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# The site and stoichiometry of the *N*-phenylmaleimide reaction with myosin when weakly-binding crossbridges are formed in skinned rabbit psoas fibers

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**Abstract**

Treatment of relaxed skinned rabbit psoas muscle fibers with 0.1 mM *N*-phenylmaleimide (NPM) for 1 h locks all of the crossbridges in a weakly-binding state resembling that of the myosin·ATP crossbridge. Under these conditions, NPM reacts mainly with myosin heavy chain (Barnett et al. (1992) Biophys. J. 61, 358–367). Here the specific sites for that reaction are explored. Small bundles of rabbit psoas muscle fibers were treated with Triton X-100 to make the fiber sarcolemmas permeable. The bundles were treated with 0.1 mM [$^{14}$C]NPM for 1 h, and homogenized for SDS-PAGE. 43 ± 2.2% of the muscle fiber protein ran in the myosin heavy chain band, the same as for untreated fibers. An alkylating stoichiometry of 2.2 ± 0.33 moles NPM per mole myosin heavy chain was determined. Exhaustive trypsin digestion followed by two-dimensional thin-layer chromatography and reverse-phase HPLC revealed two major sites on myosin heavy chain for NPM binding. The sites contained about the same amount of linked NPM, suggesting that the reaction stoichiometry of each site under the conditions studied is approx. 1 mol NPM/mol myosin heavy chain. Comparison of the labeled tryptic peptides with NPM-reacted synthetic SH1 and SH2 tryptic peptides and analysis of the treated fiber bundles' ATPase activity suggested that the sites for NPM reaction on myosin heavy chain when it locks crossbridges in a weakly-binding state are Cys-697 (SH2) and Cys-707 (SH1).

*Keywords:* Muscle; Alkylation; Essential sulfhydryl; Myosin heavy chain

## 1. Introduction

Vertebrate skeletal muscle is composed of several proteins that work together as a functional unit, myosin being one of the major proteins. The myosin molecule consists of a helical coiled region from which two globular 'heads' emerge. Each myosin molecule is composed of two heavy chains and two pairs of light chains [1]. Myosin heavy chain has a molecular mass of ~ 220 kDa and represents ~ 43% of muscle fiber protein [2].

Myosin heavy chain contains 42 cysteine residues. The two most reactive of these, SH1 and SH2, are located near the carboxy-terminal end in the head region. In solution, these sulfhydryls can be modified by alkylating agents such as *para*-phenylenedimaleimide (pPDM) or *N*-phenylmaleimide (NPM) [3,4]. This modification locks the myosin in a weakly-binding state resembling the normal myosin·ATP state.

The effect of pPDM and NPM in muscle fibers is similar to their effect upon myosin in solution. Treatment of muscle fibers with these compounds locks the crossbridges in a state that resembles that of the normal myosin · ATP crossbridge of relaxed fibers. Although analogy with the solution studies makes it likely that the effect of pPDM and NPM on muscle fibers is due to binding to the

Abbreviations: NPM, *N*-phenylmaleimide; pPDM, *para*-phenylenedimaleimide; SH1, myosin's most reactive sulfhydryl, Cys-707; SH2, myosin's second most reactive sulfhydryl, Cys-697; MHC, myosin heavy chain; SDS, sodium dodecylsulfate; PA, polyacrylamide; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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SH1 and SH2 sulfhydryls of myosin heavy chain, no direct evidence supporting this is available. Previously we showed [5] that the main site of pPDM and NPM-reactivity with muscle fiber treatment is myosin heavy chain. Here, we show that under conditions identical to those used in our previous study, conditions where 100% of a treated fiber's crossbridges are locked in the weakly-binding state, NPM alkylates with a stoichiometry of approximately 1 mol/mol to SH1 and 1 mol/mol to SH2.

## 2. Methods

### 2.1. Treatment of fiber bundles to make the sarcolemmas permeable

Before the fibers within a muscle fiber bundle can be reacted with pPDM or NPM, the plasma membranes surrounding the individual muscle fibers must be made permeable. The protocol that makes the sarcolemmas of single muscle fibers permeable [6] was not totally adequate for fiber bundles [7]. A more rigorous procedure was used, based upon the method of Magid and Reedy [8].

Strips of rabbit psoas muscle tissue ($80 \times (4–6$ mm)) were removed from 2–3 kg New Zealand rabbits and incubated in skinning solution [6] for 30 min. Muscle bundles ($12 \times (0.2–0.5$ mm)) were dissected from the strips and fixed between the arms of stainless steel staples using cyanoacrylate glue. Each small fiber bundle was placed in a 1.5 ml Eppendorf tube and incubated, with agitation, ~ 18 h at 4°C in skinning solution containing 0.5% (vol/vol) Triton X-100.

### 2.2. Treatment of fiber bundles with [$^{14}$C]NPM

After the sarcolemmas were made permeable, the skinning solution was removed and the fiber bundles were incubated ($2 \times$) for 15 min in Triton-free, dithiothreitol-free relaxing solution. [$^{14}$C]NPM (10 mM in dimethylformamide, stored for less than 30 days) was added at the desired concentration directly to the dithiothreitol-free relaxing solution. The contents of the relaxing solution, shown in Table 1, and the treatment duration for labeling the fibers, are identical to those used in our previous study [5].

Table 1
Millimolar composition of relaxing solution

| | |
|---|---|
| KCl | 125 |
| EGTA | 4 |
| $MgCl_2$ | 1 |
| MgATP | 4 |
| Imidazole | 10 |

pH 7.0 ± 0.1 5°C.

### 2.3. Homogenization of fiber bundles

Treated bundles were removed from the staples and homogenized in 0.2 ml homogenization buffer (2 mM EDTA in 20 mM Tris (pH 8)), using a micro tissue grinder (Wheaton, Millville, NJ) in an ice-water bath. After four or five specimens had been ground in the 0.2 ml, enough homogenization buffer was added to bring the protein concentration to 2 mg/ml as determined by the Lowry assay [9].

### 2.4. Quantitative SDS-PAGE

Fiber homogenates (0.5–1.5 μg) and myosin heavy chain (0.25 to 0.75 μg, kindly provided by Dr. James Sellers) were mixed 1:1 with electrophoresis sample buffer, heated in boiling water for 2 min, and electrophoresed on a 2–12% gradient SDS-PA gel [10] at 250 V for ~ 2.5 h. Gels were stained with Coomassie blue (0.063% brilliant blue R-250 in Destain I (methanol/acetic acid/water, 5:1:4)). Gels were placed in Destain I for 1 h and Destain II (methanol/acetic acid/water, 6:3:31) for 3.5 to 5 h. For trypsin digestion, gels were stained for only 10 min and placed in Destain I (15 min) and Destain II (60 min).

### 2.5. Determination of the amount of $^{14}$C-label linked to the myosin heavy chain in SDS-PA gels

Stoichiometry of the covalent reaction of [$^{14}$C]NPM with myosin heavy chain was determined from the amount of radioactivity migrating with the myosin heavy chain band using the procedure of Mahin and Lofberg [11]. Slices ($8 \times 10 \times 1.5$ mm) of gels containing the myosin heavy chain band were oxidized (0.2 ml 60% perchloric acid) in a 7 ml liquid scintillation vial. After addition of 30% hydrogen peroxide (0.4 ml) the mixture was incubated at 75–80°C for 60 min with gentle agitation. Occasionally, an additional 30 min of incubation was necessary to completely dissolve the gel slice. After cooling to room temperature, 6.25 ml of aqueous scintillation fluor (Ready-Safe, Beckman Instruments, Fullerton, CA) was added, equilibrated for more than 3 h, and counted for 10 min on a LS3801 liquid scintillation counter (Beckman Instruments, Fullerton, CA). Incubation for more than 3 h is required to allow decay of the substantial chemiluminescence generated by perchloric acid and peroxide. 3 h incubation is sufficient, since subtraction of a non-radioactive control corrects for the remaining chemiluminescence.

### 2.6. Specific activity of [$^{14}$C]NPM

NPM was labeled at the C-2 and C-3 positions of the maleimide ring (Amersham Corporation) as shown in Fig. 1 at a nominal specific activity of $27.8 \times 10^{12}$ dpm/mol. The measured specific activity of the labeled NPM was $(21.2 \pm 1.4) \times 10^{12}$ cpm/mol.

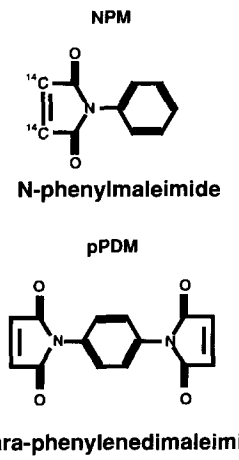


Fig. 1. Chemical structure of *N*-phenylmaleimide (NPM), showing the position of $^{14}C$ labeling. For comparison, the structure of *para*-phenylenedimaleimide (pPDM), the more commonly studied bifunctional alkylating reagent, also is shown. It is a carbon on either side of the double bond in the maleimide ring that links to the sulfur in cysteine in the alkylation reaction.

### 2.7. Tryptic digestion of the myosin from muscle bundle specimens

Homogenized muscle protein (20 $\mu$g per well) was electrophoresed and lightly stained. Strips of four myosin heavy chain bands were washed (2 ×, 25% isopropyl alcohol, 15 min, and 2 ×, 10% methanol, 15 min). The 4-band strips were divided in two; each 2-band strip was minced and air-dried overnight in an uncapped 14-ml test tube. 960 $\mu$l of 50 mM $NH_4HCO_3$ and 40 $\mu$l of 0.05 mg/ml TPCK-treated trypsin (Sigma, St. Louis, MO, T-8642) were added to the test tubes, which were capped and incubated (with agitation) at 37°C for 16–24 h. The liquid phase was pipetted to a 12-ml syringe, forced through a 0.45 $\mu$m filter (Millex-HA, Millipore), and lyophilized in a Speed-Vac Concentrator (Savant, Farmingdale, NY). The samples were re-suspended in 0.2 ml distilled water and lyophilized, two more times.

### 2.8. Two-dimensional thin-layer chromatography of tryptic digests

For the first dimension, horizontal electrophoresis, lyophilized tryptic digests were dissolved in 10 $\mu$l of electrophoresis buffer (acetic acid/formic acid/water, 3:1:16), applied to a 20 cm × 20 cm silica coated plate, and run at 1000 V for ~ 65 min at 4°C. For the second dimension of ascending chromatography, the dried plate was run for 5–6 h in 1-butanol/pyridine/acetic acid/water (13:10:2:8) [12,13]. Dried plates were scanned for $^{14}C$ for 17 h on a $\beta$-scanner (AMBIS Radioanalytic Imaging Systems, San Diego, CA).

### 2.9. Reverse phase HPLC of tryptic peptides

The trypsin-digested lyophilized myosin heavy chain was dissolved in 330 $\mu$l of solvent made up of 93% solvent A (0.1% trifluoroacetic acid in HPLC-grade distilled water) and 7% solvent B (0.1% trifluoroacetic acid, 80% acetonitrile). A small amount of the lyophilized material, containing less than 3% of the $^{14}C$-label, was insoluble. After centrifugation, 30 $\mu$l of clear supernatant were removed for $^{14}C$-counting and 250 $\mu$l were injected onto an HPLC column (0.46 × 25 cm Vydac reverse phase $C_{18}$ column 218RT54) attached to an HP1090 HPLC (Hewlett-Packard, Palo Alto, CA). Elution and separation of the peptides was accomplished with a gradient between solvents A and B. A gradient from 7% solvent B to 100% solvent B was completed in 55 min at 0.5 ml/min. The presence of peptides in the column eluate was monitored by absorbance at 214 nm. 50 $\mu$l were removed from 120 250-$\mu$l fractions collected between 0 and 60 min and analyzed for radioactivity.

### 2.10. Synthetic peptides

The SH1-containing and SH2-containing tryptic peptides were synthesized (Peninsula Laboratories, Belmont, CA) using the known amino acid sequence of rabbit skeletal myosin heavy chain and the substrate specificity of trypsin [14,15]. Since the sequence of rabbit skeletal myosin in the SH1 and SH2 containing region of myosin is RCNGVLEGIRICR and since trypsin splits peptides at arginine and lysine, the tryptic peptides that contain SH1 and SH2 are ICR and CNGVLEGIR, respectively [14,15]. The purity and identity of the peptides was checked by HPLC, thin-layer chromatography, amino acid analysis, and N-terminal sequencing.

### 2.11. Stiffness and force measurements

The stiffness of the treated muscle fibers in rigor solution (136 mM KCl, 4 mM EGTA, 4 mM $MgCl_2$, 10 mM imidazole (pH 7.0)) was measured by stretching the muscle fibers 2 nm/half-sarcomere and measuring the change in force. The stretch was applied using a loudspeaker voice coil and the force was measured with a modified Sensonor (Horten, Norway) force gauge. Directions for mounting the fibers, and descriptions of the voice coil stretcher and force transducer, are given in Schoenberg and Eisenberg, 1985. Isometric force was measured in a solution containing 130 mM potassium propionate, 2 mM $MgCl_2$, 5 mM MgATP, 1 mM EGTA, 1.5 mM $CaCl_2$, and 10 mM Mops (pH 7.0).

### 2.12. High-salt ATPase activity

Myofibrils were made by grinding psoas fiber bundles with a Tekmar Tissuemizer in 10 mM Mops (pH 7.5). The protein concentration was determined by Biuret assay and incubation tubes were prepared by dilution of the myofibrils into either EDTA-ATPase buffer (5 mM EDTA, 0.6 M KCl, 50 mM Mops (pH 7.5)) or Ca-ATPase buffer (10 mM $CaCl_2$, 0.6 M KCl, 50 mM Mops (pH 7.5)). The reaction was initiated (25°C) by addition of ATP and activity was determined by measuring the production of

inorganic phosphate [16]. Myofibril protein concentrations were chosen to maintain a constant rate of phosphate liberation for at least 5 min.

## 3. Results

Both untreated and NPM-treated fiber bundles were run on SDS-PAGE with known concentrations of pure myosin heavy chain as standards (Fig. 2). From densitometric scans of the gels, the integrated area under the myosin heavy chain band of untreated fibers, NPM-treated fibers and pure myosin heavy chain was found to be linear with amount of protein loaded (data not shown). The ratio of the slopes of these lines showed that $49 \pm 2.7\%$ of untreated fiber protein ($n = 12$) ran in the myosin heavy chain band, in agreement with previous work (cf. Yates and Greaser, 1983). The NPM-treated fiber bundles showed $43 \pm 2\%$ of treated fiber protein ($n = 8$) in the myosin heavy chain band. This amount of myosin heavy chain is not significantly different from that of the untreated control fibers (Student's $t$-test, $P > 0.05$), suggesting that, unlike the case for pPDM treatment which crosslinks myosin heavy chain [4], NPM treatment does not change the concentration of protein running in the myosin heavy chain band [5].

Before determining labeling stoichiometry, two controls were performed. Non-radioactive gel slices were added to three concentrations of [$^{14}$C]myosin (Dupont-NEN, Boston, MA) having 26 500, 53 000 and 79 500 cpm. The gel slices were dissolved and counted for radioactivity. In four experiments, an average of 89% of the counts were recovered. This was used as the counting efficiency. In a second control, 106 000 cpm of the $^{14}$C-labeled myosin were run on an SDS-PA gel; 97 190 cpm were recovered. After accounting for counting efficiency, it is seen that virtually all of the counts loaded onto an SDS-PA gel are recovered in the gel dissolving and counting procedures.

Thirteen fiber bundles had their sarcolemmas made permeable and were incubated for 1 h with 0.1 mM [$^{14}$C]NPM. After 1 h, the reaction was terminated with DTT [5]. The fiber bundles were homogenized, electrophoresed, stained with Coomassie, and the radioactivity in the myosin heavy chain band was determined. There were $91.9 \pm 11.1$ ($n = 13$) cpm in the myosin heavy chain band per $\mu$g of fiber protein loaded. From these data, the stoichiometry of NPM alkylation of the myosin heavy chain was $2.2 \pm 0.33$ mol/mol, suggesting that two sites are reacting with NPM.

To determine whether NPM was labeling two sites stoichiometrically (1:1), as opposed to more than two sites sub-stoichiometrically, two methods (two-dimensional thin-layer chromatography and reverse-phase HPLC) were used to quantitate the number of specific reaction sites for NPM on the myosin heavy chain.

Treated fiber bundles were homogenized and electrophoresed. The heavy chain was isolated, digested with trypsin, and lyophilized. For the two-dimensional chro-

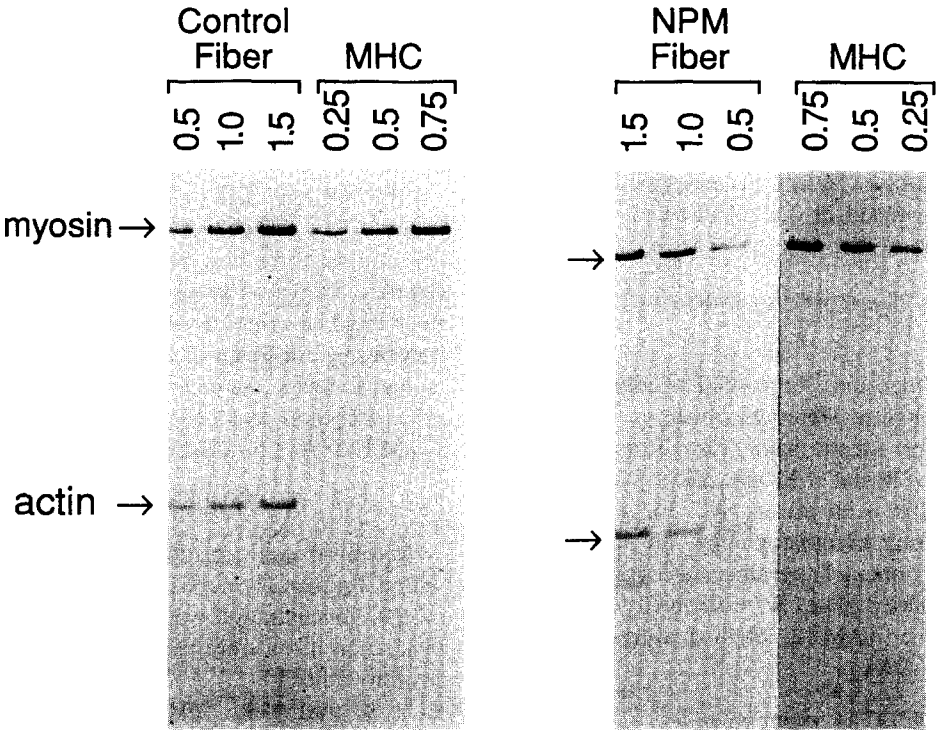


Fig. 2. SDS-PAGE of muscle fiber bundles along side myosin heavy chain standard (MHC). (a) 0.5–1.5 $\mu$g of control, untreated fiber bundles run next to 0.25–0.75 $\mu$g of myosin heavy chain. Exp 043091-1. (b) 0.5–1.5 $\mu$g of NPM-treated fiber bundles run next to 0.25–0.75 $\mu$g myosin heavy chain. Exp. 022191.

matography, material from ~ 1 mg of muscle fiber protein was applied to a silica-coated TLC plate. As shown in Fig. 3, radiolabeled trypsin-digested protein loaded at the origin separates into two main radioactive spots (B and C). A possible lightly labeled third spot (D) also is noted. Very little radioactivity appears to remain at the origin. The results are compatible with alkylation mainly at two sites. Reverse-phase HPLC (Fig. 4) similarly suggests that major labeling due to [$^{14}$C]NPM occurs at just two sites on myosin heavy chain. Due to counting noise, the existence of additional, lightly labeled sites cannot be ruled out.

We performed three types of experiment to identify the sites reacted. The most straightforward type of experiment, direct N-terminal sequencing of the eluant under the radioactive peaks, repeatedly was unsuccessful. This probably was due to the small amount of available starting material, the high molecular weight of myosin heavy chain, and the large number of tryptic peptides generated. The

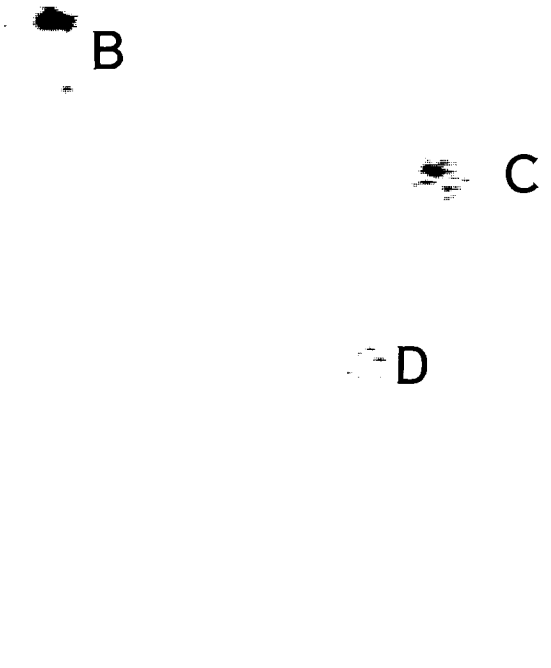


Fig. 3. $\beta$-scan of a thin-layer silica plate after two-dimensional thin-layer chromatography of [$^{14}$C]NPM-labeled myosin heavy chain tryptic digest. Electrophoresis in the horizontal direction was followed by chromatography in the vertical direction. (B) and (C) show two radioactive spots, (D), a possible third spot. The origin (A) shows little radioactivity (see text). Exp. 120391.

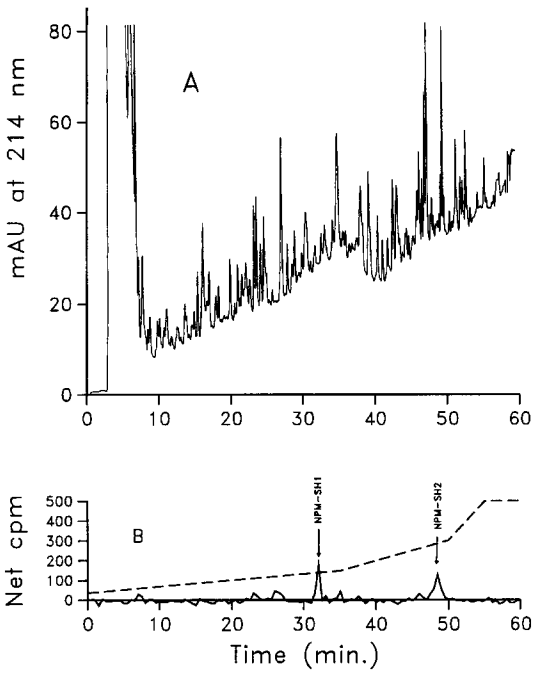


Fig. 4. Reverse-phase HPLC chromatogram of $^{14}$C-NPM-labeled, trypsin-digested myosin heavy chain. (A) Absorbance measured at 214 nm. (B) Radioactivity of aliquots collected from RP-HPLC of tryptic digest. The net cpm from the aliquot fractions are plotted versus retention time, where retention time, with two aliquots per minute, is simply twice the aliquot tube number. The dashed curve shows the elution gradient which was: 0–35 min, 7–30%B; 50 min, 60%B; 55–65 min, 100%B. The vertical arrows show the retention times of synthesized SH1 and SH2 tryptic peptides reacted with NPM and eluted from the column using the same gradient. Exp. 111792.

two other types of experiment, however, provided strong suggestive evidence that the sites reacted by NPM are Cys-707 and Cys-697, SH1 and SH2.

As seen in Fig. 4A, tryptic digestion of myosin heavy chain generates many peptides. As seen in Fig. 4B, reverse-phase HPLC elution typically separates the tryptic peptides into two main peaks of radioactivity, one having a retention time of 32 min and the other, 48.5 min. Since SH1 and SH2 are myosin's most reactive sulfhydryls, and those most likely to be reacting with NPM, and since the amino acid sequence of rabbit myosin heavy chain and the specificity of trypsin are known [14,15], we synthesized the tryptic peptides of myosin heavy chain that contain SH1 and SH2. These synthetic peptides were reacted with *N*-phenylmaleimide and subjected to our standard reverse-phase HPLC gradient elution. NPM-treated SH1 peptide showed a retention time of $32.2 \pm 0.09$ min ($n = 3$) and NPM-reacted SH2 peptide showed a retention time of $48.4 \pm 0.07$ min ($n = 3$). These [2] are identical with the

[2] It was necessary, of course, to compare the retention times of the NPM-reacted fiber peptides with those of NPM-reacted synthetic peptides. The unreacted synthetic SH1 and SH2 tryptic peptides showed very different retention times, 14.7 min ($n = 2$) and $35.6 \pm 0.1$ min ($n = 4$), respectively.

retention times of the radioactive peaks from trypsin-digested myosin heavy chain of treated fibers, strongly suggesting that under conditions where 100% of the cross-bridges are locked in a weakly-binding state, NPM reacts at Cys-697 (SH2) and Cys-707 (SH1) on the myosin heavy chain.

Another experiment suggesting that NPM reacts with SH1 and SH2 is based upon the observation that alkylation of these two cysteine residues in purified myosin affects the high salt ATPase in the presence and absence of divalent cations; modification of only SH1 results in a loss of EDTA-ATPase activity and a stimulation of the $Ca^{2+}$-ATPase activity, while modification of both SH1 and SH2 results in an inhibition of ATPase activity under both conditions [17,18]. Fibers were incubated with NPM for time periods varying from 0 to 60 min. Myofibrils were made from the fiber samples and the EDTA- and $Ca^{2+}$-ATPase of the myofibrils was measured in the presence of 0.6 M KCl. As shown in Fig. 5, the fiber's EDTA-ATPase shows a steady decline to zero activity during 60 min of treatment. The $Ca^{2+}$-ATPase (Fig. 6) shows a transient early increase and then a steady decline towards zero. The ATPase profiles that result from progressive NPM labeling of myosin in muscle fibers are indicative of modification of SH1 followed closely by modification of SH2 [17,18]. A causal relationship between the ATPase results and the mechanical properties of NPM-treated fibers is further suggested by the observation shown in Figs. 5 and 6 that the elimination of the myosin ATPase activities occurs on the same time-scale as the loss of fiber isometric tension and rigor stiffness.

We additionally made use of the EDTA- and $Ca^{2+}$-ATPase measurements to verify the efficacy of our procedure for making the sarcolemmas throughout the fiber bundle permeable. The experiment of Fig. 5 was done on a

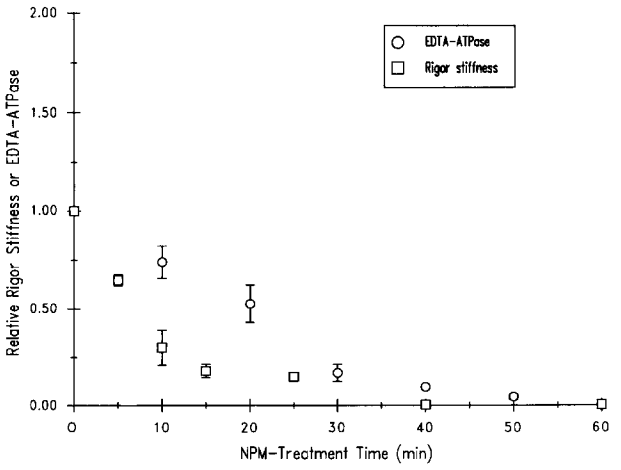


Fig. 5. Stiffness in rigor solution (□) and EDTA-ATPase (○) as a function of treatment time in 100 μM NPM-containing relaxing solution. Both values normalized by corresponding values measured before treatment, 8.2 dyn/nm for stiffness and 1.05 μmol $P_i$/mg myosin per min for EDTA-ATPase. Points and error bars show mean ± S.E. ($n = 5$).

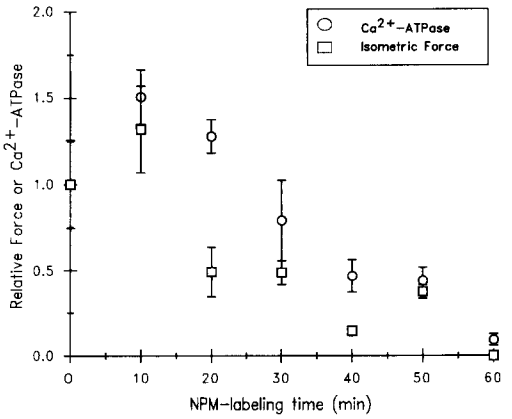


Fig. 6. Calcium activated force (□) and $Ca^{2+}$-ATPase (○) as a function of treatment time in 100 μM NPM-containing relaxing solution. Both values normalized by corresponding values measured before treatment, 1.9 kg/cm$^2$ for force and 0.09 μmol $P_i$/mg myosin per min for $Ca^2$-ATPase. Points and error bars show mean ± S.E. ($n = 5$).

fiber bundle whose diameter was comparable to those used in the other experiments. We also measured the EDTA-ATPase of treated fiber bundles having exceptionally large diameter (0.76 mm). The fact that with our standard 1-h NPM treatment the EDTA-ATPase was reduced more than 200-fold from 1.04 to < 0.005 μmol $P_i$/mg per min provides evidence that even fiber bundles considerably larger than those routinely used here have virtually all their sarcolemmas made permeable by the skinning procedure. The $Ca^{2+}$-ATPase profile in the large bundles was similar to that observed for smaller ones, an activation of the ATPase for short incubations, followed by inactivation at the 1 h end-point (data not shown).

## 4. Discussion

The coordinated movements of skeletal muscle can be attributed to the integrated action of several proteins, with actin and myosin having dominant roles. Modification of these proteins can be a useful tool for furthering our understanding of their function. Previously, Barnett et al. (1992) examined the effect of treating muscle fibers with the alkylating agents pPDM and NPM. Treatment with either of these agents produces muscles where the cross-bridges appear locked in a myosin ATP-like state characterized by weak binding to actin with very rapid detachment rate constants.

pPDM and NPM link to a number of muscle fiber proteins [5] and have major effects on the interaction of the myosin crossbridges with actin. Since the effects seen are analogous to those when pPDM covalently links to the SH1 and SH2 sulfhydryls of myosin in solution, it is likely that the physiological effects of these compounds in skeletal muscle fibers is also due to their reaction with SH1 and SH2 on myosin. The current data strongly support this hypothesis.

In order to link NPM and pPDM to muscle fiber proteins, the sarcolemmal membrane surrounding the muscle fiber first must be made permeable. Previously we showed that the relatively mild 'skinning' procedure of Schoenberg and Eisenberg, 1985 [19], which works quite well for single fibers [6], was inadequate for 1–2 mm diameter muscle bundles [7]. In the present work we showed, using NPM's ability to inhibit the fiber EDTA-ATPase, that the 'skinning' procedure of Magid and Reedy (1980) works extremely well to make sarcolemmas permeable, even in fiber bundles as large as 0.75 mm.

After treating the permeable muscle fiber bundles with [$^{14}$C]NPM, the stoichiometry of NPM alkylation of myosin was determined. The myosin heavy chain was separated from the rest of the muscle proteins using SDS-PAGE and the amount of $^{14}$C-label associated with myosin heavy chain was determined. This yielded a binding stoichiometry for NPM of $2.2 \pm 0.33$ mol/mol under conditions that lock 100% of the crossbridges in a weakly-binding state, raising the possibility that NPM is producing its effect in muscle fibers by reacting with two sites on myosin.

To further explore this, we examined where on myosin heavy chain [$^{14}$C]NPM appeared to link. We did this by using two-dimensional thin-layer chromatography and HPLC to resolve tryptic digests of the myosin heavy chain from [$^{14}$C]NPM-treated fiber bundles. Both techniques suggested that the NPM label could be found mainly at two sites on myosin heavy chain. Co-elution on HPLC of the $^{14}$C-labeled tryptic peptides with NPM-treated synthetic SH1 and SH2 peptides and inhibition of the muscle fiber's EDTA- and $Ca^{2+}$-ATPase activities with NPM treatment suggested that the reaction sites for NPM on myosin heavy chain are Cys-697 (SH2) and Cys-707 (SH1).

A number of factors influence the susceptibility of myosin's sulfhydryls to alkylation. Although our results for NPM could not have been predicted *a priori*, they are not surprising in light of previously published work. In general, the pattern of labeling depends upon the specific alkylating agent. Comparing just maleimide-based labels and iodoacetamide-derived labels, the latter are much more likely to label specifically at SH1 [20,18]. Another factor influencing the labeling pattern is nucleotide. The absence of nucleotide tends to inhibit reactivity of both SH1 and SH2 [21,4], $PP_i$ favors labeling at SH1 [22], and ADP and ATP enhance the reactivity of SH2 [23–25].

SH1 and SH2 are located in what is likely a critical region of the myosin crossbridge head, near the base of the nucleotide binding pocket and also the cleft involved in actin binding [26]. In solution, pPDM treatment of myosin in the presence of ADP, leads to crosslinking of SH1 and SH2 with inhibition of the actomyosin ATPase and locking of myosin in a weakly-binding form [3,27]. Wells et al. [28], using different length crosslinkers, concluded that it was the reaction of SH1 and SH2, and not the crosslinking per se that was responsible for pPDM's action. The present results strongly support this conclusion.

Our finding that linking NPM to SH1 and SH2 induces a weakly-binding myosin state similar to that induced by pPDM opens up the possibility for exploring further the relative contributions of the SH1 and SH2 modifications. Unlike the case for pPDM, with NPM it should be possible to alkylate SH1 or SH2 singly, rather than concurrently as in the present work. While it already has been shown in solution that linking phenylmaleimide solely to SH1 inhibits actomyosin ATPase, it was observed also that, unlike the case for pPDM, this modification increases myosin's affinity for actin in the presence of ATP [22,29].

The mechanism by which linking phenylmaleimide groups to SH1 and SH2 locks crossbridges in a weakly-binding configuration is, as yet, unknown. This modification could be inhibiting a conformational change necessary for conversion to the strongly-binding configuration, or, alternatively, it could be interfering with communication between the ATP-binding and actin-binding sites, occupancy of the former normally determining affinity of the latter.

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