Light-Directed Generation of the Actin-Activated ATPase Activity of Caged Heavy Meromyosin†

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ABSTRACT: An understanding of the molecular mechanism of muscle contraction will require a complete description of the kinetics of the myosin motor *in vitro* and *in vivo*. To this end chemical relaxation studies employing light-directed generation of ATP from caged ATP have provided detailed kinetic information in muscle fibers. A more direct approach would be to trigger the actin-activated ATPase activity from a caged myosin, i.e., myosin whose activity is blocked upon derivatization with a photolabile protection group. Herein we report that a new type of caged reagent can be used to prepare a caged heavy meromyosin by modification of critical thiol groups, i.e., a chemically modified motor without activity that can be reactivated at will using a pulse of near-ultraviolet light. Heavy meromyosin modified at Cys-707 with the thiol reactive reagent 1-(bromomethyl)-2-nitro-4,5-dimethoxybenzene does not exhibit an actin-activated ATPase activity and may be viewed as a caged protein. Absorption spectroscopy showed that the thioether bond linking the cage group to Cys-707 is cleaved following irradiation (340−400 nm) via a transient aci-nitro intermediate which has an absorption maximum at 440 nm and decays with a rate constant of 45.6 s^{-1}. The *in vitro* motility assay showed that caged heavy meromyosin cannot generate the force necessary to move actin filaments although following irradiation of the image field with a 30 ms pulse of 340−400 nm light the caged group was removed with the concomitant movement of most filaments at a velocity of 0.5−2 μm/s compared to 3−4 μm/s for unmodified HMM. The specificity and simplicity of labeling myosin with the caged reagent should prove useful in studies of muscle contraction *in vivo*.

Elucidation of the mechanism of muscle contraction will require a detailed description of the transient kinetics of the actin-activated ATPase reaction of myosin II *in vitro* and *in vivo*. Whereas light-directed activation of ATP from caged ATP (McCray & Trentham, 1989) has resulted in significant progress toward this goal, a complementary and more direct approach would be to trigger actin-activated ATPase activity from a myosin conjugate whose activity is blocked upon derivatization with a photolabile protection group, i.e., a caged myosin. We have previously shown that caged G-actin (Marriott, 1994) can be prepared by the derivatization of several lysine residues of actin with the photolabile 6-nitroveratryl chloroformate group. The employment of a similar approach to prepare caged HMM was unsuccessful (Marriott, unpublished results). Interestingly though, HMM contains two head domains each with two reactive cysteine residues (Cys-707 and Cys-697) which can be specifically modified under mild conditions using a variety of haloacetyl alkylating reagents; these modifications result in considerable perturbations of the ATPase activity of myosin (Mulhern & Eisenberg, 1978; Crowder & Cooke, 1984; Reisler, 1982; Root & Reisler, 1993). For example, HMM labeled at Cys-707 with the thiol reactive fluorophore IA-TMR or a number of other alkylating groups does not support F-actin-activated ATP-dependent sliding of F-actin filaments when assayed in the *in vitro* motility assay (Root & Reisler, 1993; data not shown). It is worthwhile to note that the *in vitro* motility assay (Kron & Spudich, 1985; Yanagida et al., 1984) is a more functional assay of the actin-activated ATPase activity of myosin than the corresponding solution-based ATPase assay which, of course, does not measure the force generating ATPase activity (Mulhern & Eisenberg, 1978; Crowder & Cooke, 1984; Reisler, 1982; Root & Reisler, 1993). On the other hand, the sliding velocity of actin filaments as measured in the *in vitro* motility assay is, intuitively, a measure of the force generating actin-activated ATPase activity. Correspondingly, the sliding velocity is dependent on the concentration of ATP (and the quality of the HMM preparation) and obeys Michaelis−Menten kinetics. As an example the sliding velocity of a single actin filament was analyzed using the Michaelis−Menten equation and exhibits a k_m for ATP of 60 μM with a V_{max} of 4 μm/s [Figure 1; see also Kron and Spudich (1985)]. We belive these data validate the use of the *in vitro* motility assay as a measure of the force producing actin-activated ATPase activity.

Since Cys-707-modified HMM does not produce movement of actin filaments in the *in vitro* motility assay, we reasoned that if the thiol group of Cys-707, or even Cys-697, could be reversibly alkylated using a photolabile protection group for mercaptans (Marriott et al., 1992), then the inactive HMM conjugate should be capable of being reactivated, at will, upon irradiation with near-ultraviolet light. We used our first thiol photodeprotection reagent, 1-(bromomethyl)-2-nitro-4-benzoic acid (Marriott et al., 1992), to cage the actin-activated ATPase of HMM, but the conjugate exhibited a poor photocleavage efficiency (data not shown). In this study we have employed 1-(bromomethyl)-2-nitro-4,5-dimethoxybenzene (BMNDMB; Figure

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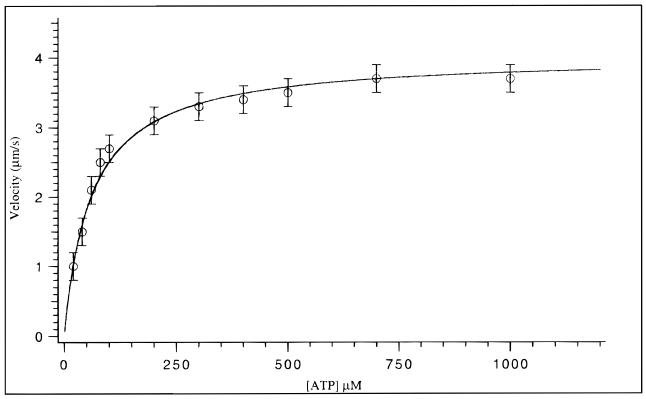

FIGURE 1: Dependence of the sliding velocity of a single actin filament on a surface of unmodified HMM as a function of the ATP concentration. Sliding distances were measured over a 2 s interval for higher ATP concentrations (>300 μM) and after 10 s for the lower concentrations of ATP. The data were fit to the Michaelis−Menten equation using an iterative fitting procedure with a k_m of 60 μM and a V_{max} of 4 μm/s. HMM preparations showing a V_{max} of <2 μm/s were discarded.

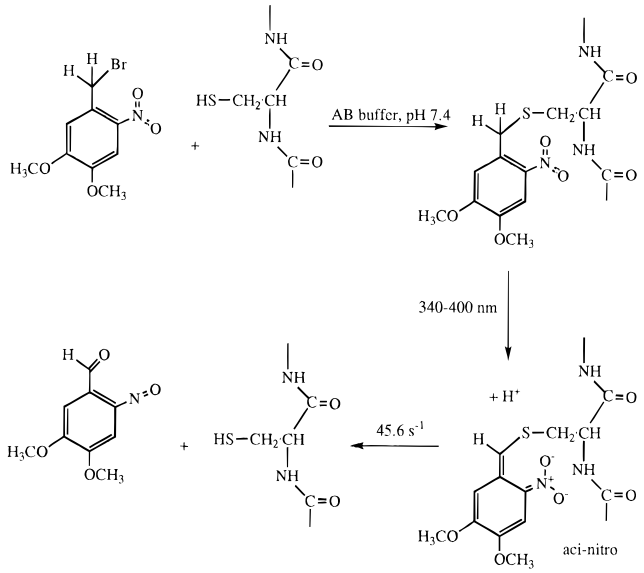

FIGURE 2: Structure of BMNDMB and proposed mechanism [after McCray and Trentham (1989)] of the protection and deprotection of the thiol group of Cys-707 of HMM.

2) chosen as a more suitable photodeprotection reagent for thiol groups for the following reasons. First, bromomethyl derivatives specifically alkylate thiol groups (Marriott et al., 1992); second, BMNDMB has a relatively broad near-ultraviolet absorption band (Figure 3) with a high molar absorptivity (E_{350nm} of 5900 $M^{-1}\cdot cm^{-1}$) which can be used to accurately quantify the protein labeling ratio; and third, 2-nitrobenzyl thioethers exhibit a reasonable quantum yield for the photocleavage reaction (Marriott et al., 1992). Herein we describe the labeling of HMM with BMNDMB and the spectroscopic and biochemical characterization of the BMNDMB−HMM conjugate and a kinetic analysis of the photodeprotection of the 3,4-dimethoxy-2-nitrobenzyl group to yield native HMM with a normal actin-activated ATPase activity.

MATERIALS AND METHODS

Proteins and Protein Labeling. HMM was prepared according to Heidecker et al. (1995), stored in liquid nitrogen, and used within 1 month of preparation. Actin was prepared according to Marriott (1994) and labeled with tetramethylrhodamine phalloidin according to Heidecker et al. (1995). All labeling reactions involving the thiol reactive caging reagent, BMNDMB (Aldrich), were performed in a darkened room or protected from external lights using aluminum foil. Since BMNDMB is a very effective alkylating reagent, it should handled with due care and should be dispensed and weighed in small quantities in a well-ventilated hood. BMNDMB-labeled HMM was prepared by treating HMM in AB buffer (25 mM imidazole/25 mM KCl, pH 7.4) at 18.6 μM with BMNDMB (added from a 9 mM stock solution in DMF) to a final concentration of 186 μM for 75 min at room temperature. After an overnight dialysis at 4 °C against AB buffer with 10 mM DTT (dithiothreitol) and clarification by centrifugation, the absorption spectrum of the protein was recorded using a HP8452 diode array spectrophotometer (Hewlett-Packard). The HMM conjugate (600 μL) was irradiated with 340−400 nm light delivered from a 100-W mercury arc lamp according to Marriott (1994). The irradiated protein was dialyzed overnight against AB buffer containing 10 mM DTT whereafter the absorption spectrum was recorded.

Kinetic Studies. Transient absorption spectroscopy was recorded using an instrument described by Uhl et al. (1984). The absorption spectra of BMNDMB−HMM as shown in Figure 4A are the average of 10 spectra recorded after 10 consecutive 50 ns pulses of 380 nm light delivered by an excimer laser. Less than 1% of the BMNDMB is photolyzed per pulse based on the change in the absorption at 350 nm. The peak of the aci-nitro transient absorption spectra at 440 nm was extracted from the data set and fit to a single exponential decay.

Microscopy. The *in vitro* assay was performed according to Heidecker et al. (1995). Basically, HMM at 50 μg/mL was laid on a coverslip coated with nitrocellulose for 90 s in AB buffer. After being washed with AB buffer the surface was treated with AB buffer containing BMNDMB freshly added to a concentration of 45 μM. After 30 min in the dark the BMNDMB was washed out of the chamber with AB buffer containing 10 mM DTT. Actin filaments labeled with tetramethylrhodamine phalloidin (Fluka) were added to the chamber at a concentration of 20 nM. After 90 s the filaments were washed out with AB/GO/CAT/DTT buffer [see Heidecker et al. (1995)], and ATP (to 1 mM) was added to the chamber in AB/GO/CAT/DTT buffer.

The dependence of the sliding velocity on the concentration of ATP was determined as described above. The velocity was measured by measuring the number of pixels traversed by the leading edge of a filament during a fixed time period. The pixel-distance calibration was determined using a calibration grid from Zeiss (Oberkochen). Simultaneous fluorescence imaging and photolysis of the image field were performed essentially as described by Mitchison (1989). Near-ultraviolet light (340−400 nm) was selected from a 100 W arc lamp aligned 90° to the microscope body (Zeiss, Axiovert 35) using a broad-band near-ultraviolet filter (Dr. Rapp Elektronic, Hamburg). The beam was directed onto an Ilex electron shutter (Melles Griot) operated under remote manual control. The photolysis beam was collimated and directed into the microscope body via a dichroic mirror that reflects light from 300 to 420 nm and transmits light above 450 nm. The 546 nm line of a second 100 W lamp

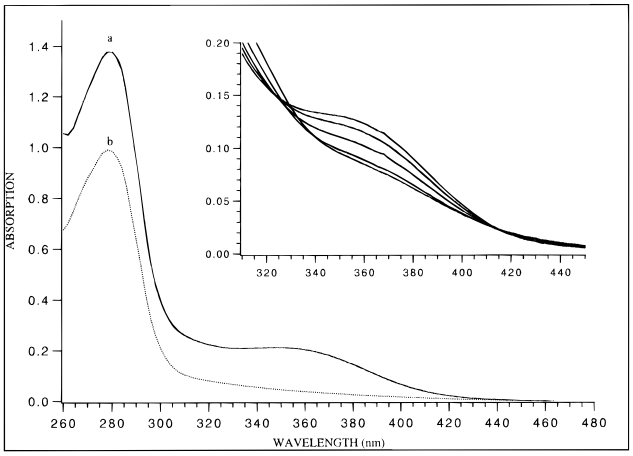

FIGURE 3: Absorption spectrum of a 600 μL solution of BMNDMB-labeled HMM (18.6 μM) in AB buffer containing 2 mM DTT before (a) and after 30 min of irradiation with 340−400 nm light and dialysis against AB buffer (b). The inset shows the time-dependent change in the absorption spectrum of a separate preparation of BMNDMB-labeled HMM (10.7 μM) at 30, 60, 90, 120, and 240 s (upper to lower trace, respectively) after irradiation with 340−400 nm light.

aligned parallel to the microscope body was used for fluorescence excitation. This light passed through the dichroic mirror described above. The excitation filter of the dichroic filter assembly in the microscope was removed. For some experiments the photolysis beam was focused onto a narrow slit and then recollimated for photolithographic investigations. Fluorescently labeled actin filaments were imaged on an image-intensified charge-coupled device camera (C2400, Hamamatsu Photonics) and simultaneously recorded on video tape and stored at 0.5 s intervals in the memory of a frame grabber board (Scion Inc. PA) in a Macintosh IIfx computer. Image processing was performed using NIH Image.

RESULTS AND DISCUSSION

Treatment of HMM in solution with a 10-fold excess of BMNDMB in the absence of both ATP and DTT at 25 °C for 75 min leads to the incorporation of two dimethoxy-2-nitrobenzyl groups per HMM molecule as determined by absorption spectroscopy or one chromophoric group per S1 head. A large number of labeling studies of myosin using alkyl halides have shown Cys-707 can be specifically labeled in the absence of ATP [reviewed by Reisler (1982)]. We believe BMNDMB also labels Cys-707 since the 1:1 labeling ratio was obtained for HMM labeled with both a 10-fold or 20-fold excess of BMNDMB (Figure 3, curve a; data not shown). Inclusion of ATP to 1 mM did not influence the labeling ratio, which suggests the SH-2 thiol is not exposed to the reagent upon binding ATP. Irradiation of the BMNDMB-labeled HMM with near-ultraviolet light (340−400 nm) in AB buffer in the presence of 10 mM DTT leads to an irradiation dose-dependent change in the absorption spectrum typical of that found for photoisomerization of the dimethoxy-2-nitrobenzyl group (Figure 3 inset). Following dialysis, absorption spectroscopy of the irradiated HMM conjugate (600 μL at 18 μM HMM) revealed that most of the protection groups had been removed (Figure 3, curve b). The structureless, residual absorption in the near-ultraviolet region might originate from secondary, photochemical reactions of the 2-nitrosobenzaldehyde photoproduct with HMM during the long exposure time (30 min) that was necessary to ensure a complete reaction of the large sample volume. However, in all subsequent experiments using BMNDMB-labeled HMM, on a surface or in solution, the exposure time for photoactivation was 0.5 s or less, and therefore we expect these side reactions would be very limited. Transient absorption spectroscopy of the modified HMM following a 50 ns pulse of 380 nm excitation showed that a new absorption band centered at 440 nm had already formed 100 μs after the photolysis pulse (Figure 4A). This transient absorption band, which most likely corresponds to the aci-nitro intermediate (McCray & Trentham, 1989; Figure 2) decayed with a rate constant of 45.6 s^{-1} at pH 7.4 (Figure 4B). The aci-nitro spectrum reported in Figure 4A is red-shifted compared to that of 2-nitrobenzyl derivatives (Walker et al., 1988) because of the presence of the two methoxy groups. The rate of the aci-nitro decay was independent of the concentration of ATP (data not shown). The rapid release kinetics will prove useful in transient kinetic studies of force generation in more complex environments such as glycerinated muscle fibers where tension is developed some 80 ms following ATP-based activation of myosin (McCray & Trentham, 1989). Here labeling conditions have been developed to obtain a relatively high degree of specificity for the SH-1 thiol (Bell et al., 1995). However, even in the presence of nonspecific labeling in the muscle fiber, we have

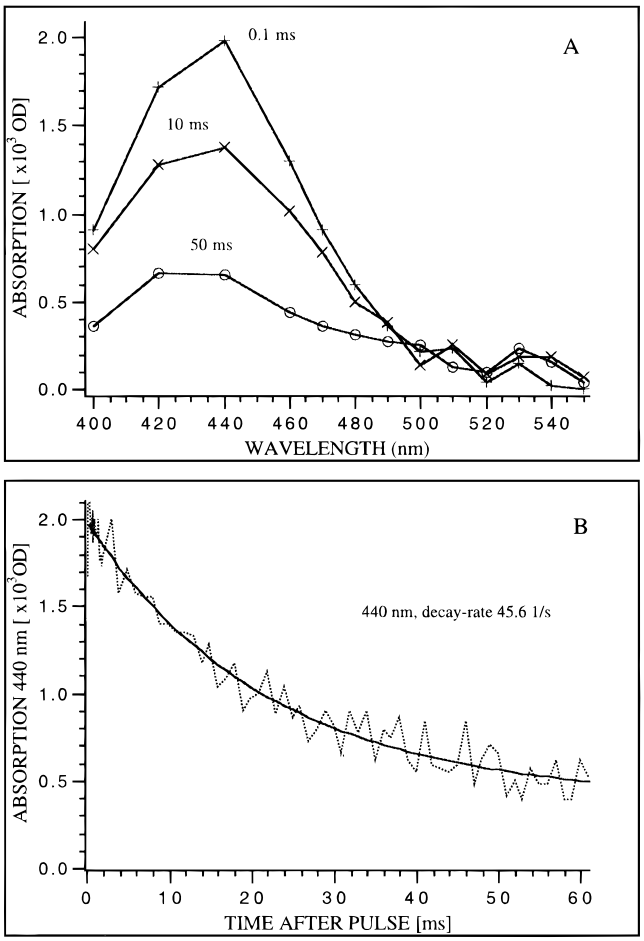

FIGURE 4: (A) Selected time-resolved absorption spectra of a 21.4 μM solution of BMNDMB-labeled HMM in AB buffer containing 5 mM DTT following irradiation with a 10 ns pulse of 380 nm light: 0.1 ms (top), 10 ms (middle), and 50 ms (bottom). (B) Time-resolved absorption decay of the sample described in (A) recorded at 440 nm.

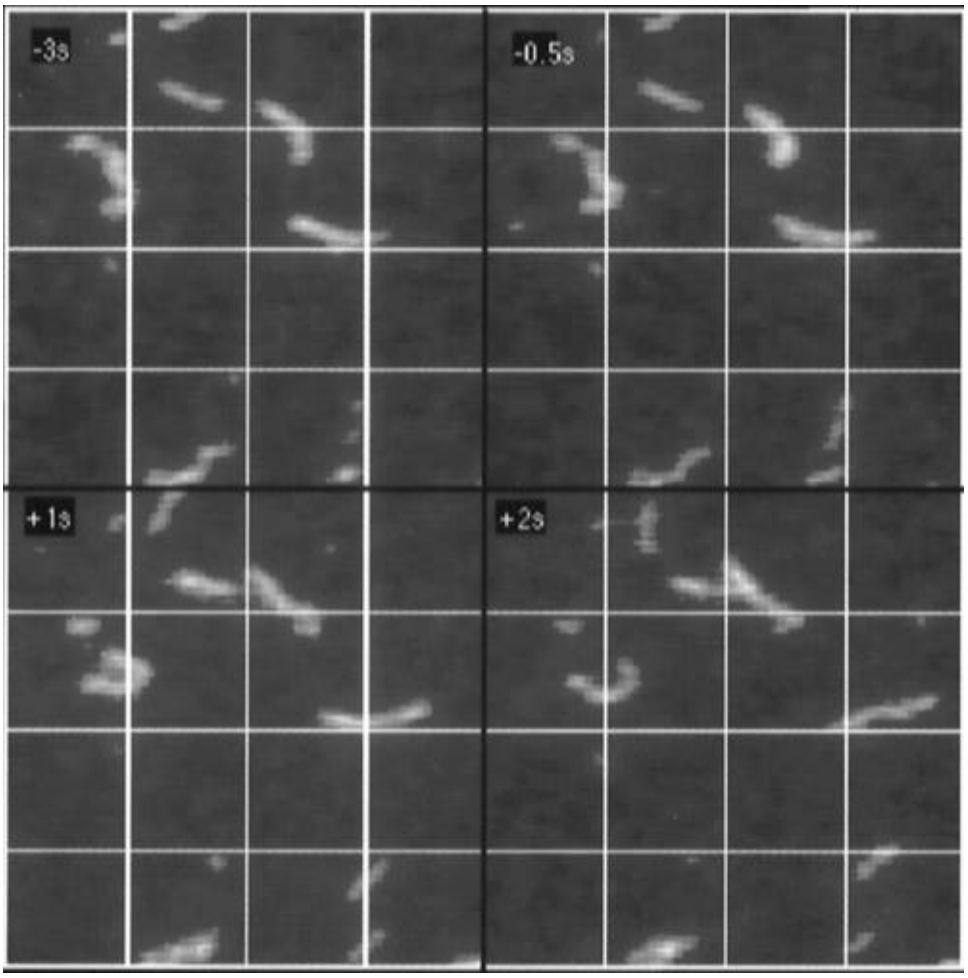

FIGURE 5: Images of single actin filaments on caged HMM before and after flash photolysis of the image field in AB/GO/CAT/DTT buffer in the presence of ATP. Images of the fluorescent actin filaments on caged HMM taken from −3 to −0.5 s do not show any actin sliding motion. Irradiation of the image field for 0.5 s initiated the movement of most actin filaments in the field. The velocity of the filaments recorded from 0 to 1 s and 1 to 2 s is between 0.5−2.0 μm/s. The grid is 5 μm × 5 μm. Actin filaments in the image field adjacent to the irradiated one did not move (data not shown).

preliminary data which suggest that BMNDMB can be photodeprotected from other thiol-labeled proteins with millisecond release kinetics. It would be interesting to investigate if, following light-directed activation of myosin, changes in the conformation of myosin can be seen before the development of tension.

HMM can also be modified with BMNDMB when immobilized on a nitrocellulose coated glass coverslip as indicated in the legend to Figure 5. BMNDMB-labeled HMM does not support motility of F-actin filaments in the *in vitro* motility assay (Figure 5). However, irradiation of the BMNDMB−HMM coated surface with 320−400 nm light for as little as 30 ms leads to the movement of more than 90% of the actin filaments with a sliding velocity of 0.5−2.0 μm/s compared to the 3−4 μm/s for unmodified HMM. An example of this effect is shown in Figure 5 in which the time-dependent position of filaments on a BMNDMB-modified HMM surface before and after a 0.5 s flash with near-ultraviolet light is shown. Multiple flashing of the image field with 30 ms pulses improved the sliding rate (data not shown). Similar results to those described above were obtained using HMM that had been modified with BMNDMB in solution as described in Figure 3 (data not shown). It was often found that the shorter filaments, typically less than 2 μm, ran smoothly almost immediately following the photolysis pulse, whereas longer filaments often buckled after the flash (Nakazaki et al., 1993) and occasionally severed in the first few seconds following the photolysis pulse whereafter they ran smoothly. Actin filaments in the image field adjacent to the photolyzed image field did not move (data not shown), and when the excitation focus of the near-ultraviolet light in the image field was restricted to a bar of 5 μm by 15 μm, filaments were seen to move only in the photolyzed rectangle, thereby demonstrating that the activity of caged HMM can be spatially manipulated by photolithography (data not shown). Irradiation of a surface of caged HMM with a polarized pulse of near-ultraviolet light to photoselect BMNDMB molecules whose absorption dipole moment lies in the direction of the electric vector of the photolysis beam resulted in filaments moving in all directions, which suggests that the head domain of the surface-bound myosin exhibits a considerable degree of rotational freedom.

The results presented herein are consistent with the interpretation that alkylation of the Cys-707 thiol group of HMM with BMNDMB inhibits the actin-activated ATPase activity of HMM; removal of the photolabile protection group with near-ultraviolet light restores the actin-activated ATPase activity. The millisecond photogeneration kinetics of actin-activated ATPase activity from caged HMM, together with the high efficiency of light-directed thioether bond breakage, will make caged HMM, and probably caged myosin, an

unique probe to study the mechanism of muscle contraction in *in vitro* model systems and in muscle fibers. The generation of actin-activated ATPase activity from caged HMM has activation kinetics similar to those of caged ATP (McCray & Trentham, 1989; Walker et al., 1995) but requires a fraction of the irradiation energy and liberates 100 times less photoproduct, and its application will overcome the problem of competitive inhibition of myosin ATPase by caged ATP (Sleep et al., 1994).

Myosin II labeled at Cys-707 has been used extensively to study the structure and dynamics of the myosin head during muscle contraction (Crowder & Cooke, 1984). However, the altered enzymic and mechanical activities of SH1-labeled myosin compared to native myosin have resulted in a controversy on the suitability of using this labeling site to probe the chemomechanical properties of actomyosin (Mulhern & Eisenberg, 1978; Crowder & Cooke, 1984; Reisler, 1982; Root & Reisler, 1993). In fact, in this study we have exploited the light-directed reversible inhibition of actomyosin-activated ATPase of Cys-707 labeled with BMNDMB. Our work supports the concern of other researchers (Mulhern & Eisenberg, 1978; Root & Reisler, 1993) on the suitability of SH1-labeled myosin conjugates to study the structure, function, and dynamics of myosin and the mechanism of muscle contraction, when it is clear that the modified conjugate (a) has drastically altered ATPase activities both *in vitro* and *in vivo*, (b) does not generate the force to move actin filaments using *in vitro* motility systems, and (c) does not develop the same tension and force as unlabeled myosin in muscle fibers (Bell et al., 1995).

We expect investigations of the mechanism of BMNDMB-modified Cys-707 inhibition of the actomyosin-activated ATPase activity may yield important information on the mechanism of the myosin motor. For example, we are now addressing the issue of whether the modification simply slows down the cross-bridge cycle or if it traps a specific intermediate in the cycle. Future studies will also include the application of caged skeletal muscle myosin to investigate the mechanism of contraction in permeabilized muscle fibers and of smooth caged myosin to study the cytomechanics of the cortical contraction reaction and to investigate properties of the cleavage furrow in living cells.

In summary, we conclude that the activity of HMM and probably myosin II, nonmuscle myosins, and other proteins with essential cysteine residues can be caged using 1-(bromomethyl)-2-nitro-4,5-dimethoxybenzene and that light-directed activation of caged proteins (Marriott, 1994) will continue to emerge as a powerful technique to study protein activity in complex molecular environments.

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