A Structural and Kinetic Study on Myofibrils Prevented from Shortening by Chemical Cross-Linking†

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Received February 11, 1993; Revised Manuscript Received April 30, 1993

ABSTRACT: In previous work, we studied the early steps of the Mg^{2+}-ATPase activity of Ca^{2+}-activated myofibrils [Houadjeto, M., Travers, F., & Barman, T. (1992) *Biochemistry 31*, 1564–1569]. The myofibrils were free to contract, and the results obtained refer to the ATPase cycle of myofibrils contracting with no external load. Here we studied the ATPase of myofibrils contracting isometrically. To prevent shortening, we cross-linked them with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). SDS–PAGE and Western blot analyses showed that the myosin rods were extensively cross-linked and that 8% of the myosin heads were cross-linked to the thin filament. The transient kinetics of the cross-linked myofibrils were studied in 0.1 M potassium acetate, pH 7.4 and 4 °C, by the rapid-flow quench method. The ATP binding steps were studied by the cold ATP chase and the cleavage and release of products steps by the P_i burst method. In P_i burst experiments, the sizes of the bursts were equal within experimental error to the ATPase site concentrations (as determined by the cold ATP chase methods) for both cross-linked (isometric) and un-cross-linked (isotonic) myofibrils. This shows that in both cases the rate-limiting step is after the cleavage of ATP. When cross-linked, the k_{cat} of Ca^{2+}-activated myofibrils was reduced from 1.7 to 0.8 s^{-1}. This is consistent with the observation that fibers shortening at moderate velocity have a higher ATPase activity than isometric fibers. Under relaxing conditions ($-Ca^{2+}$), the k_{cat} remains large (0.6 s^{-1}) presumably due to rigor activation induced by the 8% heads cross-linked to the thin filaments.

A major problem in muscle contraction is to relate the physiological events that occur to the different steps of the Mg^{2+}-ATPase of the myosin heads. Extensive kinetic studies have been carried out on dispersed molecules such as myosin and actomyosin [e.g., see Trentham et al. (1976), Taylor (1979), Adelstein and Eisenberg (1980), and Geeves (1991)]. However, in these systems the chemical activity is not coupled to mechanical work, and the organized structure of thick and thin filaments is not preserved. The use of skinned fibers allows control of the mechanical behavior, but the limited rate of diffusion of molecules into the center of the fiber prevents the transient kinetic experiments which were so powerful in elucidating the acto–myosin mechanism from being applied.

One way to overcome some of these problems is to use caged adenosine 5′-triphosphate (ATP).[1] Thus, tension measurements with caged ATP allowed for an estimate of the rate of ATP binding (Goldman et al., 1984a,b). Further, by the use of cold hammers to stop the reaction, Ferenczi et al. (1984) showed that there was a substantial P_i burst per myosin head. However, with caged ATP, it is difficult to do ATP chase experiments, so the method does not allow for the titration of ATPase sites.

Another approach is to use myofibrils. These are sufficiently small that diffusion does not limit the use of rapid reaction methods and yet large enough for their shortening to be studied under the light microscope. A range of experiments have been carried out on myofibrils, and the ATP binding and hydrolysis steps have been characterized (Taylor, 1990; Houadjeto et al., 1991, 1992; Herrmann et al., 1992). The kinetics of ATP binding and of the forward rate of the hydrolysis step are the same as for subfragment 1 of myosin (S1). One limitation of this work is that with the exception of experiments of the single-turnover type (Sleep, 1981), myofibrils shorten and overcontract. The critical point is not the overcontraction, which occurs after the events of interest, but the fact that the steady state being approached is one of shortening at moderate velocity. It would be of interest to determine the kinetics of the approach to the state of shortening at V_{max} and also to the isometric state. The first experiment is not possible because the "K_m" for ATP for shortening is 150 μM (Cooke & Bialek, 1979) and one cannot do P_i burst experiments with myofibrils at the required high ATP concentrations.

We have approached the isometric state by using chemically cross-linked myofibrils to prevent contraction. Glyn and Sleep (1985) cross-linked myofibrils with the zero-length cross-linker EDC and obtained a material that did not shorten upon the addition of ATP and Ca^{2+}. Duong and Reisler (1989) tested a number of cross-linking agents and concluded that EDC was the most effective in preventing the shortening of myofibrils. Muscle fibers have also been cross-linked with EDC (Tawada & Kimura, 1986; Tawada & Kawai, 1990).

Here, we studied the structural and kinetic properties of myofibrils cross-linked with EDC. In agreement with Duong

† This work was supported by a grant from EEC [SCI 0327-C(J)R]. C.H. was supported by an INSERM fellowship. J.S. is grateful to the Royal Society for a travel grant.

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; S1, myosin subfragment 1; Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic orthophosphate; PP_i, inorganic pyrophosphate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; NHS, *N*-hydroxysulfosuccinimide; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; MES, 2-(*N*-morpholino)-ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IgG, immunoglobulin G.

0006-2960/93/0432-7255$04.00/0

and Reisler (1989), we found that the rods were extensively cross-linked but, in addition, about 8% of the myosin heads were cross-linked to the thin filament. When activated, the ATPase activity of the cross-linked myofibrils was about half that of the native ones. However, in the absence of Ca^{2+}, their ATPase activity remained high which suggests that when cross-linked, myofibrils are no longer regulated.

MATERIALS AND METHODS

Proteins and Reagents. Myosin and its subfragment S1 and actin were prepared from rabbit muscle (Weeds & Taylor, 1975; Spudich & Watt, 1971, respectively).

NHS was from Fluka AG (Buchs, Switzerland); EDC, apyrase, and chymotrypsin (TLCK treated) were from Sigma Chemical Co; PMSF was from SERVA (Heidelberg, Germany); and leupeptin and pepstatin were from Boehringer Mannheim (Mannheim, Germany). [γ-^{32}P]ATP was from Amersham International.

Myofibril Preparation. The following procedure is a modification of that described by Knight and Trinick (1985). All the steps were carried out as near as possible to 0 °C. The myofibrils were prepared from rabbit psoas muscle which had been depleted of ATP by leaving the skinned and degutted animal at 4 °C overnight. The psoas muscles were removed, chopped into lengths of about 0.5 cm, and homogenized in 10 volumes of ice-cold buffer A (50 mM Tris, pH 7.4, 0.1 M potassium acetate, 5 mM KCl, 2 mM DTT, 0.2 mM PMSF, 10 μM leupeptin, 5 μM pepstatin, and 0.5 mM sodium azide) containing 5 mM EDTA and 0.5% Triton X-100 using a Sorval Omnimix at maximum speed (six homogenizations for 10 s with a 30-s rest between each). The myofibrils were recovered by low-speed centrifugation (2000*g* for 10 min) and washed 2 times in buffer A containing 5 mM EDTA and 0.5% Triton X-100 and then 3 times in buffer A containing only 2 mM magnesium acetate. The washed myofibrils were kept on ice in this last buffer. Typically, 12 g of psoas muscle gave 1.2 g of myofibrils. The average sarcomere length was 2.0 (±0.2) μm.

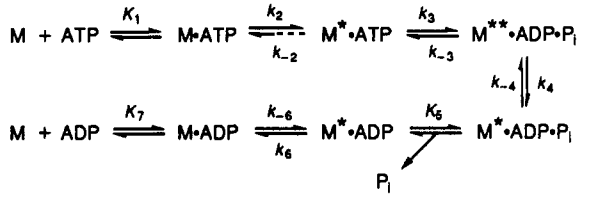
The concentrations of myosin head (by absorbance at 280 nm) and ATPase site (by cold ATP chase) were determined as described previously (Houadjeto et al., 1992).

Cross-Linking of Myofibrils. Myofibrils stored as above were washed in 0.1 M MES buffer, pH 7, containing 3 mM magnesium acetate and 3 mM EGTA. The cross-linking of myofibrils at 5–10 mg/mL was carried out at 4 °C in the same buffer containing 5 mM NHS (Staros et al., 1986) and 2 mM EDC. After incubation for different times, the reaction was stopped by the addition of 25 mM glycine (pH 8) and 10 mM DTT, followed by centrifugation. In certain experiments, the myofibrils were cross-linked in the presence of 4 mM ATP, 5 mM PP_i, 5 mM magnesium acetate, and 150 mM potassium acetate. For subsequent experimentation, the cross-linked myofibrils were washed 2 times and resuspended in the experimental buffer.

Chymotryptic Digestion of Myofibrils. Digestion of myofibrils (5–10 mg/mL) was carried out in 0.1 M MES and 10 mM EDTA (pH 7) at 25 °C for 30 min using a 1/200 w/w ratio of chymotrypsin to myofibrils. The reaction mixtures were quenched by 0.1 mg/mL PMSF and made ready for SDS–PAGE by the addition of 50% glycerol, 5% 2-mercaptoethanol, and 1.5% SDS in 50 mM Tris, pH 8.

SDS–PAGE and Western Blot Analysis. Cross-linked myofibrils were analyzed directly or following chymotryptic digestion by SDS–PAGE using a linear 3–15% gradient as already described (Mornet et al., 1981). Densitometric scanning of the gels was carried out with a Shimadzu CS-930 scanner connected to a computerized integrator.

Scheme I



Western blot studies were performed after electrophoretic transfer from acrylamide gels to nitrocellulose sheets (0.45 μm) as described in Towbin et al. (1979). Actin-containing bands were revealed by using polyclonal antibodies specifically directed against the N-terminal actin segment of residues 1–12 isolated from rabbit immune antisera raised against the DNase I–G-actin complex (Polzar et al., 1989). The presence of the S1 heavy chain was revealed by polyclonal antibodies directed against the N-terminal 27-kDa tryptic fragment of the heavy chain, obtained from rabbit immune antisera raised against the fragment isolated from SDS–PAGE.

Protein bands stained with the polyclonal antibodies were visualized by goat anti-rabbit IgG, conjugated to horseradish peroxidase with α-naphthol as substrate.

Estimation of the Amount of Cross-Linked Acto–S1 in EDC-Treated Myofibrils. The amount of S1 cross-linked to actin in EDC-treated myofibrils was estimated by densitometric scanning of SDS–PAGE. The myofibrils were treated with EDC for different times, digested with chymotrypsin, and then loaded on SDS–PAGE at two different concentrations. The higher concentration was used for the estimation of the acto–S1 adducts (e.g., Figure 2). A concentration of a tenth of this was used to determine the amounts of actin and S1 heavy chain, with reference to standard curves (Figure 3).

In the densitometric scanning, the amounts of actin and S1 heavy chain in the actin–S1 adducts were estimated assuming a stoichiometry of 1/1 and using the relationship $Q_t = Q_a + 1.14Q_s$ where Q_t is the total amount of the acto–S1 adducts and Q_a and Q_s are the amounts of actin and S1 heavy chain in the cross-linked products, respectively. The factor of 1.14 is used to correct for the relative uptake of the Coomassie blue stain by the actin and S1 heavy chains (Sutoh, 1983). The amounts of actin and S1 cross-linked were expressed as proportions of the total amounts in the myofibrils.

Microscopic Studies. In order to check the extent of contraction of the myofibrils after cross-linking, the average sarcomer length was measured under the microscope using phase-contrast optics with a 63× planapo objective. Contraction of the myofibrils was tested by the addition of 0.1 mM Ca^{2+} and 5 mM Mg^{2+}-ATP.

Kinetic Experiments. These were carried out by the rapid-flow quench method (Barman & Travers, 1985). There were two types of experiments. In *ATP chase experiments*, S1 or myofibrils plus [γ-^{32}P]ATP reaction mixture are first quenched in a large excess of unlabeled ATP–Mg^{2+} (50 mM) in a rapid-flow quench apparatus, incubated on ice for 2 min, and then quenched in 22% trichloroacetic acid (final concentration 11%), and [^{32}P]P_i is determined. In these experiments, one measures the kinetics of binding of ATP to the myosin heads with $k = k_2[ATP]/K_1 + [ATP]$ (Scheme I). For a full discussion of the ATP chase technique, see Barman and Travers (1985).

In *P_i burst experiments*, reaction mixtures are quenched directly in acid, and the [^{32}P]P_i is determined. In these, one

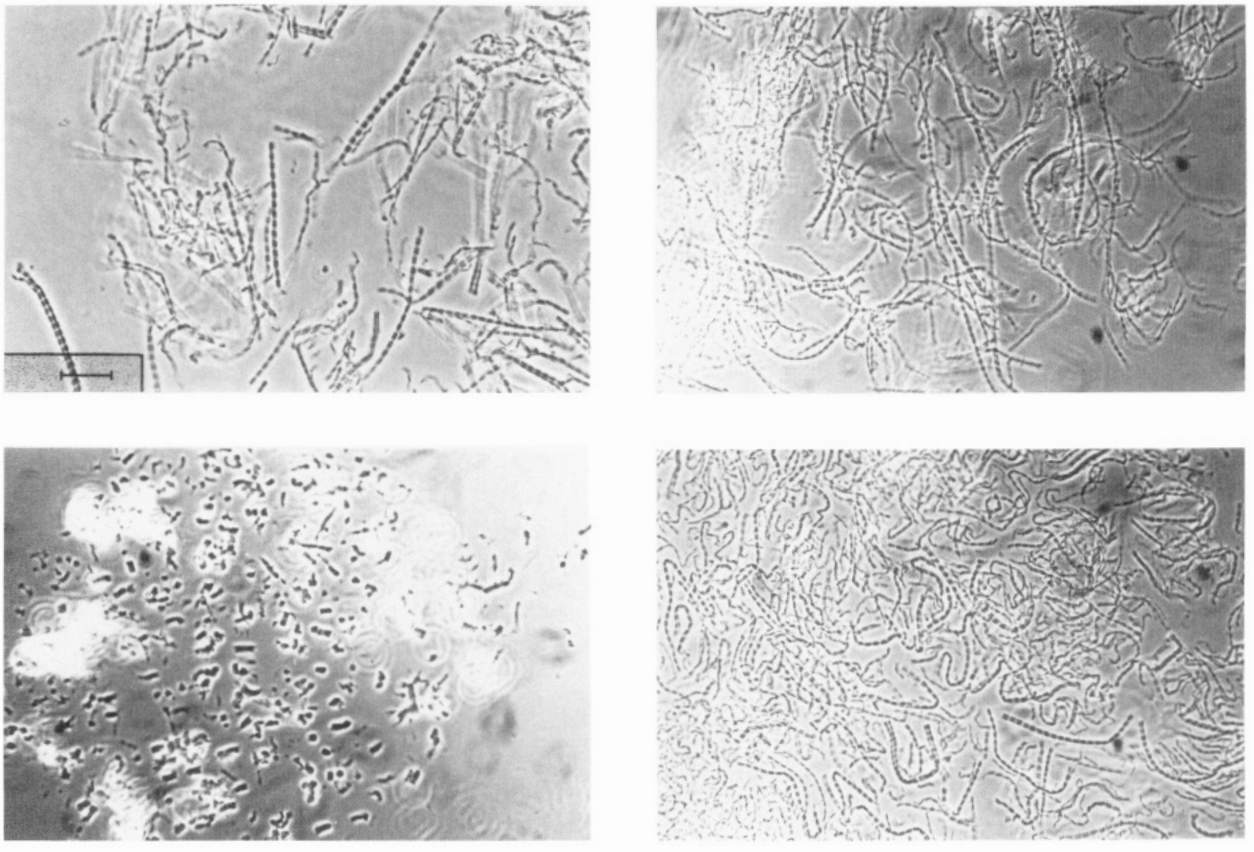


FIGURE 1: Photomicrographs of myofibrils before and after treatment with EDC. The untreated myofibrils are shown in the two panels on the left and the EDC-treated myofibrils on the right. In the top two panels, the myofibrils were in rigor; in the bottom panels, ATP and Ca^{2+} had been added. For further details, see Materials and Methods.

obtains a rapid burst of P_i which is followed by a steady-state rate.

The data obtained were interpreted by Scheme I where M represents the myosin heads in myofibrils with or without actin interaction. The scheme does not take account the interactions of the heads with actin.

Unless otherwise stated, all experiments were carried out at 4 °C in 0.1 M potassium acetate, 5 mM KCl, and 50 mM Tris adjusted to pH 7.4 with acetic acid. With *relaxed myofibrils*, the buffer included 5 mM magnesium acetate and 2 mM EGTA, and with *activated myofibrils*, 2 mM magnesium acetate and 0.1 mM $CaCl_2$.

The data were treated according to Houadjeto et al. (1992). Comparative experiments (i.e., cross-linked myofibrils versus un-cross-linked myofibrils) were carried out in the same day and on the same preparation.

RESULTS

Structural Studies on Myofibrils Cross-Linked with EDC

Our aim was to obtain the chemical kinetics of myofibrils during isometric contraction. To attain this goal, we treated them with the chemical cross-linker EDC under different conditions with the following as criteria: first, the overall myofibrillar structure should be unchanged; second, the myofibrils should no longer contract upon the addition of ATP and Ca^{2+}; and third, their ATPase sites should remain intact.

Cross-Linking Conditions. The conditions usually employed to cross-link acto–myosin or acto–S1 filaments [25 °C, short reactions times; e.g., see Bertrand et al. (1988)] were unsuitable with myofibrils. The sarcomere structure appeared to be unaffected, but the treated myofibrils still contracted upon the addition of ATP and Ca^{2+}. Increasing the reaction time did not help matters as it led to aggregation. A satisfactory material was obtained when the temperature was reduced to 4 °C with a reaction time of 90 min. As shown in Figure 1, the overall structures of the myofibrils thus treated appeared to be unchanged (although we cannot exclude a slight shortening of the sarcomers), but when the reaction time was greater than 90 min, aggregation occurred. The cross-linked myofibrils did not contract upon the addition of Mg^{2+}-ATP and Ca^{2+} (Figure 1), and their ATPase sites were apparently unaffected (from kinetic studies, below).

The Rods Are Heavily Cross-Linked. To define the specificity and the extent of protein modification caused by EDC treatment, the myofibrils were analyzed by SDS–PAGE. A time-dependent study revealed that among the different proteins only the amounts of myosin, and to a lesser extent actin and tropomyosin, were reduced (Figure 2A, left panel, and Figure 3A). These reductions were further analyzed in chymotryptic digests of the myofibrils at each cross-linking time. As shown in Figures 2A, right panel, and 3B, the amount of rod decreased dramatically during EDC treatment (up to 90% decrease after 90-min cross-linking) whereas the S1 heavy chain was only slightly reduced (up to 10%). These results show that the predominant cross-linking products involve the rods. The products were not seen in Figures 2 and 3, presumably because they were too large to enter the gels.

Some of the Myosin Heads Are Cross-Linked to the Actin Filament. When chymotryptic digests of the cross-linked myofibrils were investigated by SDS–PAGE, there appeared two new protein bands of molecular mass 165–175 kDa (Figure 2A, right). These bands increased with the cross-linking time, and from their molecular masses, they were good candidates

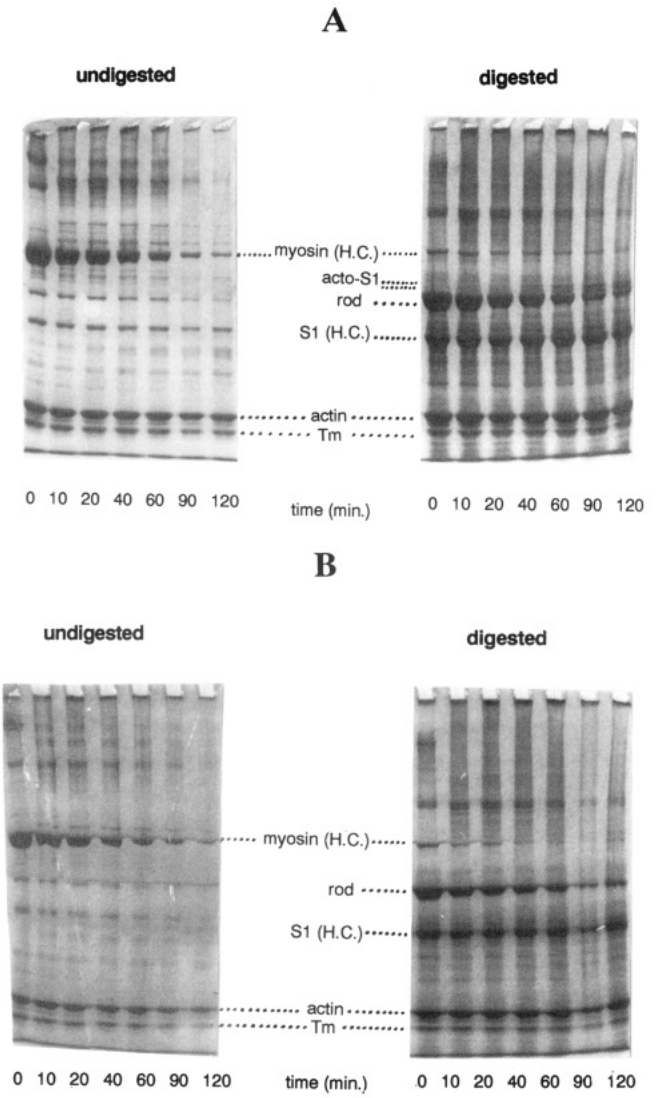


FIGURE 2: SDS–PAGE of myofibrils cross-linked under rigor conditions (A) and relaxing conditions (B) as a function of cross-linking time. The left gels refer to undigested myofibrils and the right gels to myofibrils digested with chymotrypsin. The main bands are labeled and refer to myosin heavy chain (H.C., 250 kDa), actin–S1 heavy-chain cross-linked products (acto–S1, 165–175 kDa), myosin rod (140 kDa), S1 heavy chain (S1 H.C., 95 kDa), actin (42 kDa), and tropomyosin (Tm, 33 kDa).

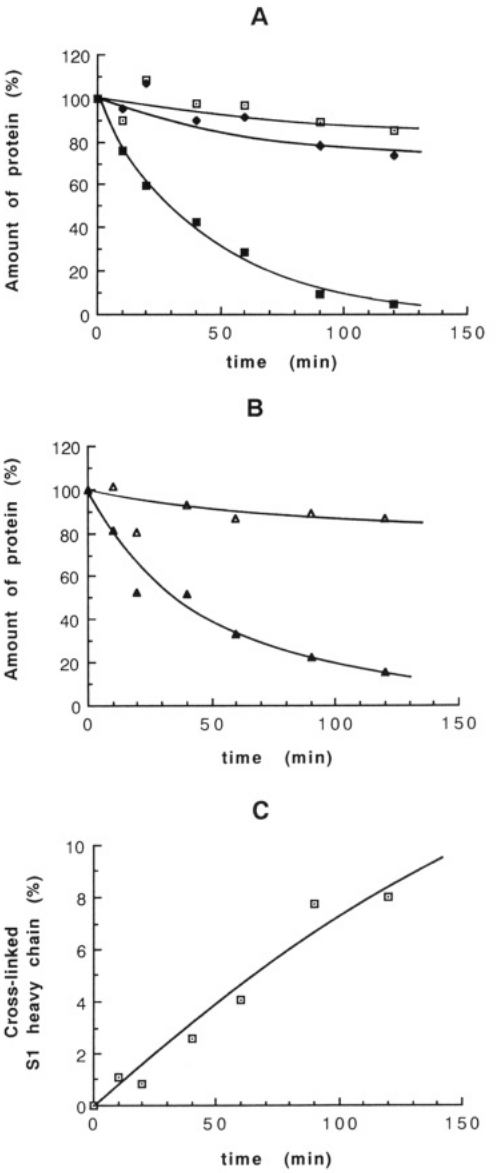


FIGURE 3: Effect of cross-linking time on certain proteins of myofibrils. The SDS–PAGEs in Figure 2 were scanned as described under Materials and Methods. (A) Actin (□), myosin (■), and tropomyosin (♦) bands in undigested cross-linked myofibrils. (B) and (C) refer to chymotryptic digests of myofibrils: (B) S1 heavy chain (△) and rod (▲) bands; (C) cross-linked acto–S1 heavy-chain products. The amount of S1 heavy chain cross-linked to actin was estimated as described under Materials and Methods and is given as a percentage of the total S1 heavy chain.

for the acto–S1 heavy-chain cross-linked products (Bertrand et al., 1988). To confirm this, we analyzed the bands immunochemically by Western blot using antibodies directed specifically against actin or S1 heavy chain as described in Figure 4.

The specificities of the antibodies were checked beforehand on a mixture of actin and S1, and the results were as anticipated (lanes d). When the actin plus S1 mixture was treated with EDC in the same way as the myofibrils, the 165–175-kDa doublet appeared (lanes e). Following these control experiments, we now subjected the myofibrillar preparations to the same analysis.

With *un-cross-linked* and undigested myofibrils, the analysis revealed as major bands the myosin heavy chain, actin, and tropomyosin. In the corresponding Western blots, the predominant bands were the myosin heavy chain and actin, revealed by anti-S1 (lanes a) and anti-actin, respectively. After chymotryptic digestion of the myofibrils (lanes Da), rods and S1 appeared, and, as expected, only S1 was revealed by anti-S1. Taken together, these results illustrate the specificity of the antibodies used: first, the anti-S1 did not cross-react with the rod fragment of myosin; second, the anti-actin did not cross-react significantly with the other myofibrillar proteins.

With *cross-linked*, undigested myofibrils, the gel patterns obtained (lanes b) were similar to those with un-cross-linked myofibrils, except for the appearance of actin oligomers. After chymotryptic digestion, actin and again its oligomers and S1 (lanes Db) were revealed. However, most important, only the 165–175-kDa doublet reacted with *both* antibodies. These data strongly suggest that the 165–175-kDa bands obtained after cross-linking myofibrils under rigor conditions contain both actin and S1 and, therefore, that they correspond to the two acto–S1 complexes obtained when purified actin and S1 are cross-linked.

Finally, the amount of acto–S1 heavy-chain adduct was quantified by scanning the gel electrophoresis pattern of the chymotryptic digest of the cross-linked myofibrils. As shown

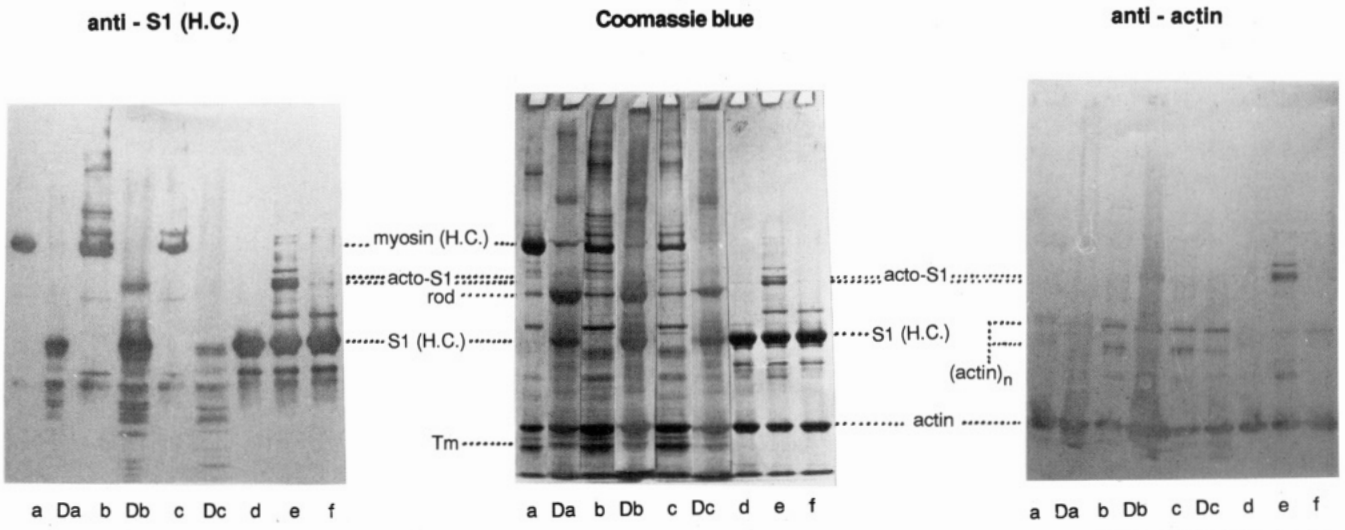


FIGURE 4: Western blot analyses of SDS–PAGE runs of myofibrils and S1 plus actin, treated or untreated with EDC. Gels stained with Coomassie blue (middle) and Western blot analyses with anti-S1 and anti-actin, respectively. Lanes a, un-cross-linked myofibrils; lanes b, myofibrils cross-linked for 90 min under rigor conditions; lanes c, myofibrils cross-linked for 90 min under relaxing conditions; lanes d, actin plus S1 (2 to 1 molar ratio); lanes e, actin plus S1 (as in lanes d) cross-linked for 90 min under rigor conditions; lanes f, actin plus S1 (as in lanes d) cross-linked for 90 min under relaxing conditions. Lanes Da, Db, and Dc represent chymotryptic digests of the materials in lanes a, b, and c, respectively. The bands are labeled as in Figure 2.

Table I: Effect of Treating S1 with EDC on Its ATPase Activity[a]

expt	burst size, ([^{32}P]P_i/S1, mol/mol)	steady-state rate (s^{-1})
1	0.46 (±0.02)	0.017
2	0.35 (±0.02)	0.015
3	0.028 (±0.009)	0.006
4	0.29 (±0.02)	0.014

[a] S1 (50 μM) was incubated for 90 min with EDC, quenching agents, and, in experiments 3 and 4, relaxing agents added in different orders. Experiment 1, EDC, S1, incubated, and then quenched; experiment 2, quenching agents, EDC then S1, and incubated; experiment 3, relaxing agents, EDC then S1, incubated, and then quenched; experiment 4, relaxing, quenching agents, EDC then S1, and incubated. The concentrations of the reagents were as follows: cross-linking agents, 2 mM EDC + 5 mM *N*-hydroxysulfosuccinimide; quenching agents, 10 mM DTT + 25 mM glycine; relaxing agents, 4 mM ATP + 5 mM PP_i + 5 mM magnesium acetate + 0.2 M potassium acetate. The other conditions were 0.1 M MES buffer, pH 7, 4 °C. After the 90-min incubations, 10 μg/mL apyrase was added, and the mixtures were kept at 4 °C overnight. For the kinetic experiments, the treated S1 mixtures were diluted 100× in 50 mM Tris, 0.1 M potassium acetate, 5 mM KCl, and 2 mM magnesium acetate pH 7.4. The final reaction mixtures (0.5 μM S1 + 2 μM [γ-^{32}P]ATP) were quenched in acid, and the [^{32}P]P_i was determined.

in Figure 3C, we estimate that after a 90-min cross-linking reaction, 8–10% of S1 was present in the 165–175-kDa bands (i.e., the acto-S1-cross-linked products). This estimate is in good agreement with the 10% loss of the S1 heavy chain during the 90-min cross-linking time (Figure 3A), suggesting that most of the S1 heavy chain that is cross-linked is covalently linked to actin. Further, we estimate that about 3% of the total actin in the myofibrils was present in the acto–S1 adducts. If we assume that the molar ratio of actin to myosin head in myofibrils is 3/1 (Yates & Greaser, 1983), this is in good agreement with the S1 estimations above.

The cross-linked myofibrils investigated above had been treated with EDC under rigor conditions. Under these conditions, the rods were cross-linked extensively, but some of the heads were also cross-linked to the actin filament. It seemed important to evaluate the relative importance of these two types of cross-link, first, in preventing shortening and, second, in modifying the myofibrillar ATPase activities (see below).

To prevent the S1–actin cross-links, the myofibrils were treated with EDC under relaxing conditions, i.e., in the presence of ATP and PP_i. As shown in Figure 4 (lanes f), when a mixture of actin and S1 was treated with EDC under these conditions, the acto–S1 adducts were not formed.

Myofibrils treated with EDC under relaxing conditions were now analyzed. As shown in Figure 2B, the rods were extensively cross-linked, as with the myofibrils cross-linked in rigor. However, the myosin heavy chain and actin bands appeared to be unchanged. When a chymotryptic digest of these myofibrils was investigated, the 165–175-kDa bands were absent (Figures 2B). This absence was confirmed by Western blot analysis (Figure 4, lanes Dc). Thus, when myofibrils are cross-linked under relaxing conditions, only the rods appear to be cross-linked. Interestingly, Mg^{2+}-ATP or Mg^{2+}-PP_i alone was less effective in reducing the 165–175-kDa bands. The myofibrils cross-linked under relaxing conditions no longer contracted upon the addition of ATP and Ca^{2+}, but disappointingly, their Ca^{2+}-activated ATPase activity was reduced greatly (see below).

Kinetic Studies on Myofibrils Cross-Linked with EDC

We now studied the chemical kinetics of the myofibrils cross-linked as described under Materials and Methods. Their structural properties are given above. However, first we determined the effect of our cross-linking procedure on S1 ATPase.

Effect of EDC Treatment on the ATPase Activity of S1. Chaussepied and Morales (1988) showed that when S1 was treated with EDC, its Mg^{2+}-ATPase activity increased 3.5×. Their experiments were carried out under different conditions from ours (10 mM EDC at 20 °C for 60 min), and it was important to determine the effect of EDC on S1 under the experimental conditions used here (2 mM EDC at 4 °C for 90 min).

As shown in Table I, S1 was treated with EDC under "rigor" and "relaxing" conditions with the reagents added in different orders. The reaction mixtures were then diluted in the kinetic buffer, and P_i burst experiments were carried out from which the parameters are a *P_i burst size* and a *steady-state rate* (see Materials and Methods). The S1 preparation used in these experiments titrated 0.6 mol of active site per mole of S1 protein, so with a K_3 of 1.7 (Houadjeto et al., 1992), the

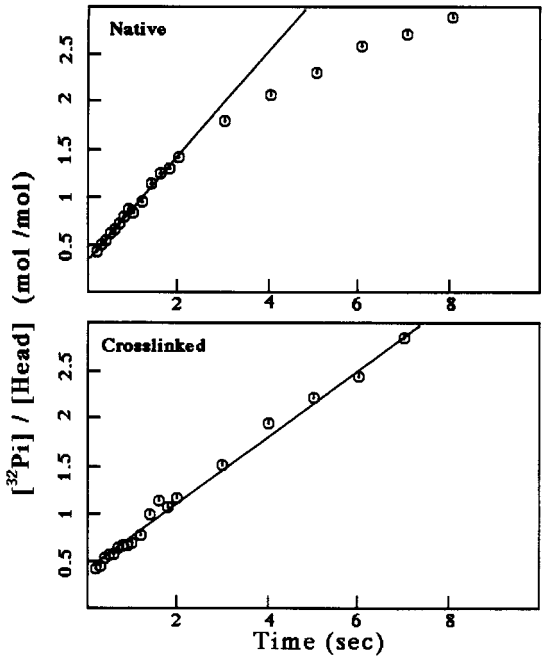


FIGURE 5: Steady-state time courses of native and cross-linked myofibrils activated by Ca^{2+} at 4 °C. The reaction mixtures (6 μM myosin heads + 30 μM [γ-^{32}P]ATP) were quenched in acid, and the [^{32}P]P_i was determined. The buffer was 50 mM Tris–acetate, pH 7.4, 0.1 M potassium acetate, 5 mM KCl, 2 mM magnesium acetate, and 0.1 mM $CaCl_2$. The steady-state parameters obtained (burst size and steady-state rates) are given in Table II (experiment A).

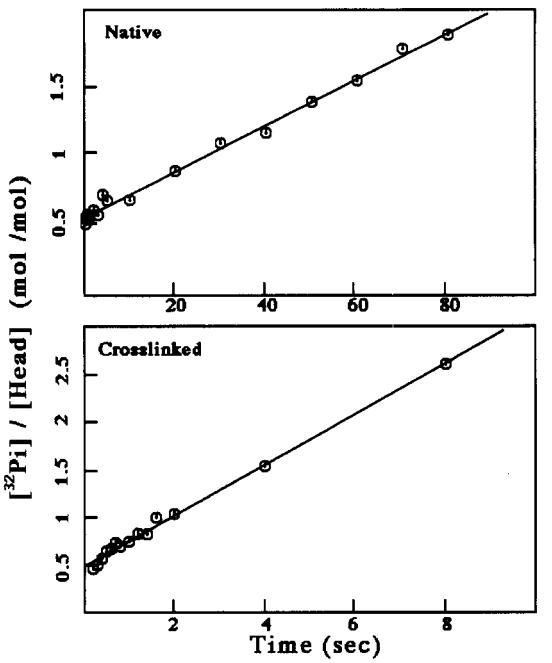


FIGURE 6: Steady-state time courses of native and cross-linked myofibrils under relaxing conditions at 4 °C. The reaction mixtures (6 μM myosin heads + 30 μM [γ-^{32}P]ATP) were quenched in acid, and the [^{32}P]P_i was determined. The steady-state parameters obtained are given in Table II (experiment A).

expected burst size is 0.38 mol of P_i/mol of S1. The results obtained are summarized in Table I.

In experiment 1, S1 was treated with EDC under the conditions used to cross-link myofibrils. Experiment 2 is a control in that the EDC quenching agents (DTT, glycine) were added before the carbodiimide. EDC treatment increased somewhat the burst size, but the steady-state rate was less affected. We conclude that under our cross-linking conditions, the ATPase site of S1 is not modified significantly.

In experiment 3, S1 was treated with EDC in the presence of ATP and PP_i (i.e., under relaxing conditions for myofibrils). In the subsequent kinetic experiments, interference from any ATP or ADP was reduced by treatment with apyrase. There was a dramatic reduction in the P_i burst size, but the steady-state rate was less affected. The reduction in the burst size is probably not due to remaining nucleotides or PP_i in the kinetic reaction mixtures. Thus, when S1 was treated in the same way but with the EDC quenching agents added before the EDC (experiment 4), the burst size was only slightly smaller than in the control experiment (experiment 2).

Effect of Cross-Linking on the Overall Myofibrillar ATPases. The effects of cross-linking on the ATPase activities of activated and relaxed myofibrils are shown in Figures 5 and 6. These experiments were carried out on the same preparation of myofibrils, and the kinetic parameters obtained (burst sizes, steady-state rates, and k_{cat}) are summarized in Table II under experiment A. k_{cat} values are usually obtained by dividing steady-state rates by cold ATP chase amplitudes (i.e., [ATPase sites]; Barman & Travers, 1985). Here we divided the steady-state rates by P_i burst amplitudes as with myofibrils chase and P_i bursts have identical sizes (see below). Also included in Table II are the steady-state parameters obtained with a different myofibrillar preparation (experiment B).

The differences in the sizes of the bursts in experiments A and B are noteworthy. Several P_i burst experiments were

Table II: Steady-State Kinetic Constants for Cross-Linked Myofibrils[a]

material	burst size ([^{32}P]P_i/S1, mol/mol)		steady-state rate (s^{-1})		k_{cat} (s^{-1})	
	expt A	expt B	expt A	expt B	expt A	expt B
un-cross-linked myofibrils						
$+Ca^{2+}$	0.35	0.57	0.56	0.98	1.6	1.7
$-Ca^{2+}$	0.36	0.57	0.016	0.016	0.044	0.028
cross-linked myofibrils						
$+Ca^{2+}$	0.44	0.55	0.35	0.43	0.80	0.78
$-Ca^{2+}$	0.44	0.53	0.29	0.26	0.66	0.49

[a] The data are from two sets of experiments on different preparations. k_{cat} values were obtained by dividing steady-state rates by burst sizes.

carried out, and the amplitudes varied in the range 0.3–0.6 mol of P_i/mol of myosin head. This variation seems to depend upon the myofibrillar preparation rather than on any treatment to which it had been subjected (e.g., cross-linking or storage for 2–3 days at 4 °C). Although fewer ATP chase experiments were carried out, there seems to be a variation in their amplitudes too. Some of the variability is probably due to the difficulties in obtaining accurate values for the myosin head concentrations, but this is not the full explanation. There was little variation in the derived k_{cat} as the steady-state rates were proportional to the burst sizes.

We have already discussed the problem of the variability of P_i burst amplitudes with un-cross-linked myofibrils (Houadjeto et al., 1992) and with myosin (Tesi et al., 1989). It required care in the interpretation of burst sizes, and when we determined the effect of cross-linking on the ATPases of myofibrils or the effect of Ca^{2+} activation or when we compared burst sizes (see below), the experiments were carried out on the same myofibrillar preparation and on the same day.

An important conclusion from Figures 5 and 6 is that as judged from the similarities of the P_i burst sizes in the different experiments, the ATPase site in the myosin head appears to

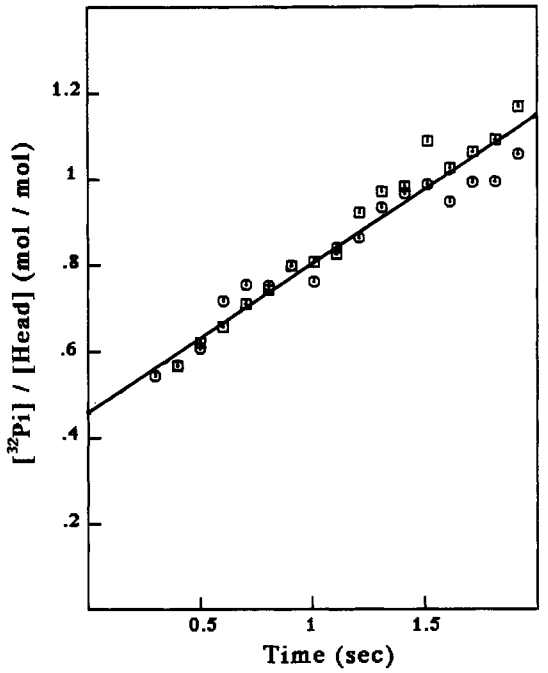


FIGURE 7: ATP chase and P_i burst experiments with cross-linked myofibrils in the steady state. The reaction mixtures (6 μM myosin heads + 15 μM [γ-^{32}P]ATP) were quenched in cold ATP (O) or acid (□), and the [^{32}P]P_i was determined.

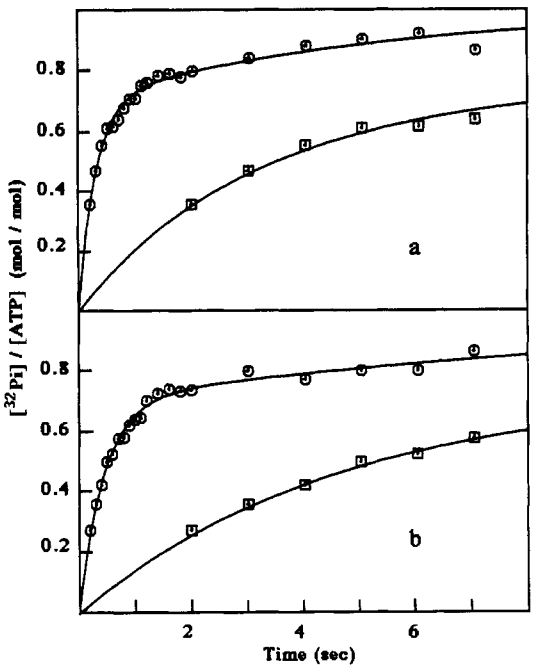


FIGURE 8: Single-turnover time courses of the hydrolysis of ATP by native (a) and cross-linked (b) myofibrils at 4 °C in activating buffer (see Figure 5). The reaction mixtures (7.5 μM myosin heads + 0.1 μM [γ-^{32}P]ATP) were quenched in acid, and the [^{32}P]P_i was determined. For points (O), the time scale is as indicated; for (□), it is divided by 10. In each experiment, the points were computer-fitted to two exponentials.

be unaffected by the cross-linking procedure. This is in agreement with the results of the S1 cross-linking experiments (Table I).

We now consider two further features of our results. First, take the overall progress curves with the activated myofibrils (Figure 5). With native myofibrils, the progress curve consists of three phases: an initial P_i burst (kinetics not obtained on the time scale used); a rapid steady-state rate; and then a break at about 2 s to a slow steady-state rate. This is in agreement with our previous results (Houadjeto et al., 1991) in which we suggested that the fast steady-state rate is that of the rapidly contracting myofibrils. This, we argued, leads to overcontraction, a loss of myofibrillar structure, and a reduction in ATPase activity. With cross-linked myofibrils, there were only two phases: a P_i burst phase followed by a rapid steady-state rate. Here there was no break in ATPase activity. Since these myofibrils did not shorten upon the addition of Ca^{2+} and ATP, this is evidence that their ATPase activity corresponds to that of myofibrils prevented from shortening.

Second, consider the regulatory properties of the cross-linked myofibrils (Figure 6). With untreated myofibrils, the ratio k_{cat} activated to k_{cat} relaxed was at least 35/1 [a minimum value for the k_{cat} of regulated myofibrils has yet to be determined; for a discussion, see Herrmann et al. (1992)]. However, with cross-linked myofibrils, the ratio was only about 1.3/1. This low ratio can almost entirely be accounted for by the high k_{cat} obtained in the absence of Ca^{2+}.

Effects of Cross-Linking on the Tight Binding of ATP and the Cleavage Step of Myofibrillar ATPase. The results of ATP chase and P_i burst experiments with activated cross-linked myofibrils are illustrated in Figure 7. To make the estimates of the two burst sizes as accurate as possible, the experiments were carried out at the low ratio of myosin heads to [γ-^{32}P]ATP of 1/2.5. The points in the two experiments could be fitted to the same progress curve, giving a burst amplitude of 0.46 mol of P_i/mol of myosin head and a steady-state rate of 0.34 s^{-1}.

We come to two conclusions from these experiments. First, from the amplitude in the chase experiment, it appears that the cross-linking procedure does not affect the tight binding of ATP to myofibrils (Houadjeto et al., 1992; Herrmann et al., 1992). This tight binding was confirmed by chase experiments carried out under single-turnover conditions. Three experiments were carried out with reaction mixtures containing 3 μM in myosin heads at three different concentrations of [γ-^{32}P]ATP: 0.17, 1.5, and 3 μM. The reaction mixtures were quenched in 25 mM cold ATP, and the [^{32}P]P_i was determined after a 2-min incubation period on ice. In the experiments, within an error range of 2%, all the [γ-^{32}P]ATP was recovered as [^{32}P]P_i, which shows that $k_{ATPase} > k_{off}$ for ATP, i.e., $k_{-2} < k_{cat}$ [for a discussion on the tight binding of ATP to activated myofibrils, see Houadjeto et al. (1992)]. In chase experiments, therefore, we assume that the amplitude of the rapid rise is equal to the ATPase site concentration. Thus, by dividing the steady-state rate by the amplitude, we obtain a k_{cat} of 0.74 s^{-1}, in good agreement with the value obtained at 30 μM ATP (Table I). This similarity of the k_{cat} at 15 and 30 μM shows that the K_m for the ATP is much less than 15 μM, as with un-cross-linked myofibrils (Herrmann et al., 1992). Second, from the amplitudes in the P_i burst and chase experiments, we were able to determine the fraction of heads in the ATP and products states. Since the amplitudes in the two experiments were equal within experimental error, less than 10% of the heads are in the ATP state in the steady state.

P_i Burst Experiments under Single-Turnover Conditions. Single-turnover experiments (i.e., experiments in which [myosin] $\gg$ [ATP]) can give information on the kinetics of the early steps (binding, cleavage) as well as on those of the release of products (Bagshaw & Trentham, 1973).

Typical P_i burst experiments with native and cross-linked myofibrils under activating conditions are illustrated in Figure 8. In each, there was a rapid phase followed by a slow one

leading to the complete hydrolysis of the ATP. The rapid phases were fitted to single exponentials with (for native myofibrils) a rate of 3.3 (±0.3) s^{-1} and an amplitude of 0.70 (±0.02) mol of P_i/mol of ATP and (for cross-linked myofibrils) kinetics of 2.3 (±0.2) s^{-1} and an amplitude of 0.70 (±0.02) mol of P_i/mol of ATP.

The rapid phases represent the formation of the ADP·P_i state (as with myosin) and/or free P_i. As the concentrations of active sites and ATP were low, the kinetics of the phases (k) are a function primarily of the second-order binding constant for ATP binding (k_2/K_1). With native myofibrils, $k_2/K_1 = 1\ \mu M^{-1}\ s^{-1}$ (Herrmann et al., 1992), and with an ATPase site concentration of 4.1 μM (titrated by the ATP chase method), $k = 4.1\ s^{-1}$. This is in reasonable agreement with the 3.3 s^{-1} obtained.

The cross-linking procedure did not significantly change k. This suggests that the kinetics of the binding of ATP to myofibrils are not affected significantly by the cross-linking procedure.

To what process can we attribute the slow phase? With myosin, a slow phase is observed in single-turnover experiments (due to a relatively small value for K_3) which is controlled by the release of P_i (Bagshaw & Trentham, 1973). However, with the activated myofibrils, we cannot attribute the slow phases to this step because it is too slow: 0.19 (±0.03) s^{-1} for native and 0.09 (±0.02) s^{-1} for cross-linked myofibrils, compared with k_{cat} values of 1.6 and 0.8 s^{-1}, respectively. Instead, we suggest that the slow phases with the myofibrils are due to the slow release of ATP from secondary ATP sites. These are probably limited to myosin as none of the other major proteins of the myofibril is capable of binding ATP in the concentration range studied (Maruyama & Weber, 1972). The reader is referred to Tesi et al. (1989) for a discussion of this problem. In our single-turnover experiments, therefore, we did not discern the kinetics of the release of products step. This confirms that the fraction of heads in the ATP state with myofibrils remains low when they are cross-linked.

ATPases of Myofibrils Cross-Linked under Relaxing Conditions. We showed above that in myofibrils that had been treated with EDC under relaxing conditions, only the rods appeared to be cross-linked. In particular, we could not detect any myosin head–actin adducts. These myofibrils did not contract upon the addition of ATP and Ca^{2+}, and we now investigated their ATPase activities by determining the two steady-state parameters (P_i burst size and steady-state rate) in P_i burst experiments. The parameters were obtained under relaxing ($-Ca^{2+}$) and activating ($+Ca^{2+}$) conditions.

In the absence of Ca^{2+}, both the P_i burst and steady-state rates were reduced to about 50%. Under activating conditions, there was the same reduction in the P_i burst size, and, in addition, there was a dramatic reduction in the steady-state rate to less than 20% of that found with myofibrils cross-linked in rigor (results not illustrated).

DISCUSSION

The objective of our work was to obtain the chemical kinetics of myofibrils that contract isometrically and to compare these with the kinetics of myofibrils that contract isotonically (i.e., that are free to contract). We prevented shortening by cross-linking them with EDC. Under our cross-linking conditions (i.e., in the absence of ATP), the ATPase site appeared to be unaffected by the treatment, whether in myofibrils or in S1.

Structural Studies. We show that when myofibrils are treated with EDC, the rods are heavily cross-linked. In addition, 8% of the myosin heads are cross-linked to the thin filament. The latter type of cross-link was first shown by SDS–PAGE of chymotryptic digests of cross-linked myofibrils and then confirmed by a Western blot study. We cannot exclude other types of cross-link that were not detected by our analytical methods.

Previous workers have prevented myofibrils from shortening by cross-linking them chemically with EDC (Glyn & Sleep, 1985; Duong & Reisler, 1989) or enzymically with a bacterial transaminase (Huang et al., 1992). Where structural studies have been carried out, it was found, as here, that the rods were extensively cross-linked. In agreement with our work, Duong and Reisler (1989) showed that the cross-linking led to a loss of myosin heads but they gave no evidence for the cross-linking of these to the thin filament. Tawada and Kimura (1986) treated single-skinned fibers with EDC and concluded, first, that the rods were extensively cross-linked and, second, that as much as 18% of the myosin heads were cross-linked, presumably to the thin filament, but they did not present evidence for the acto–S1 adducts.

Kinetic Studies: Myofibrils Cross-Linked under Rigor Conditions. (1) Overall ATPases. When the myofibrils were cross-linked, they no longer contracted upon the addition of ATP and Ca^{2+}, and their k_{cat} was reduced from 1.7 to 0.8 s^{-1}. Although we cannot exclude the possibility that this reduction is caused by EDC treatment, we note that it fits in with the observation that fibers contracting at moderate velocities of shortening have a higher ATPase rate than isometric fibers (Kushmeric & Davies, 1969). We note that the k_{cat} of 0.8 s^{-1} at 4 °C found with cross-linked myofibrils is very close to the ATPase rate of skinned psoas fibers in the isometric conditions of about 1 s^{-1} at 5 °C (Brenner, 1988).

The high k_{cat} with relaxed cross-linked myofibrils is almost certainly due to rigor activation (Bremel & Weber, 1973). The 8% of heads cross-linked to the thin filament keep the actin filaments switched on, even in the absence of Ca^{2+}. Rouayrenc et al. (1985) found that when S1 bound to thin filaments was treated with EDC, the regulatory proteins were not cross-linked to either actin or S1.

(2) Steady-State Intermediates during Myofibrillar ATPase. Activated, cross-linked myofibrils gave a P_i burst equal to 1.0 within experimental error, and thus the fraction of myosin heads in the ATP state during isometric contraction is low (<10%). This result is similar to that with relaxed and activated un-cross-linked myofibrils (Houadjeto et al., 1992; Herrmann et al., 1992) and with isometric muscle fibers (Ferenczi et al., 1984).

In the case of S1, the size of the burst gives direct information about the equilibrium constant of the hydrolysis step because the steps immediately following hydrolysis and also the steady-state ATPase are much slower than the hydrolysis step (Bagshaw & Trentham, 1973). The situation is quite different for acto–S1 ATPase as the step following hydrolysis is about 3 times faster than the hydrolysis step itself (Rosenberg & Taylor, 1984; Biosca et al., 1986; Tesi et al., 1990) and the size of the burst cannot be related directly to the equilibrium constant for the cleavage step, K_3. In these circumstances, it is unlikely that activated myofibrils are going to behave like S1. Moreover, there are strong arguments from physiological evidence that the S1 interpretation is not applicable to myofibrils. The case is strongest at 20 °C, and this condition will be considered first. The rates of the steps immediately after hydrolysis are fast relative to the isometric fiber ATPase rate (3 s^{-1}; Kawai et al., 1990). For example, the rate of tension development after release of ATP from caged ATP is 100 s^{-1} (Goldman et al., 1984b), and the rate of the caged

P_i transient is >80 s^{-1} (Dantzig et al., 1992). The steady-state ATPase must be limited by a later step in the scheme, probably ADP release or AM·ADP isomerization prior to ADP release. The case is not quite as clear-cut at 5 °C where the steady-state ATPase rate is 1 s^{-1} (Brenner, 1988) and the caged ATP experiment gives 5 s^{-1}. At this temperature, the steady-state ATPase may be limited by a combination of steps, and the hydrolysis step may partially equilibrate. Our experiments at 4 °C may imply that K_3 is somewhat larger for myofibrils than for S1, but it seems unlikely that the conditions for making a direct measurement of K_3 are met.

Kinetic Studies: Myofibrils Cross-Linked under Relaxing Conditions. When myofibrils in rigor were treated with EDC, two types of cross-link were detected: one involving the rods (extensively) and the other the myosin heads–actin filament (about 8% of the heads involved). These myofibrils did not shorten upon the addition of Mg^{2+}-ATP and Ca^{2+}, and we attempted to determine which type of cross-link was responsible.

When the myofibrils were cross-linked under relaxing conditions, only the rods appeared to be cross-linked. These myofibrils did not shorten, but their ATPase activities and P_i burst amplitudes were low. Thus, it appears that as with S1 (Table I), when myofibrils are treated with EDC under relaxing conditions, some of the heads are inactivated. It is possible that these myofibrils do not shorten because the inactive heads that do not contribute to the burst bind to the thin filament in a rigorlike state, even in the presence of ATP. Further experiments are needed to resolve this problem.

CONCLUSIONS

There is clearly a certain danger in modifying chemically a material as complex as myofibrils and then to carry out transient kinetics with it. We are not dealing with purified proteins as when cross-linking S1 to actin. It cannot be excluded that our EDC treatment, although mild, may give myofibrillar preparations that are not kinetically homogeneous. If such an inhomogeneity exists, we have no evidence for it: there is no more heterogeneity in the kinetic curves obtained with EDC-treated myofibrils (here) than there is in those obtained with untreated myofibrils (Houadjeto et al., 1992; Herrmann et al., 1992).

We conclude, therefore, by suggesting that EDC-cross-linked myofibrils are a useful model for fibers in the isometric state. Their structures can be observed directly under the microscope, and their ATPase activities can be studied by conventional rapid reaction methods. By their use, we hope to assign the step(s) on the ATPase cycle that is (are) connected with tension development.

ACKNOWLEDGMENT

We thank Nathalie Bonafé for the gift of polyclonal antibodies directed toward actin and the S1 heavy chain.

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