J. Mol. Biol. (1982) **156**, 153–173

Regulatory and Essential Light-chain Interactions in Scallop Myosin

II. Photochemical Cross-linking of Regulatory and Essential Light-chains by Heterobifunctional reagents

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(Received 19 August 1981)

From four to six lysine residues of isolated scallop regulatory light-chains were conjugated with *N*-hydroxysuccinimidyl-4-azidobenzoate; the single thiol group of isolated *Mercenaria* R-LC‡, and the two thiol groups of isolated rabbit R-LCs were substituted with *p*-azidophenacylbromide. R-LCs modified by these heterobifunctional photosensitive cross-linkers combined readily with scallop myofibrils from which R-LCs had been removed by treatment with EDTA at 35°C. The modified scallop and *Mercenaria* R-LCs restored the calcium sensitivity of the actin-activated Mg-ATPase. The complexes formed upon illumination between modified R-LCs and essential light-chains were visualized and quantitated by indirect antibody staining of electrophoretic nitrocellulose blots of sodium dodecyl sulfate/polyacrylamide gels using specific anti-scallop R-LC and anti-scallop SH-LC antibodies followed by fluorescein isothiocyanate-labeled or ^{125}I-labeled goat anti-rabbit immunoglobulin G, respectively. The presence of SH-LC in bands of higher molecular weight complexes ($M_r > 17{,}000$) formed upon photolysis was taken as evidence for cross-linking between the substituted R-LCs and scallop SH-LCs. About one-third of the scallop SH-LC cross-linked with *p*-APA-substituted *Mercenaria* R-LC, when illumination generated a single new band at M_r 35,000 consisting of one R-LC and one SH-LC. Higher molecular weight complexes were not formed. With the multi-substituted scallop and rabbit R-LCs, more than half of the scallop SH-LC was cross-linked; about one-third formed a one to one complex with the modified R-LC, and a significant amount was cross-linked to the myosin heavy-chain *via* the R-LC. Complexes of molecular weights of approximately 53,000 and 66,000 were also formed in small amounts with highly modified scallop and rabbit R-LCs, probably consisting of three and four cross-linked light-chains. Some cross-linking of the two R-LCs of the scallop myosin molecule was demonstrated with myofibrils treated with EDTA at 12°C and containing one mole

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‡ Abbreviations used: R-LC, regulatory light-chain; SH-LC, essential light-chain; HSAB, *N*-hydroxysuccinimidyl-4-azidobenzoate; *p*-APA, *p*-azidophenacylbromide; Ig, immunoglobulin; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid; FITC, fluorescein isothiocyanate.

0022-2836/82/090153-21 $03.00/0

of intact scallop R-LC and one mole of *p*-APA-modified *Mercenaria* or rabbit R-LC. R-LCs did not significantly cross-link with actin or tropomyosin. Photolysis did not inactivate the actin-activated Mg-ATPase activity, but reduced calcium sensitivity by activation in the absence of calcium. These results show that R-LCs and SH-LCs of scallop myosin are in close proximity (no more than 6 to 8 Å apart) and overlap for at least half of their lengths. Cross-linking of R-LCs and SH-LCs interferes with regulation, consistent with a regulatory role for the SH-LCs.

1. Introduction

In a previous study we observed that antibodies specific against the essential light-chains of scallop myosin abolished regulation in scallop myofibrils and myosin (Wallimann & Szent-Györgyi, 1981*a*). We concluded that the regulatory and essential light-chains may interact, and suggested that both types of light-chain are regulatory subunits of myosin.

Interaction between the two light-chain types was indicated some time ago by demonstrating that the R-LCs† protect the SH-LC on scallop myosin from degradation by papain (Stafford *et al.*, 1979). Furthermore, it has been shown that myofibrils and myosin bind considerably larger amounts of anti-SH-LC IgG if R-LCs have been removed. In turn, the re-uptake of the R-LCs was drastically reduced in myofibrils that were pretreated with anti-SH-LC IgG. As an explanation, we have suggested a model for the position of the light-chains on the myosin head in which there was at least a partial overlap between the two kinds of light-chains (Wallimann & Szent-Györgyi, 1981*a*). This interpretation, however, was limited by the large size of the antibody used as a probe (50 Å).

In this paper, we explore more directly the proximity of the R-LC and SH-LC by using short photosensitive heterobifunctional cross-linkers (6·5 Å and 8·3 Å) attached to the R-LC. We have attempted to estimate the extent of the overlap by performing cross-linking studies with foreign light-chains containing thiol groups in known positions covalently substituted with specific heterobifunctional labels. These studies complement recent structural studies on the location of the R-LC and of the SH-LC on the myosin head (Craig *et al.*, 1980; Vibert *et al.*, 1981; Flicker *et al.*, 1981), and the accompanying paper on the protective effect of the R-LC on the reactivity of the thiol groups of the SH-LC (Hardwicke *et al.*, 1982). Some of these experiments were presented at the 1981 Meeting of the Biophysical Society (Hardwicke *et al.*, 1981).

2. Experimental Procedures

(a) *Preparation of photoaffinity-labeled light chains*

All procedures were carried out in a dark room illuminated by a 45 W Kodak Safety Lamp equipped with a GBX red filter.

(i) *Modification of scallop (*Aequipecten irradians*) R-LC with the heterobifunctional reagent* N-*hydroxysuccinimidyl-4-azidobenzoate (HSAB)*

HSAB (Pierce Chemical Co., Rockford, Ill.) was dissolved in dimethyl sulfoxide to give a final concentration of 0·1 M and stored at −25°C in the dark. A 2-fold molar ratio of reagent

† See footnote to p. 153.

to the total lysine content of *Aequipecten* R-LC (15 lysine residues/mol light-chain; Kendrick-Jones *et al.*, 1976) was added to the R-LC (5 mg ml^{-1} dissolved in 2 mM-$MgCl_2$, 0·1 M-phosphate at pH 7·5). The final concentration of dimethyl sulfoxide in the reaction mixture was kept at 20% (v/v) at a pH of 7·8. The mixture containing the R-LC and the partially dissolved reagent was stirred at room temperature in the dark for 4 h. The acylation reaction was stopped by the addition of a 10-fold molar excess of lysine (1 M stock solution titrated with NaOH to pH 7·5) over the photoreagent. After stirring for an additional 30 min at room temperature, the mixture was centrifuged and the supernatant dialyzed against 1 mM-$MgCl_2$, 1 mM-lysine, 20 mM-Tris·HCl (pH 8·0). After centrifugation for 1 h at 140,000 *g*, the supernatant was lyophilized, dissolved in 50 mM-sodium borate (pH 8·2), filtered through a 0·22 μm Millipore filter and freed of any traces of unreacted photoreagent by gel filtration over a column of Sephadex G-25 equilibrated with 50 mM-ammonium acetate (pH 7·5). Fractions containing the modified R-LC were pooled, lyophilized, and dissolved in Mg^{2+}-wash (40 mM-NaCl, 2 mM-$MgCl_2$, 5 mM-phosphate, pH 7·0) and stored frozen. This procedure yielded a R-LC with 4 to 6 mol heterobifunctional reagent/mol scallop R-LC, as estimated by the number of bands seen after polyacrylamide/urea gel electrophoresis (see Fig. 1, gel of modified R-LC).

(ii) *Modification of* Mercenaria mercenaria *R-LC with* p-*azidophenacylbromide (*p-*APA)*

p-APA (Pierce Chemical Co.) was dissolved in methanol to a concentration of 0·2 M and stored at −25°C in the dark. A sample (9 mg) of *Mercenaria* R-LC was dissolved in 50 mM-sodium borate (pH 8·2), reduced under N_2 with 10 mM-dithiothreitol at room temperature for 1 h, and dialyzed against 2 l of 0·2 mM-dithiothreitol, 2·5 mM-sodium phosphate (pH 7·0), in deaerated water at 4°C overnight. Borate was added to 0·1 M (pH 8·2), followed by 0·5 vol. methanol and the preparation put under N_2. A 5-fold molar ratio of *p*-APA to the total thiol in the sample was then added and the preparation stirred under N_2 for 2 h at room temperature. The reaction was terminated by the addition of a 5-fold molar ratio of dithiothreitol to the reagent, after which the mixture was stirred again for 0·5 h. The preparation was then centrifuged at 140,000 *g* for 1 h to remove any insoluble material, and the supernatant was applied to a 1·2 cm × 16·7 cm column of Sephadex G-25 (fine) in 25 mM-ammonium acetate (pH 7·5). The pooled fractions from the void volume were lyophilized and finally dissolved in 40 mM-NaCl, 2 mM-$MgCl_2$, 0·1 mM-EDTA, 5 mM-sodium phosphate (pH 7·0), and stored frozen. Using the extinction coefficient of $2{\cdot}01 \times 10^4$ M^{-1} cm^{-1} at 300 nm in 2% (v/v) aqueous methanol given for *p*-APA by Hixson & Hixson (1975), the *Mercenaria* R-LC was estimated to be 75% labeled.

(iii) *Modification of rabbit R-LC (DTNB light-chain) with* p-*azidophenacylbromide*

A sample (20 mg) of rabbit DTNB light-chain was dissolved in 1 ml of 7 M-guanidine·hydrochloride, 2 mM-EDTA, 0·5 M-Tris·HCl (pH 8·0), and reduced under N_2 with 5 mM-dithiothreitol for 1 h at room temperature. A 4-fold molar ratio of *p*-APA was added from a 0·144 M stock solution in methanol. The mixture was stirred at room temperature under N_2 for 2·5 h. Unreacted reagent was quenched with a 100-fold molar excess of 2-mercaptoethanol. The preparation was then dialyzed extensively against 50 mM-ammonium acetate (pH 7·5), centrifuged at 3000 *g* for 0·5 h and the supernatant was lyophilized. The product was estimated to be fully labeled with the reagent.

(iv) *Incubation of desensitized scallop myofibrils with modified R-LC and photolysis*

Typically, 3 to 5 mg of scallop myofibrils from which both of the R-LCs of myosin had been removed by treatment with 10 mM-EDTA at 33 to 35°C (Chantler & Szent-Györgyi, 1980) were suspended in Mg^{2+}-wash containing 3 mM-NaN_3, and incubated with a 10-fold molar ratio of modified R-LC while gently agitating overnight at 4°C in 3·5-ml plastic centrifuge tubes or in a 5-ml disposable plastic test-tube on a rotary mixer or an Ames

rocking aliquot mixer (Miles). Unbound light-chain was removed by washing 3 times with Mg^{2+}-wash containing no NaN_3 and centrifuged in a Sorvall HS-4 bucket rotor at 5000 revs/min for 5 min. The washed myofibrils were suspended in Mg^{2+}-wash and divided into 2 equal portions, one of which was subjected to photolysis and the other kept as an unphotolyzed control. Photolysis was carried out at 4°C by exposing the magnetically stirred samples (2 to 3 mg/ml) in quartz curvettes at a distance of 20 cm from a sunlamp (General Electric, 275 W, 80 W/m^2 at 20-cm distance within the BG3 (AZI, Zeiss) filter transmission range of 280 to 400 nm) for 2 to 6 h. Later, a high-pressure mercury lamp equipped with a BG-3 filter (HBO 200 with an output of 800 W/m^2 at 20 cm distance within the BG3 filter transmission range) was used for the same purpose, and the photolysis time was reduced to between 15 and 50 min using a myofibril concentration of 1 mg/ml. Light intensities were measured with a YSI-Kettering model 65A (Radiometer) light meter. Unphotolyzed control samples were stirred in the dark for the same period. After photolysis, samples were taken for ATPase activity measurements, urea and sodium dodecyl sulfate/polyacrylamide gel electrophoresis. All operations, including electrophoresis, blotting and ATPase measurements in a pH-stat, except for the photolysis step, were either carried out in the dark or under a 45 W darkroom light equipped with a Kodak GBX red safety filter. Preparation of proteins, tube gel electrophoresis in the presence of sodium dodecyl sulfate or urea, and ATPase measurements were conducted as described earlier (Kendrick-Jones *et al.*, 1976; Chantler & Szent-Györgyi, 1980; Wallimann & Szent-Györgyi, 1981*a*).

(b) *Blotting of the proteins and staining with antibodies*

From 100 to 300 μg of protein was electrophoresed on slab gels that had two 5 cm wide wells, one used for the unilluminated control samples and the other for photolyzed samples. Urea-containing gels were run in the same solutions as described for tube gels (Kendrick-Jones *et al.*, 1976). Sodium dodecyl sulfate-containing gels were run under the conditions described by Laemmli (1970: 0·375 M-Tris·HCl (pH 8·8) gel buffer; 0·025 M-Tris (pH 8·3), 0·192 M-glycine, electrode buffer) for 3 to 3·5 h at 175 V. An approximately 1 cm wide strip of the gel of the control and photolyzed samples was cut off and stained with Coomassie blue. The remaining 4 cm wide slabs were blotted onto nitrocellulose (Millipore Lab., Bedford, MA) according to Towbin *et al.* (1979), using an apparatus built after their design. Urea-containing gels were blotted for 2 to 3 h at 100 to 120 mA in 0·064 M-Tris (pH 8·6), 0·384 M-glycine; sodium dodecyl sulfate-containing gels were blotted overnight at 70 mA, 30 V in the Tris/glycine/methanol buffer described by Towbin *et al.* (1979). After blotting, the nitrocellulose sheets were each cut into 4 strips and air-dried for 30 min at room temperature. One of the strips was stained with 0·1% (w/v) Amido black in 7% (v/v) acetic acid. The remaining strips were distributed into three 20-ml test-tubes. Each tube contained a strip from the samples before and after photolysis separated by a plastic strip. The strips were saturated with 18 ml of 3% (w/v) bovine serum albumin, 10% (v/v) horse serum in borate/saline buffer (0·15 M-NaCl, 0·01 M-borate, pH 8·0) and incubated for >2 h at 40°C or overnight at 4°C on a rocking aliquot mixer. From 100 to 200 μg of preimmune IgG and affinity-purified, cross-adsorbed anti-R-LC or anti-SH-LC antibodies were added and incubated with the strips for 2 h at room temperature on the aliquot mixer. Primary antibodies were removed by washing 6 times for 5 min with 15 ml of borate/saline buffer. The strips were then shaken with 15 ml of FITC-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.), diluted 1 : 1000 with borate/saline buffer containing 3% bovine serum albumin, 10% horse serum for 2 h at room temperature in the dark. The strips were washed 6 times, air-dried between Whatman no. 1 filter papers, and photographed with 8 to 15 s exposure on Polaroid type 667 film through a yellow filter while epi-illuminated with a long-wave Black Raymaster ultraviolet lamp with a peak radiation of 360 nm. For quantitation, 0·075 μCi ^{125}I-labeled goat anti-rabbit IgG/ml (New England Nuclear Corp.) was mixed with the diluted FITC-conjugated goat anti-rabbit IgG. The strips were washed and photographed as usual, then cut into 5 mm segments and counted in a gamma counter.

The modified scallop and foreign R-LCs were tested on 2 or more different batches of myofibrils. Both pure hybrids and mixed hybrids were constructed with the modified foreign R-LCs by combining them with myofibrils treated previously with EDTA at 35°C or at 12°C (Sellers *et al.*, 1981).

3. Results

(a) *General experimental plan*

Isolated R-LCs were modified with heterobifunctional photosensitive reagents directed against amino groups or thiol groups. The modified R-LCs were recombined with myofibrils, a portion was illuminated, and the rest kept as a control. Control and photolyzed samples were submitted to electrophoresis on sodium dodecyl sulfate/10% polyacrylamide slab gels, blotted and the positions of the R-LC and SH-LC established with the aid of specific anti-R-LC IgG and anti-SH-LC IgG, which were visualized with FITC-labeled goat anti-rabbit IgG. Since only the R-LCs carried cross-linker, the presence of SH-LC in higher molecular weight complexes indicates cross-linking of SH-LC with R-LC. Two cross-linkers were used. HSAB reacts preferentially with amino groups of lysine residues and was used to modify scallop R-LCs. *p*-APA reacts with thiol groups and was used for labeling *Mercenaria* and rabbit R-LCs. Since scallop R-LC has no cysteine residues, it could not be labeled with *p*-APA.

(b) *Cross-linking with modified scallop R-LC*

Reaction of a lysine residue with HSAB eliminates a positive charge on the R-LC, and a semiquantitative estimation of the extent of substitution can be obtained from the appearance of new bands closer to the anode on urea-containing gels (Fig. 1). Different numbers and combinations of azidobenzoate substitutions are present in the HSAB-treated scallop R-LC preparations, including a species of R-LC with about ten substitutions. The modified R-LCs recombine with myofibrils and restore calcium sensitivity (Fig. 1; Table 1). The major portion of the recombined light-chains contained four to six azidobenzoate substitutions.

On photolysis, the modified R-LC bands decrease greatly in size or disappear completely, and concomitantly the size of the SH-LC peak relative to the tropomyosin peak decreases dramatically (Fig. 1; Table 1). The increased tropomyosin to SH-LC ratio indicates that up to about 40% of the SH-LC disappears on photolysis. The myosin heavy-chains and higher molecular weight components do not enter and are not seen on urea/10% polyacrylamide gels. A new high molecular weight band appears on sodium dodecyl sulfate/5% polyacrylamide gels, moving somewhat slower than myosin heavy-chains as a result of illumination of myofibrils that contain azidobenzoate-substituted R-LC. This band becomes stronger as the period of photolysis increases (Fig. 2). Higher molecular weight bands barely entering the gels are also seen, particularly in samples that were photolyzed for longer.

Bands containing R-LC and SH-LC can be identified uniquely with the aid of specific rabbit antibodies prepared against the two types of scallop light-chains

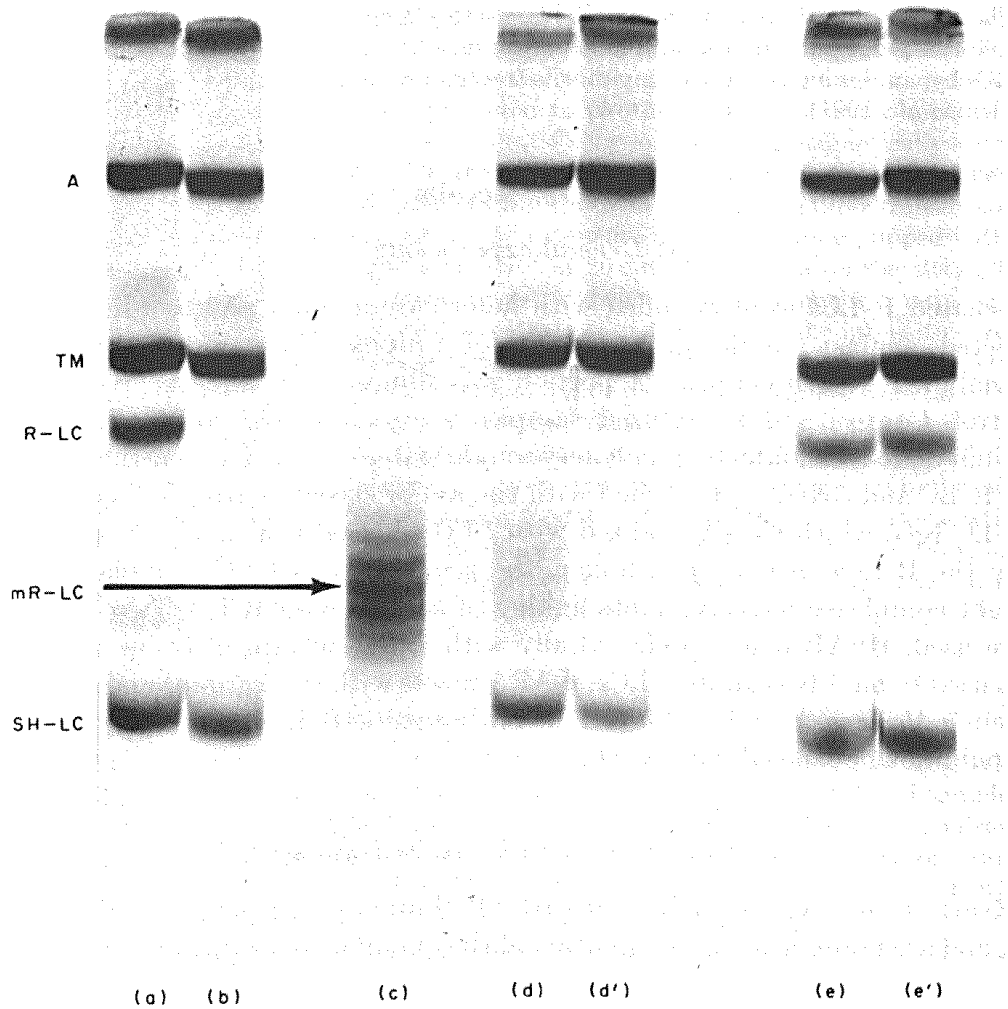

FIG. 1. Uptake of HSAB-modified scallop R-LC. Urea/10% polyacrylamide gels of: (a) untreated myofibrils; (b) desensitized myofibrils (10 mM-EDTA at 35°C for 5 min); (c) modified R-LC (mR-LC); (d) desensitized myofibrils incubated with mR-LC before photolysis; (d') same as (d) after photolysis; (e) desensitized myofibrils incubated with R-LC containing no cross-linker; (e') same as (e) after photolysis. Note that the mR-LC is taken up by densensitized myofibrils; upon photolysis, the mR-LC disappears and the SH-LC band is reduced. No decrease in the SH-LC is seen upon illumination of controls containing unmodified R-LC. A, actin; TM, tropomyosin.

(Wallimann & Szent Györgyi, 1981*b*) on nitrocellulose blots of the polyacrylamide gels. The rabbit antibodies are, in turn, visualized with FITC-conjugated goat anti-rabbit IgG secondary antibody. The specificity of the antibodies against the two types of light-chains was confirmed on nitrocellulose blots of urea-containing gels of myofibrils in conditions that separate R-LC and SH-LC. The fluorescence following treatment with anti-R-LC-IgG was restricted entirely to the R-LC band; the fluorescence following treatment with anti-SH-LC was seen only at the SH-LC band (Fig. 3). No cross-reactivity was seen between the two light-chain IgGs; or between tropomyosin, actin or paramyosin and the anti-LC IgGs.

The effect of photolysis on myofibrils containing azidobenzoate-substituted R-LCs of scallop is shown on the gels and the blots of Figure 4. One notes that,

TABLE 1

Effect of photolysis on myofibrils containing azidobenzoate-modified scallop regulatory light-chain

	ATPase†			Area‡			Ratios		
	$-Ca^{2+}$	$+Ca^{2+}$	Sensitivity	TM	R-LC	SH-LC	R-LC / SH-LC	TM / SH-LC	
Normal myofibrils	0·02	0·38	95%	17·9	8·95	9·24	0·97	1·94	Photolyzed
				17·31	8·84	9·0	0·98	1·92	Unphotolyzed
Desensitized myofibrils	0·30	0·22	—	19·18	0·66	10·25	0·06	1·87	Photolyzed
				19·0	0·67	9·7	0·07	1·95	Unphotolyzed
Desensitized myofibrils +mR-LC§	0·1	0·32	69%	20·67	<0·1	6·5	<0·02	3·18	Photolyzed
				16·80	7·15¶	9·0	0·79¶	1·87	Unphotolyzed
Desensitized myofibrils +unmodified R-LC control	0·04	0·42	90%	15·59	8·0	8·6	0·93	1·84	Photolyzed
				10·61	5·2	5·8	0·89	1·82	Unphotolyzed

† Actin activated Mg-ATPase activity in μmol min^{-1} mg^{-1}.

‡ Area under bands of urea-containing gels stained with Acid fast green, in planimetry units. TM, tropomyosin.

§ R-LC modified by a 4·5 h incubation and 30-fold molar excess HSAB at 20°C.

¶ Value for modified R-LC (mR-LC) and mR-LC/SH-LC, respectively. Myofibrils were incubated with 10-fold molar excess of azidobenzoate-substituted mR-LC overnight, washed extensively and photolyzed with a 250 W Sylvania sunlamp for 3 h at 4°C.

Note that mR-LC is bound to desensitized myofibrils and restores calcium sensitivity. Upon photolysis, mR-LC is fully cross-linked. A significant portion of the SH-LC is also cross-linked in photolyzed samples as judged from the changed TM/SH-LC ratio. In a different experiment, photolysis reduced calcium sensitivity from 82% to 32% by the activation of the ATPase activity in the absence of calcium.

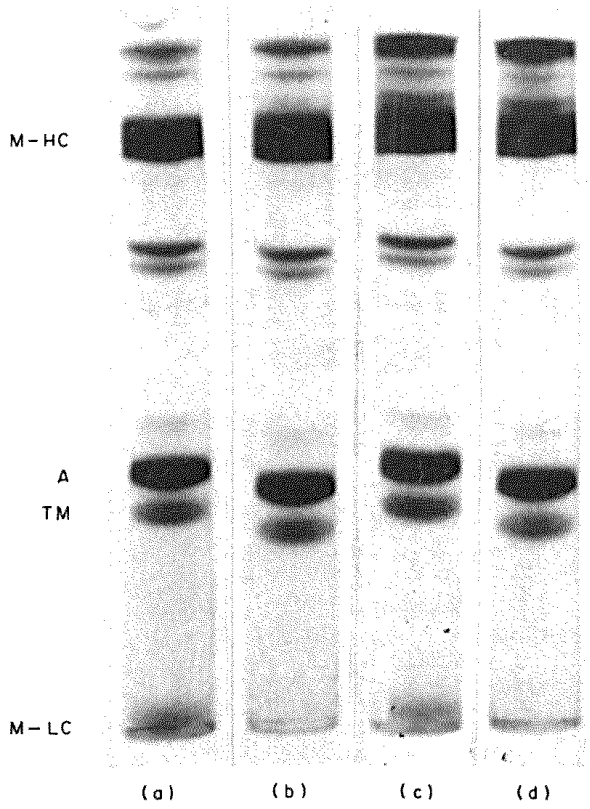

FIG. 2. High molecular weight components formed by photolysis. Sodium dodecyl sulfate/5% polyacrylamide gels of desensitized scallop myofibrils containing HSAB-modified mR-LC before photolysis (a); and after 5 min (b), 15 min (c) and 45 min (d) exposure to an HBO 200 W high-pressure mercury lamp. Note the increased formation of a band just above the myosin heavy-chain and the higher aggregates (probably 2 heavy-chains and light-chains) that barely enter the gels. M-HC, myosin heavy-chain; A, actin; TM, tropomyosin; M-LC, myosin light-chain.

although the blots are rather sharp and clearly delineated, the intensity of the bands does not correspond to the intensity of the stained bands on the gel. In particular, there is an incomplete transfer of the myosin heavy-chains and paramyosin. We tried to alter the blotting conditions of Towbin *et al.* (1979) by changing the pH and the methanol concentration of the blotting medium, or adding detergents or inorganic pyrophosphate. Conditions that improved myosin heavy-chain transfer interfered with the binding of the lower molecular weight components to nitrocellulose. When higher concentrations of protein ($>150\ \mu g/5$ cm gel) were used in order to visualize components present in small amounts, the relative stain intensity was further distorted, since major components (e.g. actin) were not fully retained due to saturation of the binding sites of the nitrocellulose.

Control preimmune IgG is not bound by unilluminated or by illuminated fibrils (Fig. 4). In unilluminated samples, R-LC and SH-LC are found only in the position characteristic of monomeric light-chains, corresponding to a molecular weight of about 18,000; i.e. the light-chains are not associated with components of higher molecular weights before photolysis (Fig. 4). In photolyzed samples, light-chains

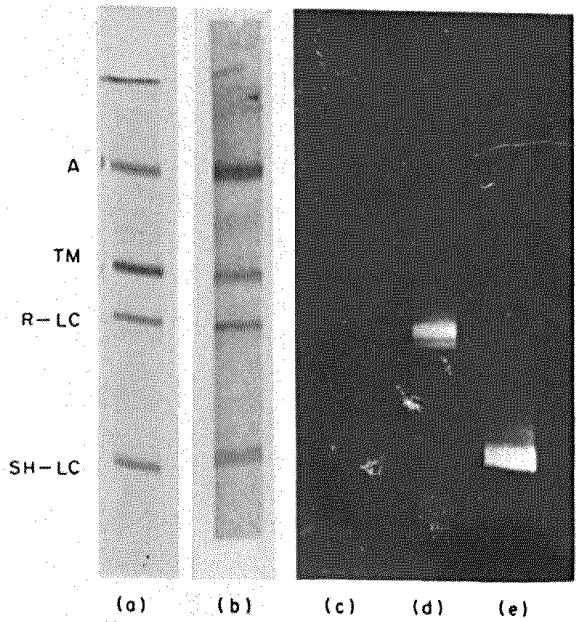

FIG. 3. Specificity of the anti-R-LC and anti-SH-LC antibodies. A sample (100 μg) of scallop myofibrils in a 5 cm well was submitted to electrophoresis on a urea/polyacrylamide slab gel, which was divided into 5 strips: (a) gel stained with Coomassie blue; (b) nitrocellulose blot stained with Amido black; (c) nitrocellulose blot treated with control preimmune IgG; (d) nitrocellulose blot treated with anti-R-LC IgG; (e) nitrocellulose blot treated with anti-SH-LC IgG. Antibody treatment in (c) to (e) was followed by staining with FITC-conjugated goat anti-rabbit IgG. Note the complete absence of cross-reactivity of both antibodies with the heterologous light-chains, even though the sensitivity of the indirect staining method is considerable. A, actin; TM, tropomyosin.

are found in significant quantitities in positions corresponding to molecular weights of 18,000, 35,000 and about 230,000. The distribution of R-LCs and SH-LCs overlap (Fig. 4). The fluorescence of both the M_r 35,000 and M_r 230,000 band is rather strong. In two experiments on a single batch of myofibrils, 25 to 35% of the radioactivity was associated with the 35,000 M_r complex after treatment with anti-R-LC or anti-SH-LC IgGs (Table 2).

The 35,000 M_r band formed on photolysis binds both types of anti-light chain IgGs, and one species that must be present in it is a complex of SH-LC with R-LC; for, since the SH-LC is not itself labelled with azidobenzoate, the only way the SH-LC can occur at this position is through cross-linking to the R-LC. In addition, a dimer of two R-LCs may also be present, because of the possibility of cross-linking to the R-LC on the other head of the same myosin molecule. Since the myosin heavy-chains blot poorly, the fluorescence seen at the 230,000 M_r position probably represents only part of this component. Both anti-R-LC IgG and anti-SH-LC IgG bind to this new band, and one species that must be present in it is a complex of myosin heavy-chain with both SH-LC and R-LC, since it is only through cross-linking *via* the R-LC that the SH-LC (which itself has no photo-activatable cross-linker) can be associated with the heavy-chain. In addition, a complex of heavy-chain with two R-LCs may also be present because of the possibility of cross-linking to the R-LC on the other head of the same myosin molecule.

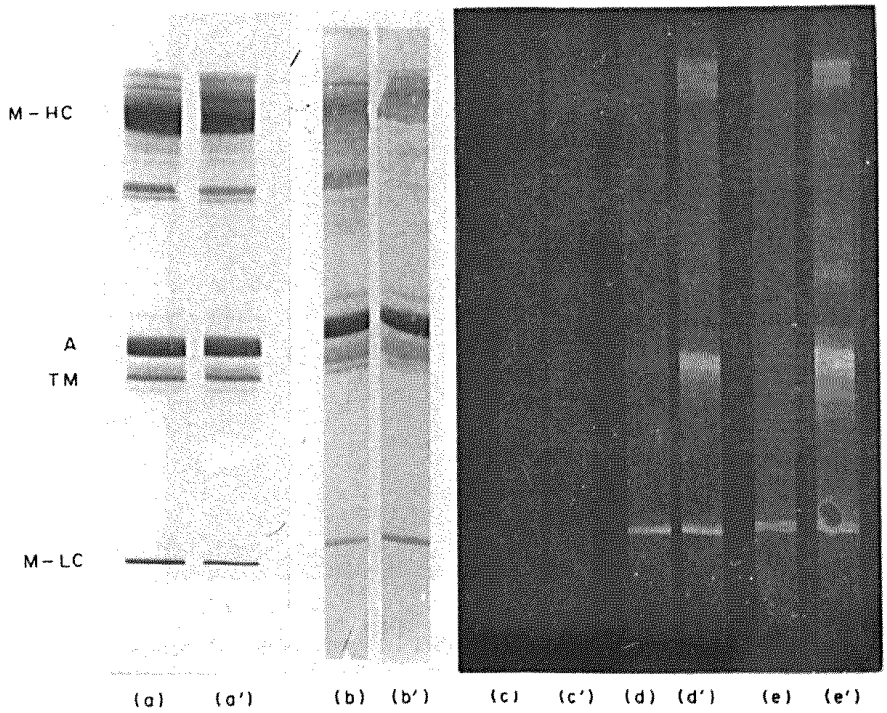

FIG. 4. Photolysis of scallop myofibrils containing HSAB-modified scallop R-LCs. (a) to (e) Unilluminated samples. (a') to (e') Samples photolyzed for 30 min with an HBO 200 high-pressure mercury lamp. (a) and (a') Gels stained with Coomassie blue; (b) and (b') blots stained with Amido black; (c) and (c') blots treated with control preimmune IgG; (d) and (d') blots treated with anti-R-LC IgG; (e) and (e') blots treated with anti-SH-LC IgG. Treatment of blots with IgG was followed by staining with FITC-conjugated goat anti-rabbit IgG. Sample loading was 100 μg myofibrils/5 cm. Sodium dodecyl sulfate/10% polyacrylamide slab gel electrophoresis. M-HC, myosin heavy chain; A, actin; TM, tropomyosin; M-LC, myosin light-chain. Myofibrils were desensitized at 35°C before addition of HSAB-modified scallop R-LC. ATPase activities and light-chain ratios are shown in Table 1.

In overloaded photolyzed samples, two additional faint fluorescent bands corresponding to chain weights of approximately 53,000±3000 and 66,000±5000 can be detected (4 measurements: not shown). These additional bands bind both anti-R-LC IgG and anti-SH-LC IgG, and thus must contain both these components. The fluorescence of the bands corresponding to molecular weights of 18,000, 35,000 and 230,000 remains strong.

Extensive cross-linking does not necessarily inhibit ATPase activity; however, it is accompanied by a significant decrease in calcium sensitivity. In a single determination, calcium sensitivity was reduced from 82% to 32% upon photolysis due to an activation of the ATPase in the absence of calcium from 0·06 to 0·29 μmol min^{-1} mg^{-1}.

(c) *Cross-linking with* Mercenaria *R-LC*

Mercenaria R-LCs contain a single thiol group lying in the link region between domains 1 and 2 towards the N-terminal end (Bagshaw & Kendrick-Jones, 1980). This thiol can be specifically and extensively labeled by *p*-APA (see Experimental

TABLE 2

Distribution of cross-linked scallop light-chains in different molecular weight complexes

Cross-linker	Anti-R–LC IgG				Anti-SH-LC IgG			
	18K	35K	50–70K	>200 K	18K	35K	50–70K	>200K
Scallop HSAB–R-LC								
Control	97	2	1	0	91	5	2	3
Photolyzed	13	25	16	46	26	38	12	24
Rabbit *p*-APA–R-LC								
Control		<8% of SH-LC			91	5	0	4
Photolyzed					44	34	3	19
Rabbit *p*-APA–R-LC								
Control		<3% of SH-LC			90	10	0	0
Photolyzed					34	39	17	10
Mercenaria p-APA–R-LC								
Control		Not measurable			100	0	0	0
Photolyzed					65	35	0	0
Mercenaria p-APA–R-LC								
Control		Not measurable			100	0	0	0
Photolyzed					80	20	0	0

The values are the percentage distribution of ^{125}I-labeled goat anti-rabbit IgG in different bands, where K = $M_r \times 10^{-3}$.

Myofibrils treated with 10 mM-EDTA at 33 to 35°C were combined with modified R-LCs, blotted, treated with rabbit anti-scallop IgGs and with secondary [^{125}I]FITC-conjugated goat anti-rabbit IgG. The blots were cut into 5-mm long segments and counted. HSAB–R-LC, HSAB-modified R-LC; *p*-APA–R-LC, *p*-APA-modified R-LC.

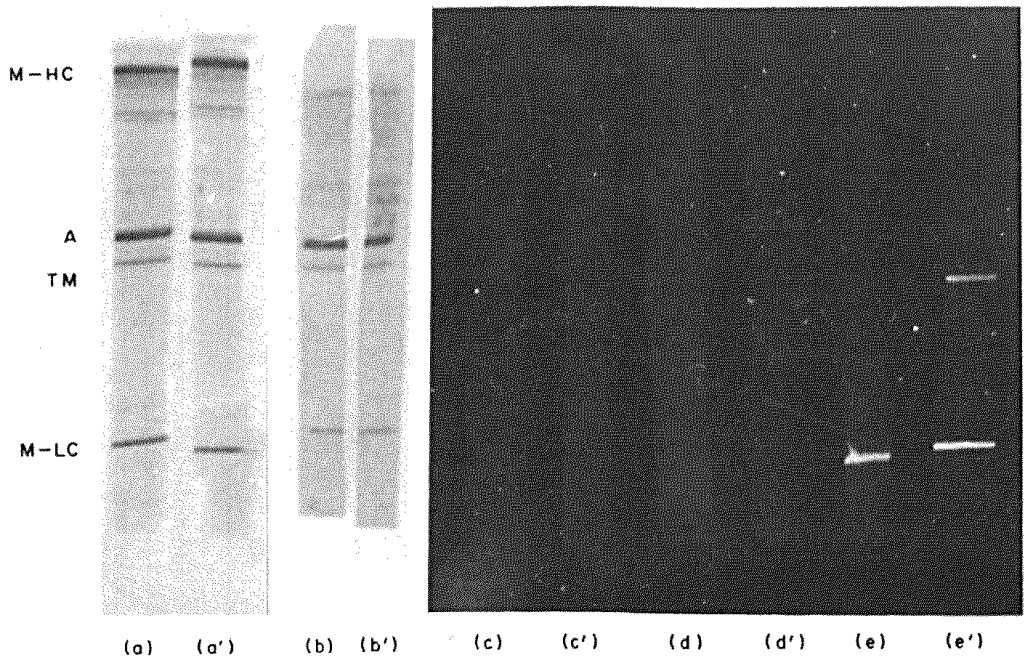

FIG. 5. Photolysis of scallop myofibrils containing *p*-APA-modified *Mercenaria* R-LC (pure hybrids). Samples were loaded at 100 μg myofibrils/5 cm of sodium dodecyl sulfate/acrylamide slab gel for electrophoresis. (a') to (e') Samples photolyzed for about 15 min with an HBO 200 high-pressure mercury lamp. (a) and (a') Gels stained with Coomassie blue; (b) and (b') blots stained with Amido black; (c) and (c') blots treated with preimmune control IgG; (d) and (d') blots treated with anti-R-LC IgG; (e) and (e') blots treated with anti-SH-LC IgG. Treatment of blots with IgG was followed by staining with FITC-conjugated goat anti-rabbit IgG. M-HC, myosin heavy-chain; A, actin; TM, tropomyosin; M-LC, myosin light-chain. Myofibrils were densensitized at 35°C before addition of *p*-APA-modified *Mercenaria* R-LC. Actin-activated Mg-ATPase activities in calcium (μmol min^{-1} mg^{-1}) and ATPase activity ratios in EGTA/Ca^{2+} were as follows: intact myofibrils, 0·55 and 0·06; desensitized controls, 0·28 and 1·22; hybrid before photolysis, 0·37 and 0·12; hybrid after photolysis, 0·35 and 0·34 (hybrid values from single time-courses).

Procedures). Scallop R-LCs lack thiol groups, and it has not been possible to label a single particular amino acid residue in that light-chain. The modified *Mercenaria* R-LC bound readily to myofibrils from which all the native scallop R-LCs had been removed by treatment with EDTA at 35°C (Sellers *et al.*, 1981). The pure hybrid formed with this foreign R-LC regained its calcium sensitivity; the ratio of the actin-activated Mg-ATPase in EGTA to that in 0·1 mM-Ca^{2+} was changed from 1·26 to 0·12 on binding of the *Mercenaria* R-LC to the desensitized scallop myofibrils. Upon illumination, the specific ATPase activity remained the same, although the ratio of ATPase activities in the absence and presence of calcium increased to 0·34 in this experiment (see the legend to Fig. 5).

No new band could be detected on the gels as a result of photolysis (Fig. 5). The 230,000 M_r band trailing the myosin heavy-chain in photolyzed samples of myofibrils with bound HSAB-modified scallop R-LC was not seen when *p*-APA-modified *Mercenaria* R-LC was used. The preimmune IgG control blots showed no fluorescence. The anti-scallop R-LC IgG did not bind to the *Mercenaria* R-LC, and no fluorescence was seen on the blots of either the unphotolyzed or photolyzed

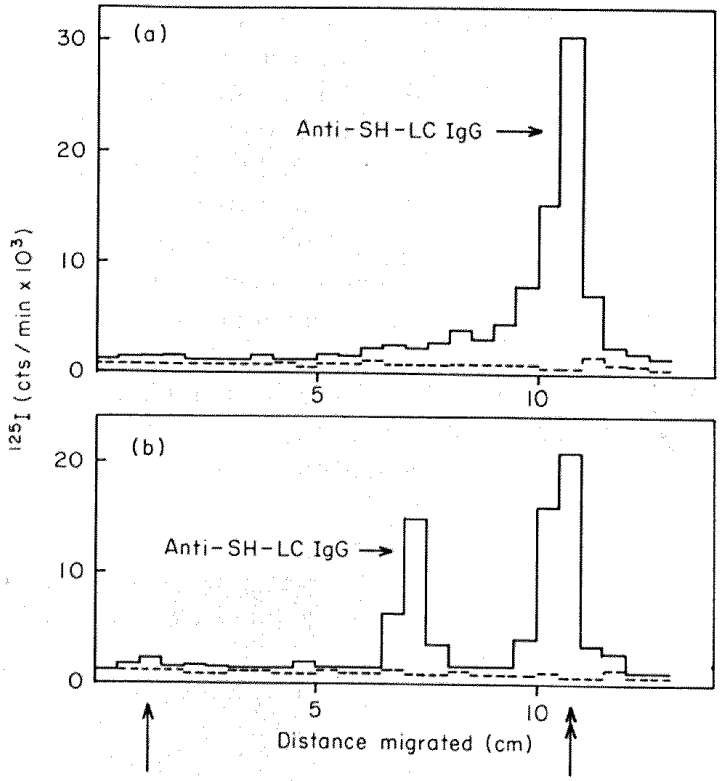

FIG. 6. Extent of cross-linkage of scallop SH-LC and *p*-APA-modified *Mercenaria* R-LC in scallop myofibrils (pure hybrids). Preparation and procedures were as described for Fig. 5, except the FITC-conjugated goat anti-rabbit IgG contained 0·075 μCi ^{125}I-labeled IgG/ml. The blots were cut into 5 mm long segments and counted. Broken lines, anti-R-LC-treated strips; continuous lines, anti-SH-LC-treated strips. Control IgG-treated samples had the same low counts as the anti-R-LC IgG-treated samples and are not shown. (a) Unphotolyzed sample; (b) photolyzed samples. The single arrow indicates the position of the heavy-chain on the gel. Monomeric light-chains move with the dye front on the sodium dodecyl sulfate/10% polyacrylamide slab gel (double arrow).

samples after treatment with that antibody. This is in agreement with previous studies, which showed that *Mercenaria* R-LC does not cross-react with rabbit anti-scallop R-LC IgG (Wallimann & Szent-Györgyi, 1981*b*), and also indicates the complete removal of scallop R-LCs by the treatment with EDTA at 35°C. The blot of the sodium dodecyl sulfate/polyacrylamide gel of the unphotolyzed control after incubating with anti-scallop SH-LC IgG showed SH-LC only in the 18,000 M_r band corresponding to the light chain monomer position. Blots of the photolyzed sample, however, showed an additional SH-LC-containing band at a position corresponding to M_r 35,000 with a mobility similar to tropomyosin (Fig. 5). The amount of SH-LC present within this 35,000 M_r complex was 20 to 35% of the total SH-LC, as estimated by the binding of ^{125}I-labeled secondary antibody, whereas the radioactivity of unphotolyzed samples was confined to the 18,000 M_r band (Fig. 6; Table 2). The SH-LC could occur at this position only through cross-linking to the *Mercenaria* R-LC. No SH-LC was detected in higher molecular weight complexes. The lack of a cross-linked SH-LC complex of 230,000 M_r is consistent with the *Mercenaria* R-LC having only a single photo-activatable cross-linking group.

The lack of cross-reactivity of the *Mercenaria* R-LC with anti-scallop R-LC IgG allows the detection of cross-linking between R-LCs in mixed hybrids, i.e. containing both scallop and *Mercenaria* R-LCs, since the cross-linked complex of the scallop R-LC with *Mercenaria* R-LC can be distinguished from the cross-linked complex of scallop SH-LC with *Mercenaria* R-LC. To obtain such preparations, *Mercenaria* R-LC labeled with *p*-APA was incubated with scallop myofibrils that had been treated with EDTA at 12°C and retained, on an average, one of their native R-LCs on the head of each myosin molecule. Part of the preparation was photolyzed and the remainder was kept as the unphotolyzed control. The gels and Amido black-stained blots looked very similar to those obtained with pure *Mercenaria* R-LC hybrids (Fig. 6). Blots of gels of unphotolyzed and photolyzed samples incubated with anti-scallop SH-LC IgG both showed fluorescence at the light-chain monomer position while, in addition, the photolyzed sample also had a

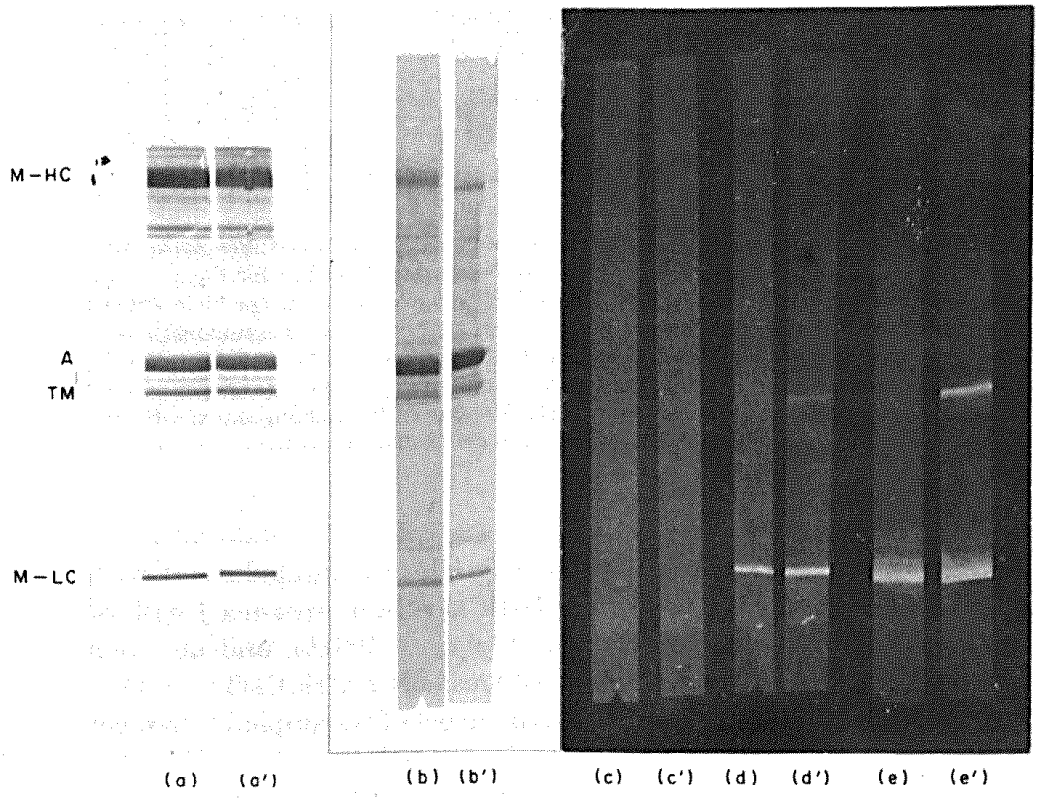

FIG. 7. Photolysis of scallop myofibrils containing scallop R-LC and *p*-APA-modified *Mercenaria* R-LC (mixed hybrids). Sample loading was 250 μg myofibril/5 cm of sodium dodecyl sulfate/10% polyacrylamide slab gel for electrophoresis. (a') to (e') Samples photolyzed for 45 min with an HBO 200 high-pressure mercury lamp. (a) and (a') Gels stained with Coomassie blue; (b) and (b') blots stained with Amido black; (c) and (c') blots treated with control preimmune IgG; (d) and (d') blots treated with anti-R-LC IgG; (e) and (e') blots treated with anti-SH-LC IgG. Treatment of blots with IgG was folllowed by staining with FITC-conjugated goat anti-rabbit IgG. M-HC, myosin heavy-chain; A, actin; TM, tropomyosin; M-LC, myosin light-chain. Myofibrils were desensitized at 12°C before addition of *p*-APA-modified *Mercenaria* R-LC. Actin-activated Mg-ATPase activities in calcium (μmol min^{-1} mg^{-1}) and ATPase ratios in EGTA/Ca^{2+} were as follows: intact myofibrils, 0·35 and 0·07; desensitized, 0·25 and 0·62; hybrid before photolysis, 0·23 and 0·06; hybrid after photolysis, 0·27 and 0·17 (hybrid values from single time-courses).

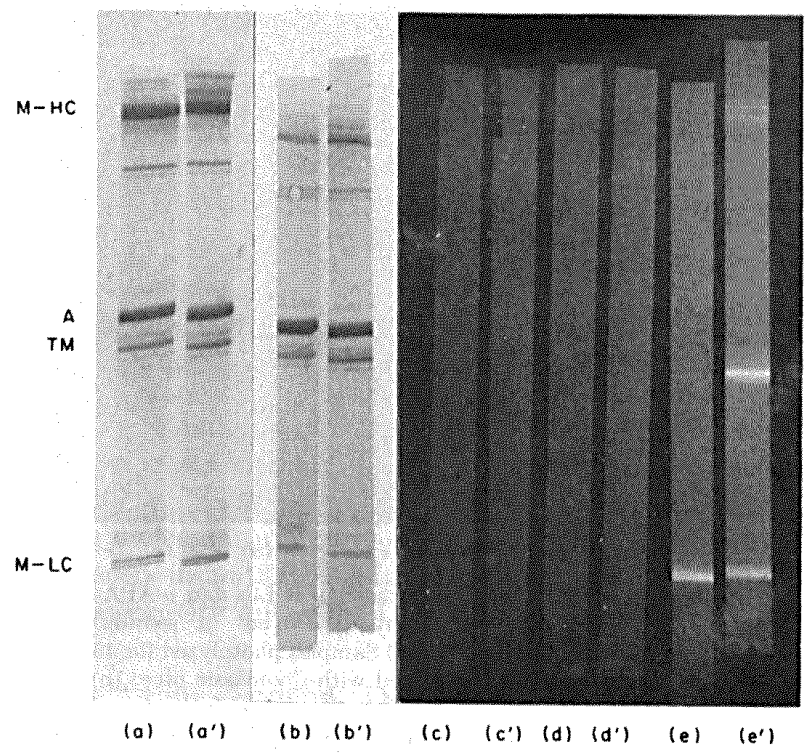

FIG. 8. Photolysis of scallop myofibrils containing *p*-APA-modified rabbit R-LC (pure hybrids). Sample loading was 250 μg myofibrils/5 cm of sodium dodecyl sulfate/10% polyacrylamide slab gel for electrophoresis. (a') to (e') Samples photolyzed for 45 min with an HBO 200 high-pressure mercury lamp. (a) and (a') Gel stained with Coomassie blue; (b) and (b') blots stained with Amido black; (c) and (c') blots treated with control preimmune IgG; (d) and (d') blots treated with anti-R-LC IgG; (e) and (e') blots treated with anti-SH-LC IgG. Treatment of blots with IgG was followed by staining with FITC-conjugated goat anti-rabbit IgG. M-HC, myosin heavy-chain; A, actin; TM, tropomyosin; M-LC, myosin light-chain. Myofibrils were desensitized at 35°C. Actin-activated Mg-ATPase activities in calcium (μmol min^{-1} mg^{-1}) and ATPase ratios in EGTA/Ca^{2+} were as follows: in intact myofibrils, 0·32 and 0·02; desensitized, 0·17 and 1·11; hybrid before photolysis, activity 0·02; hybrid after photolysis, 0·19 and 0·58 (hybrid values from single time-courses). The low ATPase activity of the hybrid myofibrils may reflect a solubilizing or some other inhibitory action of the modified rabbit R-LC after the 16 h incubation. This inhibition was significantly reversed by photolysis.

strong fluorescent band at a position corresponding to M_r 35,000, exactly as was found when the *p*-APA-labeled *Mercenaria* R-LC was incubated with fully desensitized (35°C, EDTA-treated) myofibrils. However, when blots of the unphotolyzed and photolyzed samples were incubated with anti-scallop R-LC-IgG, the antibody not only bound to the light-chain monomers in both cases, but in the photolyzed sample scallop R-LC was also detected at the 35,000 M_r position as a very faint but reproducible fluorescent band (Fig. 7). The scallop R-LC had no photo-activatable cross-linker itself and could appear at this position only if it had been cross-linked to the *Mercenaria* R-LC.

(d) *Cross-linking with rabbit R-LC*

Rabbit R-LCs contain two cysteine residues in the C-terminal region of the molecule (Cys128 and Cys157, out of 169 residues: Collins, 1976). Both of these

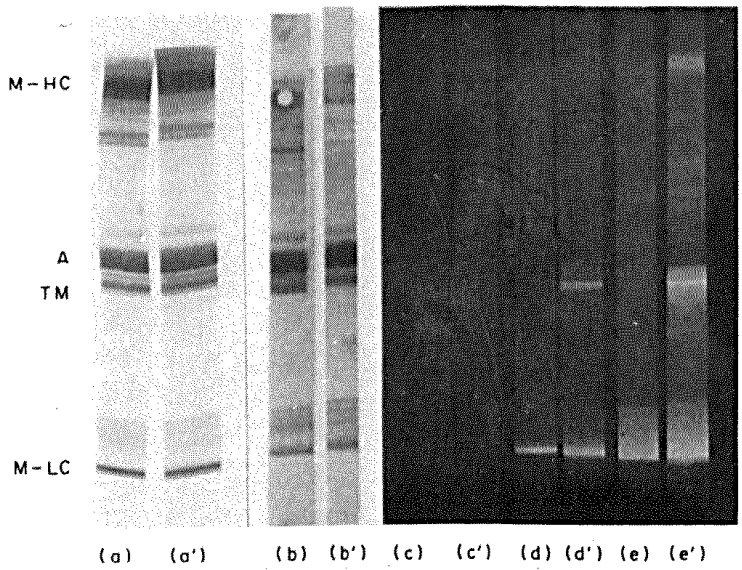

FIG. 9. Photolysis of scallop myofibrils containing scallop R-LCs and *p*-APA-modified rabbit R-LCs (mixed hybrids). Sample loading was 300 μg myofibrils/5 cm of sodium dodecyl sulfate/10% polyacrylamide slab gel for electrophoresis. (a') to (e') Samples photolyzed for 45 min with an HBO 200 high-pressure mercury lamp. (a) and (a') Gels stained with Coomassie blue; (b) and (b') blots stained with Amido black; (c) and (c') blots treated with control preimmune IgG; (d) and (d') blots treated with anti-R-LC IgG; (e) and (e') blots treated with anti-SH-LC IgG. Treatment of blots with IgG was followed by staining with FITC-conjugated goat anti-rabbit IgG. M-HC, myosin heavy-chain; A, actin; TM, tropomyosin; M-LC, myosin light-chain. Myofibrils were desensitized at 12°C. Actin-activated Mg-ATPase activities in calcium (μmol min^{-1} mg^{-1}) and ATPase ratios in EGTA/Ca^{2+} were as follows: intact myofibrils, 0·41 and 0·02; desensitized, 0·27 and 0·87; hybrid before photolysis, 0·13 and 0·05; hybrid after photolysis, 0·26 and 0·23 (hybrid values from single time-courses).

residues were labeled with *p*-APA. The modified rabbit R-LCs were combined with fully desensitized scallop myofibrils. Upon illumination of these pure hybrids, a distinct new band was seen on the gel trailing the myosin heavy-chains, similar to that observed with HABS-modified scallop R-LC. No fluorescent bands were obtained on strips incubated with preimmune or anti-scallop R-LC IgGs before or after illumination (Fig. 8). The absence of binding of anti-scallop R-LC antibody demonstrates the effectiveness of the removal of scallop R-LC light chains by treatment with EDTA at 35°C, and is in agreement with the lack of cross-reactivity between rabbit and scallop R-LCs. With anti-SH-LC IgG, fluorescent bands were seen. In unphotolyzed myofibrils, the fluorescence was restricted to the position corresponding to light-chain monomer (Fig. 8). In photolyzed samples, additional fluorescent bands appeared at positions of about 35,000 M_r, together with several closely spaced bands trailing the myosin heavy-chain (Fig. 8). At higher myofibril concentrations, faint fluorescent bands were also seen at about 53,000 and 66,000 M_r (Fig. 8). Since the rabbit R-LCs contained two photo-activatable cross-linkers, cross-linking of the SH-LC with the heavy-chain *via* the rabbit R-LC could occur as with the HABS-modified scallop R-LC. About one-third of the bound ^{125}I-labeled goat anti-rabbit IgG was associated with the 35,000 M_r complex after treatment with anti-SH-LC IgG (Table 2). Overnight incubation with modified rabbit R-LC

reduced extensively the actin-activated Mg-ATPase activities, both in the presence and in the absence of calcium (cf. Scholey *et al.*, 1981). These activities were regained upon photolysis (see the legend to Fig. 8).

Mixed hybrids of scallop myosin with rabbit-R-LC were also obtained by combining EDTA-treated (12°C) myofibrils with *p*-APA-modified rabbit R-LC. Such preparations contained an approximately equal number of scallop and rabbit R-LCs. The gels showed the formation of a new band trailing the heavy-chain as a result of photolysis (Fig. 9). The fluorescent band patterns of the blots of mixed hybrids treated with anti-SH-LC IgG were very similar to the patterns obtained with pure hybrid, showing SH-LC monomers in the unilluminated samples, and additional fluorescent bands at positions corresponding to molecular weights of about 35,000 and >200,000 formed by photolysis. Staining with anti-scallop R-LC IgG showed scallop R-LC monomers in unphotolyzed samples, while in photolyzed samples it also detected scallop R-LC as a weak fluorescent band in a position corresponding to a molecular weight of about 35,000. The >200,000 M_r fluorescent bands were not seen in anti-R-LC IgG-treated photolyzed mixed hybrids, in contrast to the results with anti-SH-LC IgG.

4. Discussion

The purpose of this study was to test the proposition that R-LC and SH-LC can functionally interact (Wallimann & Szent-Györgyi, 1981*a*). We intended to establish the close proximity of the two kinds of light-chains by cross-linking with short reagents, the length of the azidobenzoate cross-linker being 6·5 Å and that of the azidophenacyl 8·3 Å, indicating the largest estimate of separation between the cross-linked light-chains. Since the cross-linker was attached only to R-LC, the presence of SH-LC in bands of higher molecular weights as a result of photolysis could be explained only by cross-linkage between the two light-chains, and is taken as direct evidence that such cross-linking indeed takes place. It was important that the cross-linker be introduced only by the R-LC, and great care was taken to quench and remove the unreacted free photosensitive reagents and to use light-chains of the highest purity.

With scallop and *Mercenaria* R-LCs it has been possible to demonstrate that the light-chains carrying cross-linker remained functional and restored calcium sensitivity. The functionality of the scallop R-LC is particularly noteworthy, since four to six of its lysine groups had been substituted. The ability of the modified R-LCs to regulate indicates that the R-LCs carrying the cross-linker rebound in the same way as intact light-chains, and their position with respect to the SH-LC was not altered. Thus the modified R-LCs are tightly bound to their specific binding sites on the myosin heads. We have also tried to reduce the chances of cross-linking R-LC and SH-LCs from different myosin heads or myosin molecules by using myofibrils and not solutions of pure myosin. In myofibrils, especially at lengths somewhat shorter than rest length, the cross-bridges are extensively immobilized by rigor link formation with actin (Mendelson & Cheung, 1976; Thomas *et al.*, 1980). The separation between the heads of the same myosin molecule at the point of actin attachment exceeds the length of the cross-linker (Offer & Elliott, 1978;

Craig *et al.*, 1980). The distances between the different myosin molecules are even greater. Thus, extensive cross-linking of R-LC and SH-LC of different myosin heads during the lifetime (of a few milliseconds) of the diradical appears to be unlikely. Illumination exceeded the lifetime of the diradical considerably (>15 minutes) to ensure that a major portion of the photoreagent in the myofibrillar suspension was exposed to light. Although one cannot completely exclude cross-linking of SH-LC and R-LC situated on different myosin heads away from the hinge region, the use of myofibrils should considerably reduce this possibility. We believe that any cross-linking between the light-chains associated with different heads may derive from special structural features of myosin, such as the location of part of the light-chains in the hinge region. Thus, cross-linking in the hinge region between the R-LC associated with one head and the SH-LC associated with the other head, such as occurs between the two R-LCs, may occur on the present evidence, and an R-LC could even be in contact at different points with both SH-LCs. However, the results from the *Mercenaria* R-LC with its single photolabel and the rabbit R-LC, where the two photolabels are close together near one end of the molecule, can probably be attributed to a preferential cross-linking of the R-LC to only one of the two SH-LCs on the myosin.

The restricted sizes of molecular weights of cross-linked R-LC and SH-LC also indicate that the reaction is not random. Cross-linked R-LC and SH-LC appear as a single band using photoreagent-labeled *Mercenaria* R-LC on which a single thiol group is substituted. This complex has an apparent molecular weight of about 35,000, corresponding to the sum of the weights of a single R-LC and a single SH-LC. Although random cross-linkage between the R-LCs of different myosin heads could, theoretically, lead to complexes containing two or more *Mercenaria* R-LC cross-linked with a single scallop SH-LC, fluorescent bands were not detected in the 50,000 or higher molecular weight ranges. In contrast to the results obtained with modified *Mercenaria* R-LC, modified scallop R-LC and rabbit R-LC that are conjugated with two or more of the photosensitive labels give rise to strong fluorescent bands, corresponding to molecular weights greater than 200,000 in addition to the strong band at around 35,000 M_r. All of these bands contain both R-LC and SH-LC. The cross-linked high molecular weight complexes are formed in sufficiently high concentrations to be observed in Coomassie blue-stained gels; the 35,000 M_r complex, however, comigrates with tropomysin. The faint fluorescent bands seen with the modified scallop R-LC at molecular weights of about 53,000 and 66,000 are likely to be due to complexes consisting of three and four cross-linked light-chains, respectively. The presence of these two faint bands thus indicates that some limited cross-linking between the R-LCs of the two myosin heads can take place. The lack of other higher molecular weight complexes of light-chains (not involving heavy-chains) suggests the absence of extensive cross-linkage between R-LCs of different myosin molecules.

Cross-linking of the R-LC on the two heads of a single myosin molecule was demonstrated with the aid of mixed hybrids. The presence of scallop R-LC in the 35,000 M_r band of photolyzed myofibrils that contained one scallop R-LC and one modified *Mercenaria* or rabbit R-LC was due to cross-linkage between the two different R-LCs. One notes that the band was very faint in the experiments with *p*-

APA-conjugated *Mercenaria* R-LC and, although perhaps somewhat stronger with the modified rabbit R-LC, in both cases the band representing cross-linked R-LC and SH-LC complex was significantly more intense. Cross-link formation between R-LC and SH-LC is therefore a more likely event than one between two R-LCs. The R-LCs may be joined as a result of some residual mobility of myosin heads even in rigor; it is possible, however, that portions of the two R-LCs on the two myosin heads, possibly close to the hinge region, are sufficiently near to each other to be cross-linked. If so, one would expect that cross-linking would depend strongly on the position of the cross-linker along the R-LC. The differences between the *Mercenaria* R-LC and rabbit R-LC results do not appear to be sufficiently decisive to choose among the alternatives.

Although molecular weights obtained from sodium dodecyl sulfate/polyacrylamide gel electrophoresis of cross-linked chains are subject to even greater uncertainties than those of fully dissociated chains, none of the major fluorescent bands formed by photolysis can readily accommodate complexes with actin or with tropomyosin. While the presence of tropomyosin in the 53,000 and 66,000 M_r complex that are seen with modified scallop R-LCs after photolysis cannot be excluded at present, one notes that these bands are faint and, in the case of the modified scallop R-LC, contain both R-LCs and SH-LCs and thus are more likely to consist of three or four cross-linked light-chains. Thus, the light-chains seem to be at a distance from the actin binding region of the myosin head. This interpretation is in agreement with the three-dimensional reconstruction of scallop thin filaments decorated with scallop myosin subfragment-1 containing and lacking regulatory light-chains (Craig *et al.*, 1980; Vibert *et al.*, 1981). One notes that each band is likely to consist of a heterogeneously cross-linked population of the same two light-chains, particularly of complexes involving the multi-substituted scallop and rabbit R-LCs. Such heterogeneity may explain the diffuseness of the fluorescent bands obtained with the multi-conjugated scallop and rabbit R-LCs in contrast to the relatively sharp fluorescent band formed with the singly substituted *Mercenaria* R-LC.

Cross-linking does not greatly inactivate the actin-activated Mg-ATPase activities. The major effect of photolysis is a loss of calcium sensitivity, a result of activation of the ATPase activity in the absence of calcium. Although the evidence is fragmentary, there appears to be some proportionality between the degree of cross-linking of SH-LCs and the loss in calcium sensitivity. Calcium sensitivity was reduced from 88% to 66% when about one-third of the scallop SH-LCs were cross-linked to *Mercenaria* R-LC; and about 70% cross-linking with scallop R-LC lowered calcium sensitivity from 82% to 32%. The findings are in line with previous studies indicating that scallop light-chains do not interact directly with ATPase sites (Chantler & Szent-Györgyi, 1980; Wallimann & Szent-Györgyi, 1981*a*). It appears that regulation with cross-linked R-LCs and SH-LCs is lost by the inability of the myosin to switch off.

With the use of ^{125}I-labeled secondary goat anti-rabbit IgG, a remarkable degree of cross-linking between R-LCs and SH-LCs can be demonstrated. The data obtained with the *p*-APA-modified *Mercenaria* R-LC are the most straightforward, since this R-LC contains a single cross-linker in a well-defined position. The fact

that about one-third of the scallop SH-LC forms a 35,000 M_r complex upon photolysis is strong evidence that R-LCs and SH-LCs adjoin each other in scallop myosin. A figure of about 30% cross-linking is very substantial, considering that not all the photolabels on the R-LC will be successfully involved in reactions leading to cross-linkage between the peptide chains. The cross-linking with the multi-substituted scallop and rabbit R-LCs may exceed 50%, but even with these R-LCs, about one-third of the scallop SH-LC forms 35,000 M_r complexes. It appears, therefore, that modified R-LCs are cross-linked with the same or possibly greater ease with SH-LCs than with myosin heavy-chains. It is of interest that in the studies reported by d'Albis & Gratzer (1976) rabbit light-chains did not cross-link to rabbit myosin heavy-chain when the cross-linking reagent dithiobis-succinimidyl propionate was used. It is also possible that the calcium-specific site (Chantler & Szent-Györgyi, 1980) may involve both the SH-LC and the adjoining R-LC. Other data agree with the high estimate of cross-linking. The band consisting of cross-linked myosin heavy chain, R-LC and SH-LC can be detected easily on the gels by staining with Coomassie blue, using photoreactive scallop and rabbit R-LCs (Figs 3, 4, 5, 8 and 9). The fluorescence of the 35,000 M_r band containing one SH-LC and one R-LC is strong, although a corresponding band is not resolved from tropomyosin by staining with Coomassie blue. The altered tropomyosin to SH-LC ratio after illumination of myofibrils containing modified scallop R-LC also indicates that a significant fraction, possibly 40%, of the SH-LC may participate in cross-linking.

Cross-linking with HSAB-modified scallop R-LC demonstrates that there are regions on the R-LC that are no more than 6·5 Å from the SH-LC. Since the substituted lysine groups on the R-LC are not identified, this modification, although useful to establish the proximity of the two light-chain types, does not give information on how extensively these light-chains may overlap.

The results with *p*-APA-modified *Mercenaria* and rabbit R-LCs indicate that the overlap between the R-LC and SH-LCs is extensive and, assuming an elongated shape for these molecules (Stafford & Szent-Györgyi, 1978; Hartt & Mendelson, 1979; Wallimann & Szent-Györgyi, 1981*b*), 50% or more of the R-LC may be in close proximity ($\leq$8·3 Å) to SH-LCs. *p*-APA reacts specifically with thiol groups. In rabbit R-LC, both of the cysteine residues are near the carboxyl terminus of the sequence (residues 129 and 157, out of a total of 169) in domains 3 and 4 (Collins, 1976). In *Mercenaria* R-LC, the cysteine residue is near the amino terminus and is found between domains 1 and 2 (Bagshaw & Kendrick-Jones, 1980). Assuming that the positions of these light-chains are analogous to those of scallop R-LCs on myosin, the stretch between the labels of the *Mercenaria* and rabbit R-LC exceeds 50% of the molecule. This interpretation is consistent with the finding that two to three thiol groups on the SH-LC are protected by the R-LC (Hardwicke *et al.*, 1981) and with the observation that in trypsin-digested scallop myosin subfragment-1, R-LC and SH-LC are both attached to a common 14,000 M_r peptide (E. M. Szentkiralyi, unpublished results).

The interpretation is also consistent with electron microscopic studies showing that Fab antibodies specific against R-LC and against SH-LC both bind to similar regions of the myosin molecule corresponding to the distal third (towards the

subfragment-2 region) of the subfragment-1 head (Flicker *et al.*, 1981). The findings support the model of R-LC and SH-LCs in myosin with an extensive overlap between these light-chains that was proposed on the basis of immunological studies, and is in line with the suggestion that the SH-LCs may also participate in the regulatory switch of myosin (Wallimann & Szent-Györgyi, 1981*a*).

We thank Regina Niebieski for expert technical assistance. We thank Drs Peter Chantler and Eva M. Szentkiralyi for discussions and for critically reading the manuscript. We acknowledge the help of Mr Howard Shpetner in some of the exploratory studies. We also thank Dr Raymond E. Stephens for suggesting possible cross-linkers used in this study.

This research was supported by grants from the Public Health Service (AM 15963), Institutional Grant (B.R.S.G. 6781), the Muscular Dystrophy Association (to A.G.S.G.) and by postdoctoral fellowship from the Muscular Dystrophy Association (to T.W. and to P.M.D.H.).

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Edited by H. E. Huxley