as a specific inhibitor of actomyosin-dependent phenomena.

MATERIALS AND METHODS

Preparation of Proteins

Myosin was extracted from back and leg muscles of New Zealand white rabbits according to Kielley and Bradley (15) and actin from an acetone powder according to Spudich and Watt (30). HMM was prepared from myosin by the method of Lowey and Cohen (17). For binding studies, HMM at 7-10 mg/ml was reacted with 1 [1-14CIodoacetamide (IAM)/HMM "head" overnight at 4°C in 10 mM imidazole-Cl, pH 7.0 (20). Excess label was removed by dialysis against 0.2 mM dithiothreitol (DTT) in 10 mM imidazole-Cl, pH 7.0. NEM-HMM was produced by incubating HMM at 5-10 mg/ml in 10 mM imidazole-Cl and 0.2 mM DTT, with 1.0 mM freshly dissolved NEM at pH 7.0 and room temperature. The reaction was stopped by adding DTT to a final concentration of 10 mM, and unreacted NEM was removed by dialysis.

Hog neurotubulin, prepared as described previously (29), was resuspended in 100 mM piperazine-N,N'-bis(2-ethane sulfonic acid) (PIPES), pH 6.94, 1 mM MgSO4, and 1 mM GTP, warmed slowly to 37°C, and assembly of microtubules was monitored by following changes in turbidity at 350 nm. For these studies, 1.5 mg/ml tubulin and either 1.0 mg/ml NEM-HMM, 1.0 mg/ml NEM-labeled bovine serum albumin (BSA), or 1.0 mg/ml BSA were used.

Protein concentrations were determined spectrophotometrically using E280 of 5.4 for myosin, 6.0 for HMM, and 10.9 for actin (19, 39), or by the method of Lowry et al. (18). Glycerinated muscle myofibrils were a gift from Dr. Roger Cooke prepared according to Ellinger et al. (8).

ATPase Assays

All ATPase assays were run at 25°C and started by addition of Na2ATP to a final concentration of 1.0 mM. Assays for determining K+-EDTA ATPase activity contained 0.6 M KCl, 5 mM EDTA, and 10 mM imidazole-Cl, pH 7.2, and for determining calcium-activated activity contained 0.6 M KCl, 5 mM CaCl2, and 10 mM imidazole-Cl, pH 7.2. Assays for actin-activated ATPase activity were run in a solution of 4 mM MgCl2 and 10 mM imidazole-Cl, pH 7.0. Inorganic phosphate was determined by the procedure described by Pollard and Korn (24).

In competition studies the rate of ATP hydrolysis by HMM with actin minus the rate with HMM alone is taken as 100% activation, and a correction is made for the activity of NEM-HMM when present. Because of the low rate of ATP hydrolysis by actin-activated NEM-HMM, this correction is not crucial to the results.

Binding Assay

Approx. 5 x 10−9 mol of HMM, labeled with 1 IAM/molecule, were mixed with a 10-fold molar excess of pure actin in a volume of 1.0 ml, held for 10 min at room temperature, and then chilled on ice. The solution contained 0.1 M KCl, 4 mM MgCl2, and 10 mM imidazole-Cl, pH 7.0. Na2ATP was added to a final concentration of 2.0 mM immediately before sedimenting the actin at 100,000 g for 15 min at 2°C. Under these conditions over 95% of the actin pellets, as measured spectrophotometrically. The amount of labeled HMM sedimenting with actin was determined by liquid scintillation counting of aliquots of the initial solutions and final supernates solubilized in NCS Tissue Solubilizer (New England Nuclear, Boston, Mass.).

Electron Microscopy

Proteins were negatively stained with 2% uranyl acetate on 300-mesh copper grids coated with a carbon film over a holey cellulose acetobutyrate membrane. Grids were viewed in a Siemens 1A electron microscope at an accelerating voltage of 80 kV.
Superprecipitation

Actomyosin was prepared by mixing purified actin and myosin at high ionic strength (0.6 M KCl) and stirring for 3 min at room temperature before dilution. The final solution contained $2.4 \times 10^{-6}$ M actin, $4 \times 10^{-7}$ M myosin, 0.115 M KCl, 5 mM MgCl$_2$, $10^{-3}$ M CaCl$_2$, 10 mMimidazole-Cl, and 0.02% sodium azide at pH 7.0. Superprecipitation was initiated by addition of Na$^+$ATP to a final concentration of 0.1 mM, and monitored at 350 nm with a Beckman Acta III spectrophotometer (Beckman Instruments, Inc., Cedar Grove, N. J.) equipped with a cuvette stirrer.

Preparation of Cell Models

Actively moving Chaos were incubated for 5 min in the low-calcium stabilization buffer of Taylor et al. (33) and induced to effuse their endoplasm by rupturing the plasmalemma with negative hydrostatic pressure applied through a glass microneedle. Stretched cytoplasm contracted on exposure to Taylor's contraction buffer containing $10^{-3}$ M free calcium which was gently perfused into the chamber.

Bracken fern (Pteridium aquilinum) spermatozoids have 32 cilia clustered at one end of the cell, and the cilia will continue beating normally for hours after the cells are stuck to polylysine-coated glass slides at their proximal end. Cells were demembranated and reactivated as described by Wolniak and Cande (36).

RESULTS

Properties of NEM-HMM

ACTIN-BINDING AND ATPASE ACTIVITIES: Results in Table I demonstrate that treatment with the sulfhydryl reagent NEM has little effect on the ability of HMM to bind actin filaments, yet largely abolishes the ability to dissociate in the presence of MgATP. $[^{14}C]$iodoacetamide-labeled HMM was mixed with actin, and the percentage of total counts in the pellet after the actin had been sedimented at 100,000 g was measured. After a 45-min NEM treatment, the percentage of labeled HMM bound to actin in the presence of 2 mM ATP increased from $11 \pm 6$% to $66 \pm 9$% (Table I).

Table I

|                | % of counts in 100,000 g pellet* |
|----------------|---------------------------------|
| HMM + actin    | 85 ± 4                          |
| NEM-HMM + actin| 79 ± 5                          |

* Experiments used HMM or NEM-HMM preparations as described in Materials and Methods, with ~1.0 $[^{14}C]iam$ molecule/HMM.

DECORATION OF ACTIN FILAMENTS: Negatively stained actin filaments previously exposed to NEM-HMM (Fig. 2b) display the same decoration pattern as filaments exposed to untreated HMM (Fig. 2a). Arrowheads along a single microfilament are unidirectional and exhibit a measured periodicity of ~35 nm, reflecting the polarity and axial repeat distance of the underlying actin filament as do native HMM arrowheads.

Retention of the ability to form apparently normal arrowhead complexes after NEM treatment indicates that disruption of the ATPase activity of HMM can be accomplished with little or no effect on actin-binding. Decoration by untreated HMM is completely reversible by MgATP (Fig. 2c); only bare actin filaments remain. However, filaments exposed to NEM-HMM remain decorated after extensive rinsing with 1.0 mM MgATP (Fig. 2d). Such filaments display periodic interruption of the decoration pattern perhaps because of that portion of the NEM-HMM population which still responds to ATP (Table I). The persistence of arrowhead-like complexes in the presence of ATP confirms the ability of NEM-HMM to form ATP resistant complexes with actin filaments.

NEM-HMM INHIBITS THE ACTIN-ACTIVATION OF UNTREATED HMM ATPASE ACTIVITY: In the presence of NEM-HMM the ability of actin to increase untreated HMM's MgATPase activity is inhibited (Fig. 3). 50% inhibition occurs at an NEM-HMM:actin molar ratio of ~0.7. Because binding studies indicate that ~66% of the NEM-treated HMM population forms ATP-resistant complexes with actin (Table I), there are probably between 0.4 and 0.5 NEM-HMM molecules bound/actin at 50% inhibition.
FIGURE 1  Effect of NEM treatment on ATPase activities of HMM. Assays run at 25°C as described in Materials and Methods. Control rates for actin-activated (O), K⁺-EDTA (●), and calcium ATPase activities (△), were 2.4, 4.0, and 2.4 ATP head⁻¹ s⁻¹, respectively. HMM treated with 1.0 mM NEM at pH 7.0 and room temperature.

FIGURE 2  Negatively stained actin filaments decorated with 0.2 mg/ml HMM (2a) or NEM-HMM (2b). HMM (2c) and NEM-HMM (2d) decorated filaments after rinsing with 1.0 mM MgATP. Note persistent decoration of ATP-washed filaments treated with NEM-HMM (arrows). × 100,000.

NEM-HMM INHIBITS CONTRACTION OF GLYCERINATED SKELETAL MUSCLE MYOFIBRILS: Upon addition of MgATP, glycerinated myofibrils in a calcium-containing buffer actively shorten to a fraction of their relaxed length (Fig. 4a and b). A 5- to 10-min pre-incubation with 3-mg/ml NEM-HMM blocks this contraction, and the elements remain linear and sarcomeres shorten <30% as far as buffer controls (Fig. 4c). Myofibrils which have been exposed to NEM-HMM remain inhibited after being centrifuged out of the NEM-HMM solution and resuspended in buffer before addition of MgATP. Neither untreated HMM, heat-denatured NEM-HMM (60°C, 10 min), nor NEM-treated BSA affects contraction (Fig. 4d).

NEM-HMM INHIBITS SUPERPRECIPITATION: NEM-HMM blocks superprecipitation of rabbit muscle actomyosin (Fig. 5). Inhibition cannot be a result of ATP depletion by added NEM-HMM because untreated HMM has no effect despite its far greater ATPase activity. Nor can inhibition be attributed to nonspecific adsorption of denatured protein, because neither heat-denatured NEM-HMM (60°C, 10 min), BSA, nor NEM-treated...
FIGURE 3  Effect of NEM-HMM on the activation of native HMM-ATPase activity by actin. Conditions as described in Materials and Methods. 100% activation defined as rate of ATP hydrolysis by 5 x 10^{-7} M HMM in the presence of 2 x 10^{-4} M actin minus the rate for HMM alone (0.78 and 0.12 ATP head's^{-1} s^{-1}, respectively). Correction is made for the activity of added NEM-HMM (see text).

BSA inhibits superprecipitation under these conditions. 50% inhibition is generally seen with approx. one NEM-HMM molecule for every three actin monomers, and we have been able to detect inhibition at ratios as low as one NEM-HMM per eight actins.

FIGURE 4  Phase-contrast micrographs of rabbit skeletal muscle myofibrils (4a) before and (4b-d) after exposure to 0.1 mM MgATP. (4c) Myofibrils at 0.1–0.2 mg/ml incubated 5 min in 3 mg/ml NEM-HMM contract only partially with MgATP, while those incubated with native HMM (4d) contract as fully as do fibrils incubated in buffer alone (4b). X 800.

FIGURE 5  Effect of NEM-HMM on the superprecipitation of muscle actomyosin. Conditions as described in Materials and Methods. Results indicate total change in absorbance after addition of MgATP. Actin = 2.4 x 10^{-10} M; myosin = 4 x 10^{-7} M.
NEM-HMM INHIBITS CONTRACTILITY OF CHAOS CAROLINENSIS CYTOPLASM: When the plasmalemma of the soil amoeba Chaos is ruptured in a low-calcium buffer (10⁻² M free calcium), cell contents rapidly effuse to form a naked droplet of cytoplasm. On exposure to micromolar levels of free calcium, this cytoplasm undergoes a vigorous and rapid contraction which is evident when the cytoplasm has been previously stretched with a microneedle (33). A 5- to 10-min pre-incubation with NEM-HMM inhibits this contraction (Fig. 6). Contraction is not inhibited by untreated HMM. Inhibition is not caused by unreacted NEM contaminating the NEM-HMM because dialysate from the NEM-HMM preparation does not prevent contraction.

NEM-HMM DOES NOT INHIBIT IN VITRO MICROTUBULE POLYMERIZATION OR CILIARY BEAT: The rate and extent of tubulin polymerization are not affected by inclusion of NEM-HMM, BSA, or NEM-treated BSA in the polymerization medium (Fig. 7). Demembranated spermatozoid cilia beat at a frequency approaching 8 Hz in the presence of 1 mM ATP and 4 mM MgSO₄ (36). Addition of 2 mg/ml NEM-HMM to the medium does not alter the beat frequency of reactivated cilia.

DISCUSSION

Properties of NEM-HMM

In the absence of nucleotides, HMM binds actin tightly in “rigor” complexes (3). In the presence of MgATP, HMM undergoes cyclic binding to and release from the actin filament (10, 12). Using a modification of the procedure reported by Pemrick and Weber (23), we show that HMM treated with the sulphydryl reagent NEM cannot complete this cycle and remains bound to actin in the presence of MgATP (Table I). In addition, we find that the modified HMM retains its actin-binding activity even when NEM treatment is carried out in the absence of actin, indicating that the groups modified by NEM are not essential for acto-HMM complex formation but are required for MgATP-induced dissociation of the complex. Under our conditions, NEM treatment of HMM produces a mixed population of molecules, most of which bind actin in MgATP-insensitive complexes. A small portion (13%) of the population still responds to MgATP while another fraction (21%) no longer binds actin at all (Table I).

Other workers have demonstrated the existence of two cysteine sulphydryls (SH₁ and SH₂) in the ATPase sites of myosin which are essential for enzymatic activity, react with NEM, and are probably involved directly in nucleotide binding (2, 15, 26, 27). We find that NEM treatment increases HMM’s calcium-activated ATPase activity and inhibits its actin and EDTA-activated activities (Fig. 1). We do not know how extensively our procedure modifies HMM, or whether modification is restricted to sulphydryl groups alone. However, the pattern of ATPase activities we observe is consistent with the pattern reported in the literature for HMM modified at the SH₁ site (2, 15, 26–28).

NEM-HMM produces a decoration pattern on negatively stained actin filaments that is indistinguishable from that of untreated HMM (Fig. 2), implying that NEM treatment has effected little or no change in the nature of the HMM:actin bond. Persistence of NEM-HMM decoration after extensive rinsing with MgATP confirms the existence of ATP-insensitive complexes similar to rigor complexes seen after native HMM decoration in the absence of MgATP. Interruption of the NEM-HMM arrowhead pattern after MgATP rinsing is consistent with the interpretation that there is a small fraction of the NEM-HMM population which still responds to MgATP (Table I).

We find that NEM-HMM prevents actin from activating native HMM-ATPase activity with ~1:1 stoichiometry (Fig. 3). This result suggests that NEM-HMM binds to actin with an affinity sufficiently high to exclude ATP-loaded myosin “heads” from the actin filament. Silverman et al. have reported an increase in the apparent affinity of HMM for actin in the presence of ATP after NEM treatment (28). Pemrick and Weber demonstrated that HMM treated with NEM in the presence of actin could bind to untreated, regulated actin filaments in very tight “ATP-insensitive rigor-like” bonds (23). It seems likely, then, that NEM-treated HMM remains locked in its high-affinity rigor state even in the presence of MgATP.

Model Actomyosin Systems

NEM-HMM inhibits two model actomyosin systems which differ greatly in the organization of their component proteins, i.e., superprecipitation and glycerinated muscle myofibril contraction (Figs. 4 and 5). NEM-HMM also inhibits contractility of cytoplasm of the amoeba C. carolinensis (Fig. 6), which is thought to depend upon acto-
FIGURE 6  Naked cytoplasm from *C. carolinensis* stretched with a microneedle and pre-incubated 5 min in 3 mg/ml untreated HMM (6a) contracts on exposure to 10^{-6} M free calcium (6b) as does cytoplasm in buffer alone (6c). Cytoplasm pre-incubated in 3 mg/ml NEM-HMM for 5 min (6d) fails to contract on exposure to calcium (6e). × 100.
myosin but in which the organization of contractile elements is not understood (33).

Superprecipitation constitutes a well-studied but imperfectly understood in vitro analogue to muscle contraction in which actin and myosin in suspension "contract" with MgATP into large, insoluble aggregates (7). We believe that inhibition of superprecipitation by NEM-HMM results from its ability to form high-affinity, ATP-insensitive complexes with actin which physically exclude myosin heads from binding sites on the actin filament. Inhibition is not attributable to depletion of MgATP by NEM-HMM because native HMM, which possesses an ATPase activity over 10 times that of NEM-HMM, does not prevent superprecipitation.

Arrangement of filaments in the muscle sarcomere is more highly ordered than in the superprecipitation assay (10). Nevertheless, a 10-min preincubation with NEM-HMM inhibits myofibril contraction (Fig. 4). Partial contraction does occur after exposure to NEM-HMM but is typically <30% of the length change observed with controls.

Incomplete inhibition may be caused by the small portion of the NEM-HMM population which still responds to MgATP or by incomplete penetration by NEM-HMM during the 10-min incubation period. The amount of rhodamine-labeled HMM seen in the I bands of these myofibrils increases for up to 1 h during incubation, suggesting some diffusion barrier for molecules the size of HMM (Meesen and Cande, unpublished data). Alternatively, actin in the overlap region between myosin and actin filaments could be protected from NEM-HMM by natural myosin rigor bonds during incubation. We do not know whether NEM-HMM prevents myofibril contraction solely by preventing binding of myosin to actin, by forming a steric block to sliding of the closely packed filaments, or by some combination of the two.

NEM-HMM may inhibit Chaos contractility in a manner similar to its effect on myofibril contraction, i.e., NEM-HMM may block native actomyosin interactions. However, it is possible that NEM-HMM also affects complex systems similar to Chaos cytoplasm by promoting polymerization of cytoplasmic actin or by perturbing the association of actin with other proteins. Like HMM, NEM-HMM would be expected to induce actin polymerization under conditions which favor the monomeric state (5, 37). However, ionic conditions in the cytoplasm of Chaos and in the isolation medium greatly favor the polymerized state, making it unlikely that NEM-HMM inhibits contractility by further promoting actin polymerization. A number of actin-binding proteins, some from amoebae, have recently been described which are involved in the formation of actin-based "gels" (14, 21, 32, 34, 35). At least one such protein has been shown to be displaced from the actin filament by the binding of HMM (35). Another, Filamin, may compete with myosin for sites on the actin filament because it inhibits actomyosin ATPase activity (6). Not enough is known about these "gelation factors" to determine whether a disruption of their association with actin by NEM-HMM could account for the observed inhibition of cytoplasmic contractility.

**Suitability as a Probe for Actomyosin-Based Systems in Nonmuscle Cells**

We believe that NEM-HMM provides a unique tool for studying the involvement of actomyosin in nonmuscle cell motility. By physically occupying sites on the actin filament, NEM-HMM prevents the association between actin and myosin that is necessary for force production. Inhibition does not appear to be affected by the organization of the actomyosin system being studied because poorly organized systems such as dissolved actomyosin, highly structured systems such as muscle, and systems of unknown organization such as Chaos cytoplasm are all blocked by NEM-HMM. Because NEM treatment seems to have little or no effect on HMM's ability to bind actin, NEM-HMM can be expected to display the same broad applicability to various cell types as does native
HMM (12). The possibility that NEM-HMM inhibits a given process by depleting ATP pools is unlikely because of its very low physiological ATPase activity, and can be controlled for with untreated HMM.

Unlike the p-chloromercuribenzoate-treated myosin fragment S, reported by Ishiura et al. (13), NEM-HMM does not inhibit polymerization of tubulin (Fig. 7). Nor does it block the beating of demembranated cilia of P. aquillum spermatzooids reactivated in a MgATP solution. Therefore, NEM-HMM should permit identification of those cellular motile events which rely upon actomyosin systems for their driving force, as opposed to those driven by tubulin polymerization-depolymerization or microtubule-dynein interactions.

We believe that NEM-HMM constitutes a powerful probe for the involvement of actomyosin force-generating systems in nonmuscle cells. We are currently applying it to the study of such complex phenomena as chromosome movements, cytokinesis, and cytoplasmic streaming.

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