Binding of Myosin to Actin in Myofibrils during ATP Hydrolysis†

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ABSTRACT: Measurements of cross-bridge attachment to actin in myofibrils during ATP hydrolysis require prior fixation of myofibrils to prevent their contraction. The optimal cross-linking of myofibrils was achieved by using 10 mM carbodiimide (EDC) under rigor conditions and at 4 °C. The fixed myofibrils had elevated MgATPase activity (150%) and could not contract. As judged by chymotryptic digestions and subsequent SDS gel electrophoresis analysis, less than 25% of myosin heads were cross-linked in these myofibrils. The isolated, un-cross-linked myosin heads showed pH-dependent Ca^{2+}- and EDTA(K^+)-ATPase activities similar to those of standard intact S-1. For measurements of myosin binding to actin, the modified myofibrils were digested with trypsin at a weight ratio of 1:50 under rigor, relaxed, and active-state conditions. Aliquots of tryptic digestion reactions were then cleaved with chymotrypsin to yield isolated myosin heads and their fragments. Analysis of the decay of myosin heavy-chain bands on SDS gels yielded the rates of myosin cleavage under all conditions and enabled the measurements of actomyosin binding in myofibrils in the presence of MgATP. Using this approach, we detected rigorlike binding of 25 ± 6% of myosin heads to actin in myofibrils during ATP hydrolysis.

Muscle contraction is recognized to involve the cyclic interaction between myosin cross-bridges and actin filaments. This interaction results in the sliding of myosin and actin filaments past each other and the generation of force during the power stroke step of each cycle (Huxley & Niedergerke, 1954; Huxley & Hanson, 1954). The molecular modeling of these events in terms either of changes in myosin cross-bridges (Huxley, 1969; Huxley & Simmons, 1971; Huxley & Kress, 1985) or of helix–coil transition within the S-2 region (Harrington, 1971, 1979) depends on the size of the tension-generating part of the cross-bridge stroke and thus, in turn, on the extent of actomyosin binding during active contraction. Consequently, considerable efforts have been focused on the measurements of the fraction of myosin heads bound to actin during force generation in muscle. However, estimates of cross-bridge binding to actin by several techniques showed a broad range of values. In activated skeletal fibers, interpretations of EPR spectra (Cooke et al., 1982), X-ray diffraction patterns (Huxley & Kress, 1985), dichroic measurements (Burghardt et al., 1983), and polarized fluorescence observations (Yanagida, 1981) yielded values between 20 and 90% of heads bound to actin. The obvious need for a new and independent approach to determinations of fractional association of myosin heads with actin during active contraction prompted the application of proteolytic methods to such measurements.

Recently, the tryptic rates method has been employed in measurements of actin binding to myosin subfragment 1 (S-1),[1] HMM, myosin filaments, and myosin in myofibrils (Duong & Reisler, 1987a,b; Chen & Reisler, 1984; Azarcon et al., 1985). As tested in model experiments (Chen et al., 1987), and by comparison with ultracentrifugation binding measurements (Duong & Reisler, 1987a), this method accurately determines the fraction of myosin heads bound to actin in the presence of nucleotides. Briefly, the proteolytic rates method is based on the fact that the 50K/20K junction of myosin heads is protected against tryptic proteolysis upon binding to actin (Balint et al., 1978; Yamamoto & Sekine, 1979; Mornet et al., 1979). Therefore, the cleavage rate of this junction can be correlated to the degree of actin binding to myosin heads.

In the present work, we have extended the tryptic rates method to the measurements of myosin binding to actin in cross-linked myofibrils during ATP hydrolysis. Carbodiimide (EDC) fixation of myofibrils was required to prevent their rapid contraction in the presence of MgATP. The rates of tryptic cleavage at the 50K/20K junction of myosin were measured in rigor, relaxed, and activated (+MgATP) myofibrils. We determined from these measurements that a relatively small fraction of myosin heads (25%) formed rigorlike bonds to actin during ATP hydrolysis in myofibrils.

Materials and Methods

Reagents. Trypsin, soybean trypsin inhibitor, α-chymotrypsin, phenylmethanesulfonyl fluoride, the cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), β-mercaptoethanol, orthovanadate, ADP, ATP, and DTT were purchased from Sigma Chemical Co. (St. Louis, MO). *p*-Nitrophenylenemaleimide (pNPM) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other reagents used were analytical grade.

Proteins. Myofibrils were prepared from rabbit psoas muscle according to the procedure of Rome (1967). Ap-

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[1] Abbreviations: S-1, myosin subfragment 1; HMM, heavy meromyosin; LMM, light meromyosin; DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; pNPM, *p*-nitrophenylenemaleimide; pPDM, *N*,*N*′-*p*-phenylenedimaleimide; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; V_i, orthovanadate; kDa, kilodalton(s); MES, 2-(*N*-morpholino)ethanesulfonic acid.

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proximately 20 g of muscle was ground and suspended in 200 mL of 20 mM KCl, 10 mM Bis-Tris, and 4 mM EDTA (pH 6.8). The suspension was homogenized in a Waring blender for 5 s at low speed and then at high speed for 60 s. The homogenate was centrifuged in a Sorval RC-5 centrifuge at 5000 rpm for 15 min. The pellet was washed 3 times in 10× volume of the above buffer. After the pellet was washed, the myofibrils were resuspended in the same buffer containing 50% glycerol and stored at −20 °C.

Experimental Requirements for Tryptic Digestion of Myofibrils. The proteolytic rates method for measuring the binding of myosin to actin in myofibrils involves determinations of myosin cleavage rates in rigor and relaxed myofibrils (Azarcon et al., 1985) as well as under the chosen "nucleotide conditions" (ATP hydrolysis in this work). The relaxation of myofibrils is normally achieved in the presence of MgATP and the absence of Ca^{2+}. However, troponin C is rapidly cleaved in tryptic digestion experiments, resulting in the desensitization of myofibrils and their ATP-supported contraction in the absence of Ca^{2+}. Thus, the relaxation of myofibrils during proteolysis can be best achieved in the absence of ATP hydrolysis by myosin. In this work, the relaxed state was induced by the MgADP·V_i complex or by chemical inactivation of myosin heads and subsequent dissociation of actomyosin by MgATP. As described below, the inactivation of myosin heads was carried out by modification of the reactive thiols (SH_1 and SH_2) on myosin.

A second, related requirement imposed on myofibrils was to prevent their rapid supercontraction under active-state conditions. To this end, chemical cross-linkings of myofibrils with dimethyl suberimidate, glutaraldehyde, and carbodiimide were tested. The last reagent yielded optimal cross-linking results in terms of minimal changes in ATPase activity of myofibrils, minimal cross-linking of myosin heads, and effective blocking of myofibrillar contraction.

Modification of Relaxed Myofibrils with pNPM. Modification of the reactive SH_1 and SH_2 groups on myosin provides a convenient way for reducing the affinity of this protein for actin. Since the standard reaction with *p*-phenylenedimaleimide (pPDM) introduced cross-links between myosin heavy chains and decreased the rate of S-1 production by chymotrypsin, a monofunctional analogue of that reagent, pNPM, was used instead. Experiments with isolated S-1 samples showed that pNPM modification of myosin heads caused the complete loss of their Ca^{2+} and EDTA(K^+)-ATPase activities, a feature which had been normally identified with the labeling of SH_1 and SH_2 groups (Reisler, 1982).

Myofibrils stored in 50% glycerol were washed 5× in 5 volumes of relaxation buffer containing 0.1 M KCl, 25 mM Tris, 4 mM EGTA, 2 mM $MgCl_2$, and 1 mM MgATP (pH 8.0). The washings were performed by centrifugation at 4000 rpm for 5 min in a Sorval RC-5 centrifuge. The protein concentration of a myofibrillar suspension was determined spectrophotometrically in 5% (w/v) SDS using $E^{1\%}_{280nm} = 7.0$ cm^{-1} (Sutoh & Harrington, 1977). Myofibrils (1 mg/mL) were modified in the relaxation buffer with 1.2×10^{-4} M pNPM for 60 min at 4 °C. The reaction was stopped by the addition of 6 mM DTT. The modified myofibrils (pNPM–myofibrils) were washed 3 times by centrifugation in 5 volumes of standard buffer containing 0.1 M NaCl, 10 mM imidazole, and 2 mM $MgCl_2$, pH 7.0. These myofibrils could not contract and did not have any Mg^{2+}-ATPase activity as expected for SH_1- and SH_2-modified myosin. When the pNPM–myofibrils were chymotryptically digested in the presence of EDTA and then centrifuged in the presence of MgATP, more than 95% of the generated S-1 remained in the supernatant. This showed that, as required for relaxation conditions, myosin heads in pNPM–myofibrils remained dissociated from actin filaments in the presence of MgATP.

EDC Fixation of Rigor Myofibrils. To prevent contraction, myofibrils were cross-linked with EDC using procedures similar to those of Glyn and Sleep (1985). Myofibrils stored in 50% glycerol were washed as described above in a buffer containing 0.1 M NaCl and 100 mM MES, pH 6.5. The cross-linking of 1 mg/mL myofibrils was carried out for 10–30 min at 4 °C in the same buffer containing 10 mM EDC. The reaction was stopped with the addition of 0.3 M β-mercaptoethanol, followed by three washings in 0.1 M NaCl, 10 mM imidazole, and 2 mM $MgCl_2$, pH 7.0, buffer. As observed under a light microscope, myofibrils cross-linked for 30 min or longer did not contract upon activation with MgATP and Ca^{2+}. Chymotryptic digestions and subsequent SDS gel electrophoretic analysis of such myofibrils revealed that virtually all myosin rods and less than 25% of the myosin heads were cross-linked after 30 min (Figure 1).

Mg^{2+}-ATPase activity of cross-linked myofibrils was measured in the presence of 5 mM MgATP and 1 mM $CaCl_2$ at 22 °C. Mg^{2+}-, Ca^{2+}-, and EDTA(K^+)-ATPase activities of S-1 isolated from cross-linked myofibrils were assayed according to the procedures of Kielley and Bradley (1956). S-1 was extracted from EDC-cross-linked myofibrils by digesting the myofibrils with chymotrypsin at a weight ratio of 1:50 for 45 min at 22 °C and their subsequent centrifugation in the presence of 5 mM MgATP. The supernatant containing S-1 was dialyzed against a buffer containing 0.1 M NaCl and 10 mM imidazole, pH 7.0, before ATPase measurements.

Tryptic Digestions of Myofibrils. All digestions of myofibrils were performed in the presence of 0.1 M NaCl, 10 mM imidazole, and 2 mM $MgCl_2$, pH 7.0, unless indicated otherwise. Rigor, relaxed, and activated myofibrils (0.5 mg/mL) were digested at 22 °C using a 1:50 weight ratio of trypsin to myofibrils. Rigor digestions were carried out with native myofibrils in the presence of 1 mM Ca^{2+}. For relaxed-state digestions, cross-linked and pNPM–myofibrils were tryptically cleaved in relaxation buffer containing 8 mM EGTA. Cross-linked myofibrils were relaxed by 10-min incubations in 0.2 M NaCl containing 3 mM MgADP·V_i and 0.1 mM ATP. pNPM–myofibrils were relaxed by 5 mM MgATP. Under active-state conditions, EDC–myofibrils were digested in the presence of 5 mM MgATP and 1 mM Ca^{2+}. In all digestion experiments, the reactions were stopped at given time intervals by the addition of soybean trypsin inhibitor (3× enzyme concentration). The digested myofibrils were washed with 0.1 M NaCl and 10 mM imidazole, pH 7, and then adjusted to 3 mg/mL for chymotryptic digestions (1:50 chymotrypsin:myofibril ratio, w/w). Chymotryptic digestions to generate S-1 were carried out in the presence of 8 mM EDTA for 60 min at 22 °C and were stopped with PMSF (3× enzyme concentration). The resulting samples were denatured and run on SDS–polyacrylamide gel electrophoresis.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out according to the procedure of Laemmli (1970) using 7.5% (w/w) polyacrylamide gels.

Densitometric Measurements and Digestion Rates. The optical densities of Coomassie Blue stained protein bands and the respective mass distributions were determined with a Biomed Model SL-504-X soft laser scanning densitometer (Fullerton, CA) interfaced with an Apple II-e personal computer. Since actin is resistant to tryptic digestion under the

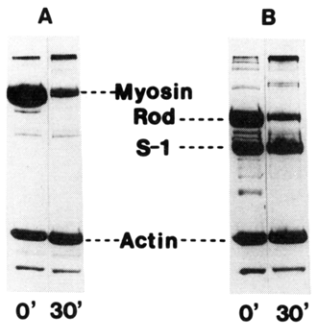

FIGURE 1: Representative SDS–polyacrylamide gels showing the cross-linking of (A) myosin and (B) S-1 and myosin rods due to EDC treatment of myofibrils. Myofibrils were cross-linked with 10 mM EDC in 0.1 M NaCl and 100 mM MES, pH 6.5 (4 °C). Aliquots of the cross-linking reaction were run on SDS gels to monitor myosin cross-linking (A). The same reaction samples were also digested with chymotrypsin (1:50 chymotrypsin:myofibrils ratio, w/w, in the presence of 4 mM EDTA) to generate S-1 and rods and then run on SDS gels (B) to measure the cross-linking of these fragments.

employed conditions, the intensities of all protein bands were normalized to that of the actin band to account for gel loading variations. The differences in dye absorption were corrected by dividing the normalized band intensities by the molecular weights of the respective protein species.

Digestion rates were obtained by monitoring the decay in intensity of the respective bands as a function of digestion time. All decay curves were fitted by single exponentials and yielded first-order rate constants for cleavage of S-1 and myosin (Applegate et al., 1984; Duong & Reisler, 1987b).

Measurement of the Binding of Myosin Heads to Actin in Myofibrils by the Tryptic Rates Method. The rigorlike binding of myosin to actin in cross-linked myofibrils during ATP hydrolysis was measured in tryptic digestion experiments as previously described (Applegate et al., 1984; Duong & Reisler, 1987a,b). Actomyosin binding was determined from the measured cleavage rates, k_2, at the 50K/20K junction of the myosin head. These rates were obtained by monitoring the combined intensities of the 95- and 70-kDa protein bands for S-1 and the 225- and 200-kDa bands for myosin. The two reference rates, $k_{2,A}$ and $k_{2,D}$, were determined by measuring the cleavage rates of rigor and relaxed myofibrils, respectively. The percentage (β) of myosin heads bound to actin during ATP hydrolysis was calculated according to

$$\beta = 100[(k_{2,D} - k_2)/(k_{2,D} - k_{2,A})] \quad (1)$$

Results

Properties of EDC-Cross-Linked Myofibrils

Cross-Linking of Myosin and MgATPase Activity of Myofibrils. According to the previous study of Glyn and Sleep (1985), the contraction of rabbit skeletal myofibrils could be blocked by cross-linking with EDC. This effect was achieved without substantial changes in the active-state ATPase of myofibrils and major cross-linking of myosin to actin. The results of Glyn and Sleep (1985) are confirmed in the present work under rather similar myofibril cross-linking conditions. As shown in Figure 1, EDC treatment of myofibrils results in extensive cross-linking of myosin heavy chains through their rod portions. This is evident from the large decrease in the intensity of myosin and myosin rod bands on SDS gels (Figure 1) following EDC treatment of myofibrils. It is also clear from Figure 1 that the parallel cross-linking of myosin heads is much smaller than that of the rods. Time profiles of EDC reactions

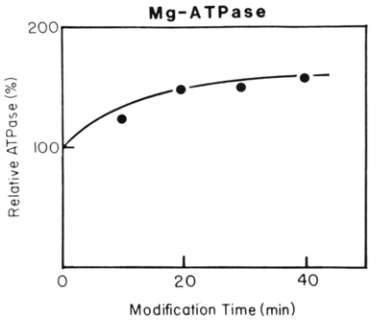

FIGURE 2: Effect of EDC modification on Mg^{2+}-ATPase activity of myofibrils. The modification was carried out as described in Figure 1, and Mg^{2+}-ATPase activity of cross-linked myofibrils was measured in the presence of 5 mM MgATP and 1 mM $CaCl_2$ at 22°C.

with myofibrils (after appropriate digestions, SDS gel electrophoresis, and densitometric analysis of protein bands) reveal the cross-linking of about 80% myosin and myosin rods and at most a 25% loss of S-1 within the first 30 min of the cross-linking (similar to Figure 1; not shown). It should be noted that very little (<5%) if any actin was cross-linked during these reactions. Thus, while some of the observed S-1 cross-linking may be attributed to its reaction with actin, part of S-1 is probably "lost" due to its covalent bonding to the thick filament backbone and the myosin light chains.

The MgATPase activity of myofibrils increases with their cross-linking under rigor conditions (0.1 M NaCl and 0.1 M MES, pH 6.5), reaching a maximum activity level (150%) within 20 or 30 min into the reaction (Figure 2). The elevated ATPase activity is very likely due to the limited cross-linking of myosin heads to actin (see above). Previous solution studies indeed established that cross-linked actomyosin complexes have greatly increased ATP turnover rates (Mornet et al., 1981).

Observation of the EDC-cross-linked myofibrils under the light microscope revealed a complete loss of their capability to contract in the presence of MgATP and Ca^{2+} after a 20- or 30-min fixation reaction. Shorter cross-linking reactions resulted in local, nonuniform loss of myofibrillar contraction in any examined sample.

ATPase Activities of S-1 Isolated from EDC-Cross-Linked Myofibrils. Earlier work indicated the presence on S-1 of an essential carboxylic group which could be blocked by using high concentrations of EDC (between 50 and 100 mM) in the presence of nucleophiles (Lacombe et al., 1981). Such modification inactivated the S-1. In view of this observation, it was necessary to verify that EDC treatment of myofibrils did not inhibit or alter the enzymatic function of myosin heads. It was conceivable that enzymatic changes in un-cross-linked myosin heads could be masked by the activation of MgATPase activity in the cross-linked molecules. Thus, the experimental test of myosin ATPase activities involved the isolation of S-1 from EDC-cross-linked myofibrils and the comparison of its activities with those of control S-1 preparations. Since the pH dependence of Ca^{2+}- and EDTA(K^+)-ATPases in myosin is particularly sensitive to S-1 modifications (Sekine & Kielley, 1964), the activity assays were done over a range of pH values.

Figure 3 shows that the ATPase activities of the two preparations of S-1, from cross-linked myofibrils (for 30 min) and unmodified myosin, are very close and similarly affected by pH conditions. The pH profiles of the Ca^{2+}- and EDTA-(K^+)-ATPase activities resemble those reported by Sekine and Kielley (1964). The MgATPase activities of these S-1 preparations (at pH 7.0) differed by between 10 and 20%. It appears then that the ATPase activities of the isolated myosin heads, which are the same as the un-cross-linked heads

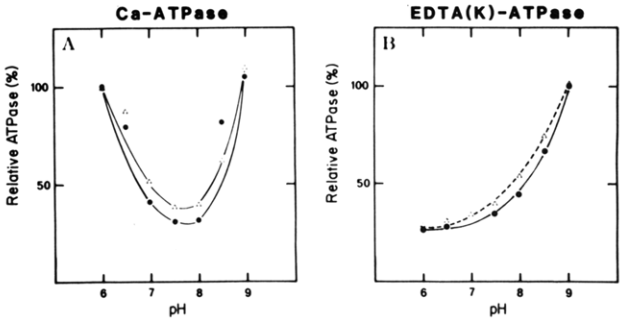

FIGURE 3: Effect of pH on the relative (A) Ca^{2+}- and (B) EDTA-(K^+)-stimulated ATPase activities at 37 °C of S-1. (●) Control S-1; (△) S-1 isolated from myofibrils cross-linked for 30 min with EDC. Cross-linking conditions are described in Figure 1. 100% ATPase activities correspond to turnover rates of 1.9 and 10 s^{-1} for Ca^{2+}- and EDTA(K^+)-stimulated ATPase activities, respectively.

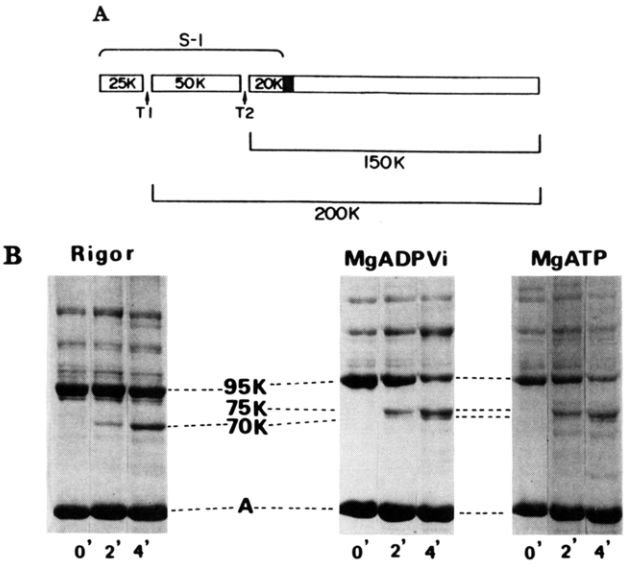

FIGURE 4: (A) Scheme for the limited tryptic digestion of the myosin heavy chain in myofibrils. Under the conditions of this work, the tryptic digestion is limited to myosin heads with very little, if any, cleavage occurring in the HMM/LMM hinge region. (B) Representative SDS–polyacrylamide gels showing tryptic cleavage of myofibrils (1:50 trypsin:myofibrils ratio, w/w) under rigor, relaxed ($MgADP{\cdot}V_i$), and activating (MgATP) conditions. The digestions were carried out in 0.1 M NaCl, 2 mM $MgCl_2$, and 10 mM imidazole, at pH 7.0 and 22 °C. In EDC-cross-linked myofibrils, the tryptic digestion of myosin heads could be followed on SDS gels after chymotryptic cleavage of all the processed samples to generate free S-1. The relaxed state was obtained in the presence of 0.2 M NaCl by adding 3 mM $MgADP{\cdot}V_i$ and 0.1 mM MgATP to cross-linked myofibrils (or by adding 5 mM MgATP and 8 mM EGTA to pNPM-modified myofibrils). Cross-linked myofibrils were activated with 5 mM MgATP and 2 mM $CaCl_2$. Rigor digestions were performed with native and cross-linked myofibrils. Myofibrils were cross-linked with EDC for 30 min under conditions described in Figure 1. "A" stands for actin.

monitored in tryptic digestion experiments, are little affected by the EDC cross-linking conditions employed in this work.

Tryptic Digestions of Myofibrils

Rigor Conditions. As shown before (Lowell & Harrington, 1981), tryptic cleavage of myosin in rigor myofibrils occurs mainly at the 25K/50K head junction (T1 site in Figure 4a) and yields the 200- and 25-kDa heavy-chain fragments. Accordingly, S-1 prepared by chymotryptic digestion of such material is composed of the 70K and 25K fragments (Figure 4B; the smaller fragment is not displayed on this gel).

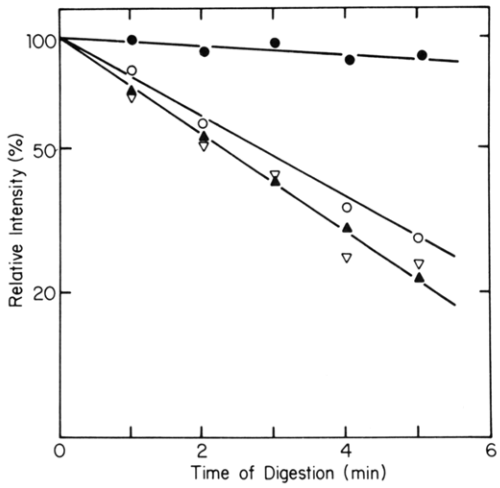

FIGURE 5: Time course of tryptic cleavage of myosin in myofibrils under rigor (●), relaxed [(△) MgADPVi; (▽) pNPM], and activating conditions (○). Relative intensities under the 225- and 200-kDa (▽) or the 95- and 70-kDa (●, ▲, ○)protein bands from a separate and complete set of electrophoretic patterns similar to those shown in Figure 4 were combined and plotted against digestion time to measure the cleavage rate at the 50K/20K junction (band intensity = 100% at time = 0).

In contrast to the T1 proteolytic site, the cleavage at the 50K/20K head junction in myosin (T2 site) is greatly inhibited by the binding of cross-bridges to actin (Lowell & Harrington, 1981; Chen & Reisler, 1984). This is evident from the virtual absence of the 75K S-1 or the 150K myosin fragments in digestions of rigor myofibrils (Figure 4B). Nevertheless, as documented before (Azarcon et al., 1985; Duong & Reisler, 1987a,b), the information on the rate of cleavage ($k_{2,A}$) at the T2 site in myosin bound to actin is required for normalization purposes in all proteolytic determinations of actomyosin binding (see eq 1).

The rate $k_{2,A}$ was obtained by plotting the combined relative intensities of the 95K and 70K myosin heavy-chain bands as a function of digestion time of rigor myofibrils (Azarcon et al., 1985; Duong & Reisler, 1987a,b). The slope of the resulting decay curve (Figure 5) yields the rate of cleavage $k_{2,A}$ and shows a slow proteolysis at the 50K/20K site in myosin attached to actin.

Relaxed, pNPM-Modified Myofibrils. One consequence of the proteolytic susceptibility of troponin is that a truly relaxed state of myofibrils can be achieved in tryptic digestion experiments only in the absence of any significant enzymatic activity of myosin (see Materials and Methods). The complete inactivation of myosin ATPase activities was attained through modifications of relaxed myofibrils (in the presence of MgATP) with pNPM, a monofunctional analogue of pPDM. Control modifications of S-1 with this reagent (not shown) followed the familiar pattern of changes in ATPase activities as expected for the labeling of the reactive SH_1 and SH_2 groups on myosin (Reisler, 1982). On this basis, it was assumed that the modification of the reactive cysteine residues was indeed responsible for the inactivation of myofibrils by pNPM. Since the inactivated cross-bridges could be completely dissociated from actin by MgATP, the modified myofibrils were suitable for tryptic digestion experiments carried out under relaxed conditions.

SDS gel patterns of tryptic digestions of relaxed, pNPM-modified myofibrils revealed (in contrast to rigor digestions) a rapid decay in the intensity of the 225K band followed by the accumulation of a 150K fragment (not shown). This

cleavage pattern is due to a rapid tryptic attack at the T2 site (the 50K/20K junction), as expected for detached myosin heads (Chen & Reisler, 1984; Azarcon et al., 1985). The rate of tryptic cleavage of detached myosin at this site, $k_{2,D}$, was determined by plotting the combined intensities of the 225- and 200-kDa bands vs the time of reaction (Figure 5). It may be noted from Figure 5 that the cleavage of dissociated heads is about 10-fold faster than that of attached heads, $k_{2,A}$, in rigor myofibrils.

Relaxed (ADP·V_i), EDC-Cross-Linked Myofibrils. Previous reports showed that MgADP·V_i dissociated S-1 from actin (Goodno & Taylor, 1982) and relaxed muscle fibers (Goody et al., 1980). In our hands, the tryptic cleavage of myosin heads in myofibrils equilibrated with 3 mM MgADP·V_i in standard salt conditions (0.1 M NaCl) was slower than that observed for pNPM-modified myofibrils in the presence of MgATP. This indicated incomplete dissociation of myosin cross-bridges from actin by MgADP·V_i.

A separate set of tryptic digestion experiments carried out on myofibrils under different ionic strength conditions suggested that complete dissociation of myosin from actin by 3 mM MgADP·V_i was attained in the presence of 0.2 M NaCl (or somewhat below). Similarly, complete dissociation of S-1 from actin was observed under these conditions in ultracentrifugation experiments. In order to avoid any extraction of myosin under these salt conditions, the myofibrils were cross-linked with EDC. Since such cross-linking affects via myosin rods a major fraction of myosin molecules (Figure 1), digestions of relaxed, EDC-cross-linked myofibrils were followed directly at the level of myosin heads. The S-1 bands monitored in these experiments were generated by a second chymotryptic digestion of the tryptically cleaved myofibrils (Materials and Methods).

The rapid cleavage of myosin heavy chain in myofibrils relaxed in 0.2 M NaCl with 3 mM MgADP·V_i (and 0.1 mM MgATP) is evident from the fast decay in the intensity of the 95K S-1 heavy-chain band (Figure 4B). The ready attack of the 50K/20K junction by trypsin is also indicated by the appearance of the 75K intermediate product (Chen et al., 1987; Applegate et al., 1984). The rate of cleavage at the 50K/20K junction of detached heads was determined by plotting the combined intensities of the 95- and 70-kDa bands against time of digestion. As expected, the $k_{2,D}$ rates determined for cross-linked myofibrils incubated with MgADP·V_i (in 0.2 M NaCl) and pNPM-modified myofibrils in the presence of MgATP were similar if not identical (Figure 5).

Activated Myofibrils. Tryptic digestions under active-state conditions (0.1 M NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM MgATP, and 10 mM imidazole, pH 7.0) were carried out on EDC-cross-linked myofibrils which could not contract. The time course of tryptic reactions was monitored by following the time-dependent cleavage of S-1 (see above and in Materials and Methods). As shown in Figure 4B, the cleavage of myosin in the presence of MgATP occurred mainly at the 50K/20K head junction, yielding the 75K fragment.

The cleavage rate, k_2, was determined as described above for the digestion of relaxed, EDC-cross-linked myofibrils. As evident from Figure 5, k_2 observed for activated myofibrils is closer to $k_{2,D}$ than to $k_{2,A}$. This indicates that a relatively small fraction of heads is bound to actin in myofibrils during ATP hydrolysis. The actual percentage of myosin binding to actin under such conditions was calculated according to eq 1, from the measured rates of myosin cleavage k_2, $k_{2,A}$, and $k_{2,D}$. By using the two reference relaxation states, one induced by MgADP·V_i and the second by pNPM modification of myofibrils and the addition of MgATP, we calculated the rigorlike binding of myosin to actin in the presence of ATP at 25 ± 7% and 23 ± 6%, respectively (Table I). Similar binding of myosin to actin in activated muscle fibers was reported by Cooke et al. (1982).

Table I: Binding of Myosin Heads to Actin in Cross-Linked Myofibrils during ATP Hydrolysis[a]

method of relaxation	binding during ATP hydrolysis (%)
3 mM MgADP·V_i, 0.1 mM MgATP	25 ± 7 (n = 4)
pNPM modification, 5 mM MgATP	23 ± 6 (n = 3)

[a] The binding of myosin heads to actin during ATP hydrolysis in EDC-cross-linked myofibrils at 22 °C was determined from the rates of tryptic cleavage of myosin heads under such conditions relative to cleavage rates in rigor and relaxed myofibrils (eq 1). The relaxed state of myofibrils was induced either by 3 mM MgADP·V_i + 0.1 mM MgATP in 0.2 M NaCl or by *p*-nitrophenylenemaleimide (pNPM) modification of myofibrils and the addition of MgATP.

Discussion

The main objective of this work was to obtain an independent measurement of cross-bridge binding to actin in myofibrils during ATP hydrolysis by using the previously developed proteolytic rates method (Azarcon et al., 1985). The method was recently tested on an acto–S-1 system (Chen et al., 1987) and shown to yield the same data on the binding of actin and S-1 in the presence of nucleotides as those dervied from standard ultracentrifugation experiments (Duong & Reisler, 1987a).

Proteolytic experiments with myofibrils present several difficulties. The first complication, caused by contraction of myofibrils in the presence of MgATP, could be circumvented by their cross-linking with EDC. The cross-linking reaction blocked the contraction of myofibrils and yet satisfied the crucial requirement for minimal perturbation of actomyosin interactions which were the very subject of our digestion studies. The majority of myosin heads (at least 75%) were left un-cross-linked after the EDC treatment, and the MgATPase activity of myofibrils was only moderately (up to 50%) increased in that process. Moreover, it was previously shown (Glyn & Sleep, 1985) that EDC cross-linking of muscle fibers or myofibrils did not significantly affect the V_{max} (maximal ATPase rate) and K_m (MgATPase concentration needed for half the maximal ATPase rate) of these systems.

Because of the unavoidable partial cross-linking of myosin heads, only the remaining 75 or 80% unfixed heads were sampled in tryptic digestion experiments. When isolated under nondenaturing conditions, the myofibrillar S-1 showed all the kinetic properties of standard S-1 preparations. Thus, the very heads analyzed in our experiments appeared unaffected by EDC. Also, judging from the time course of EDC reactions (not shown), the modification did not involve any biased attack on a population of myosin molecules. The selective tryptic analysis of the un-cross-linked heads was attained by chymotryptic cleavage of myofibrils. Only unfixed heads would yield isolated S-1 species. The properties of this 75–80% unaffected heads were considered representative of the entire myosin head population.

Another complication inherent in the proteolytic approach to measurements of actomyosin binding in relaxed myofibrils (or fibers) was related to the rapid degradation of troponin by trypsin. This necessitated the inhibition of myosin's MgATPase activity (by modification or with MgADP·V_i) in order to maintain the relaxed state of myofibrils during their tryptic digestions.

In the context of this work, a more important consequence of troponin degradation is the implicit assumption that such an event does not affect actomyosin interactions during active-state conditions in myofibrils. This assumption is supported by the previous observations that myosin ATPase shows the same activation (V_{max}) by unregulated actin and regulated actin in the presence of Ca^{2+} [e.g., see King and Green (1987)]. Thus, irrespective of the actual mechanism by which Ca^{2+} removes the inhibitory effects of the regulatory system, the actomyosin interactions in myofibrils activated by Ca^{2+} and in those containing damaged, desensitized troponin–tropomyosin complexes should be similar if not identical.

It should be emphasized that the calculations of cross-bridge binding to actin in active myofibrils are normalized to a scale in which rigor interactions are equated with 100% binding and those under relaxed conditions are set at zero binding of myosin to actin. Solution airfuge experiments with actin and S-1 justify such procedure (this work; Duong & Reisler, 1987a). Moreover, sufficient evidence has been obtained in previous studies to show that in the rigor state virtually all myosin heads are bound to actin (Cooke & Franks, 1980; Lovell & Harrington, 1981). Regarding the relaxed state, most recent estimates of weakly attached cross-bridges in relaxed skinned rabbit psoas fibers (in μ = 170 mM) range between 2 and 10% (Brenner et al., 1986). Under the conditions of the present work, such binding would be further reduced. Thus, it is unlikely that anything but a minor numerical error is involved in the choice of the above normalization scale for measurements of actomyosin binding. More significantly, however, according to eq 1 (i.e., by choosing rigor and relaxedlike conditions for the binding reference states), the 23 or 25% attachment of cross-bridges to actin in the presence of ATP represents rigorlike interactions. Thus, any attached states of cross-bridges that have different (relaxedlike?) proteolytic susceptibility would not be accounted for in these measurements. Although the experiments on acto–S-1 solutions in the presence of ATP and other nucleotides do not reveal any proteolytically unusual acto–S-1 binding states, their existence in myofibrils cannot be ruled out.

The binding of cross-bridges to actin in active myofibrils agrees well with some recent observations and interpretations of actomyosin binding. Huxley and Kress (1985) estimated from X-ray diffraction studies that no more than 20–30% of myosin heads were attached in rigor configuration in contracting frog muscle and perhaps up to 40% could be weakly attached to actin. Low estimates (20%) of rigorlike actomyosin binding in activated fibers were also obtained in electron paramagnetic resonance measurements (Cooke et al., 1984) and through a combination of biochemical and physiological data (Brenner et al., 1986). It is likely that the high-range estimates, >70%, of cross-bridge attachment to actin in active fibers stem from the apparent contributions of weakly bound heads (Burghardt & Ajtai, 1985). Similarly, the weakly attached cross-bridges might be responsible for the enhancement of the 5.9-nm actin layer line in diffraction patterns of activated fibers (Matsubara et al., 1984; Kress et al., 1984).

The information on the binding of myosin to actin in contracting muscle is valuable for the interpretation of many structural, mechanical, and biochemical results, and the modeling of cross-bridge action in muscle. The main value of the present investigation is that it provides an independent measurement of cross-bridge attachment to actin in active myofibrils. The obvious advantages of the proteolytic rates method used in this work are the easy interpretation of digestion data, the lack of any fluorescent or spin-label probes on the myosin heads, and the fact that this method involves a different set of assumptions and constraints than those applicable in other studies. The results of the present work confirm the finding that only a small fraction of myosin heads (~25%) is tightly bound to actin in active myofibrils (Cooke, 1986) and may help to resolve the dispute on this important issue.

Registry No. ATPase, 9000-83-3; ATP, 56-65-5.

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Detection of Nearest Neighbors to Specific Fluorescently Tagged Ligands in Rod Outer Segment and Lymphocyte Plasma Membranes by Photosensitization of 5-Iodonaphthyl 1-Azide†

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ABSTRACT: Lima bean agglutinin–fluorescein 5-isothiocyanate conjugate (FluNCS–lima bean lectin) interacts with specific receptor molecules on membranes both from the rod outer segment (ROS) of the frog retina and from S_{49} mouse lymphoma cells. When [^{125}I]-5-iodonaphthyl 1-azide (^{125}I-INA), which freely and randomly partitions into the lipid bilayer, is added to membranes and the suspension is irradiated at 480 nm, the FluNCS-conjugated lectin photosensitizes the [^{125}I]INA but only at discrete sites. This results in the selective labeling of specific proteins: an 88-kDa protein on ROS membranes and a 56-kDa protein on S_{49} plasma membranes. Labeling is dependent upon the interaction of the FluNCS–lectin with glycosylated receptor sites, since *N*-acetylgalactosamine, but not methyl α-mannoside, blocked labeling of the 56-kDa protein on S_{49} membranes. In contrast, a random labeling pattern of membrane proteins was observed upon irradiation at 480 nm using other fluorescein conjugates, such as FluNCS–bovine serum albumin (FluNCS–BSA) or FluNCS–soybean trypsin inhibitor (FluNCS–STI), which interact with cell membranes in a nonselective manner, or with *N*-(fluorescein-5-thiocarbamoyl)-*n*-undecyclamine (FluNCS-NHC_{11}), which is freely miscible in the membrane lipid. Random labeling was also obtained by direct photoexcitation of [^{125}I]INA at 314 nm, with no distinct labeling of the 88- and 56-kDa proteins in the respective membranes. These results suggest that protein ligands can be used to guide sensitizers to discrete receptor sites and lead to their selective labeling by photosensitized activation of [^{125}I]INA [Raviv, Y., Salomon, Y., Gitler, C., & Bercovici, T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6103–6107]. Site-directed labeling is obtained by an amplification process that locally and time-dependently intensifies the radioactive signal, thus revealing minor membranal components that could not otherwise be visualized by random labeling. This approach provides a method that offers new possibilities for application in different fields of chemical and biological research.

Elucidation of the dynamic pattern of interactions among molecules in highly complex cell membranes, or other biological structures, is an extremely difficult task. Progress in the understanding of the processes that underly these interactions requires methods of ever higher resolving power in terms of both time and space. For this purpose, techniques that permit the analysis of spatial proximity of macromolecules and identification of the nearest-neighbor components have been developed. These include chemical and photochemical cross-linking, affinity or photoaffinity labeling, and topological labeling. These techniques use NaDodSO$_4$–PAGE[1] as a tool for the resolution and isolation of the proteins which have been radioactively labeled or otherwise tagged. In chemical cross-linking, bifunctional reagents are reacted with the membrane and the cross-linked products formed are analyzed by NaDodSO$_4$–PAGE. Cleavable cross-linking reagents enable the regeneration of the cross-linked components. This technique has been very successfully applied in biological

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[1] Abbreviations: αMM, methyl α-mannoside; BSA, bovine serum albumin; Con A, concanavalin A; FluNCS, fluorescein 5-isothiocyanate; FluNCS-NHC_{11}, *N*-(fluorescein-5-thiocarbamoyl)-*n*-undecylamine; INA, 5-iodonaphthyl 1-azide; NaDodSO$_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NAGA, *N*-acetylgalactosamine; PSL, photosensitized labeling; PBS, phosphate-buffered saline; ROS, rod outer segment.

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