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Regulatory and Essential Light-chain Interactions in Scallop Myosin

I. Protection of Essential Light-chain Thiol Groups by Regulatory Light-chains

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The reactivity of the thiol groups of the essential light-chains of scallop myosin is greatly reduced by the presence of regulatory light-chains on myosin. The thiol groups of the essential light-chains react with iodoacetate only if the regulatory light-chains have been removed by treatment with EDTA. No alkylation of the essential light-chains could be detected in myosins containing regulatory light-chains (untreated or reconstituted myosins) after an overnight incubation with excess iodoacetate at 4°C. In contrast, similar treatment alkylated two to three thiol groups of essential light-chains in desensitized myosins from which the regulatory light-chains had been removed. In addition, up to seven of the 20 heavy-chain thiols were also alkylated; however, the reactivity of the heavy-chain thiols did not depend on the presence of the regulatory light-chains. ATPase activities were not inhibited by alkylation with iodoacetate. Regulatory light-chains also protected essential light-chain thiols against reaction with *N*-iodoacetyl-*N'*-(1-sulfo-5-naphthyl) ethylenediamine and against dansylation at pH 6·7, although treatment with these reagents caused a considerable loss of ATPase activities. Rebinding of the regulatory light-chains was impaired by alkylation. The results indicate an extensive interaction between the regulatory and the essential light-chains in scallop myosin.

1. Introduction

Scallop myosin is a regulatory myosin (Kendrick-Jones *et al.*, 1970) that consists of two heavy-chains and four light-chains of two kinds, regulatory light-chains and essential light-chains (Kendrick-Jones *et al.*, 1976). These light-chains are associated with the myosin head, each subfragment-1 containing one R-LC‡ and one SH-LC. Regulation and calcium binding depend on the presence of R-LCs (Szent-Györgyi *et al.*, 1973; Chantler & Szent-Györgyi, 1980). Unlike the R-LC,

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‡Abbreviations used: R-LC, regulatory light-chain; SH-LC, essential light-chains; IAEDANS, *N*-iodoacetyl-*N'*-(1-sulfo-5-naphthyl)-ethylenediamine.

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removal of the SH-LC from scallop myosin has not been possible without irreversibly denaturing the molecule, and its function could not be directly studied. On the basis of immunological evidence, it has been suggested that the SH-LC may also have a regulatory role, since binding of antibody specific against the SH-LC overrides the inhibitory effect of the R-LC on the actin-activated Mg-ATPase in the absence of calcium ions (Wallimann & Szent-Györgyi, 1981). The SH-LC may also be associated structurally with the R-LC, since the presence of R-LC on the myosin protects the SH-LC from digestion by papain (Stafford *et al.*, 1979), and the amount of specific anti-SH-LC immunoglobulin G binding depends on the presence of R-LC (Wallimann & Szent-Györgyi, 1981). Thus, the two types of light-chains possibly interact.

In this study we further explored the interaction between the two kinds of light-chains. We have found that the presence of R-LC reduces the reactivity of the thiol groups of the SH-LCs, and we describe this protective action of the R-LC towards several different reagents in some detail.

Some of the results were reported in the 1981 Meeting of the Biophysical Society (Hardwicke *et al.*, 1981).

2. Experimental Procedures

Aequipecten irradians was obtained from the Marine Biological Laboratory, Woods Hole, Mass., and the adductor muscles were stored at −30°C in 50% (v/v) ethylene glycol, 20 mM-NaCl, 0·5 mM-$MgCl_2$, 0·05 mM-EDTA, 1·5 mM-NaN_3, 2·5 mM-sodium phosphate (pH 7·0) containing 0·005% (w/v) sulfadiazine and 0·05 mM-phenylmethylsulfonyl fluoride (Sellers *et al.*, 1980). Dansyl chloride was purchased from Pierce Chemical Co., IAEDANS from the Aldrich Chemical Co. or Molecular Probes, Inc., and [^{14}C]iodoacetic acid from New England Nuclear, Inc.

(a) *Preparation of myosin*

Intact myosin was prepared from the adductor muscle of *A. irradians* as described previously (Chantler & Szent-Györgyi, 1978). Fully desensitized scallop myosin was made by extracting intact myosin with 10 mM-EDTA at 23°C for 5 min (Wallimann & Szent-Györgyi, 1981). Desensitized myosin was resensitized by incubation overnight at 4°C with a 6-fold molar ratio of scallop R-LC in Mg^{2+}-wash (40 mM-NaCl, 1 mM-$MgCl_2$, 5 mM-sodium phosphate, pH 7·0). Unbound R-LC was removed by a brief centrifugation in the Sorvall RC-2B centrifuge, and the myosin was washed 3 times and resuspended in Mg^{2+}-wash. Light-chains were removed by treatment of myosin with guanidine·HCl as described (Kendrick-Jones *et al.*, 1976). The addition of Mg^{2+}-wash instead of water to the guanidine·HCl extract before addition of ethanol led to a heavy-chain precipitate that was easier to disperse. The precipitate was pelleted at 5000 ***g*** in the Sorvall centrifuge, resuspended and washed 3 times in Mg^{2+}-wash.

(b) *Carboxymethylation of scallop myosin*

(i) *At high ionic strength*

Intact, 23°C-desensitized and resensitized myosins were incubated for 18 h at 4°C in the dark on a rotary mixer at a concentration of 5 to 6 mg/ml with a 5-fold molar ratio of iodoacetic acid (0·1 M-iodoacetic acid in 0·3 M-sodium phosphate, pH 7·5) to the total protein thiol content (46 mol thiol/mol myosin; Table 1). Equal volumes of 0·3 M-sodium phosphate (pH 7·5) were added to the respective controls. The reaction was carried out in 0·4 M-NaCl,

2 mM-$MgCl_2$, 25 mM-sodium phosphate (pH 7·5), and stopped by the addition of a 50-fold molar excess of 2-mercaptoethanol. The solutions were centrifuged at 100,000 *g* for 0·5 h to remove denatured material, and the samples were then dialyzed against 20 mM-NaCl, 1 mM-$MgCl_2$, 3 mM-NaN_3, 5 mM-sodium phosphate (pH 6·5) for 4 h to precipitate the myosins. The precipitated myosins were then centrifuged briefly in the Sorvall RC-2B centrifuge and resuspended and washed 3 times in 20 mM-NaCl, 1 mM-$MgCl_2$, 3 mM-NaN_3, 5 mM-sodium phosphate (pH 6·5), before finally resuspending in Mg^{2+}-wash.

(ii) *At low ionic strength*

Intact, 23°C-desensitized and resensitized myosins were incubated for 18 h at 4°C in the dark on a rotary mixer in 40 mM-NaCl, 2 mM-$MgCl_2$, 5 mM-sodium phosphate (pH 7·2 or 7·5) with iodoacetic acid in different molar ratios ranging from 2·5 to 13·2 to total thiol (0·1 M-iodoacetic acid in 0·3 M-Na_2HPO_4 (pH 7·2 or 7·5); equal volumes of 0·3 M-Na_2HPO_4 (pH 7·2 or 7·5) were added to the respective controls). Sometimes, before treatment with iodoacetic acid, the myosin was first incubated with 5 mM-dithiothreitol in Mg^{2+}-wash on ice for 0·5 h and the dithiothreitol was removed by centrifuging the myosins briefly, discarding the supernatants and washing quickly 3 times in Mg^{2+}-wash. The reaction was stopped by the addition of a 50-fold molar excess of 2-mercaptoethanol over the added iodoacetic acid, and the myosin was pelleted and washed 3 times in 40 mM-NaCl, 2 mM-$MgCl_2$, 5 mM-sodium phosphate (pH 7·2) before finally resuspending in Mg^{2+}-wash.

The extent of carboxymethylation after treatment with iodoacetic acid was determined either from carboxymethylcysteine contents obtained by amino acid analysis of the purified light-chain and heavy-chain fractions, or by measuring the incorporation of [^{14}C]iodoacetic acid. In the latter method, 0·5-ml samples containing about 1 to 2 mg of protein were dissolved in 7 ml of Aqueous Liquid Scintillant (ACS, Amersham Radiochemicals Co.) and counted in a Beckman LS-255 liquid scintillation counter. The same amount of "cold" protein was added to the [^{14}C]iodoacetic acid standard to obtain a quenching correction. The extent of the carboxymethylation of the SH-LC of intact, desensitized and resensitized myosins was also determined by submitting 100 to 150 μg samples of light-chain fractions from the respective myosins to electrophoresis on 10% polyacrylamide/8 M-urea gels. Gels were sliced at 4 mm intervals with a Joyce–Loebl gel cutter. The slices were transferred into liquid scintillation vials, to which 0·5 ml of 30% (v/v) H_2O_2 was added. Overnight incubation at 60 to 70°C and the subsequent addition of 7 ml of scintillant completely solubilized the gel sample. [^{14}C]iodoacetic acid was added to 4 mm slices of the gels containing no radioactivity to correct for quenching by acrylamide and urea.

(c) *Dansylation of scallop myosin*

Samples (10 mg) of intact, desensitized and resensitized myosins were reacted overnight at 4°C with 5% (w/w) dansyl chloride (corresponding to a molar ratio of 1·89 to the total protein thiol) in 3·5 ml of 40 mM-NaCl, 1 mM-$MgCl_2$, 0·1 mM-EDTA, 3 mM-NaN_3, 10 mM-sodium phosphate (pH 6·7). Next day, the samples were centrifuged at 5000 revs/min in the HS-4 rotor of the Sorvall RC-2B centrifuge for 5 min. The supernatants were discarded and the pelleted myosin samples were washed 3 times with Mg^{2+}-wash.

(d) *Reduction, carboxymethylation and dansylation of purified scallop SH-LC*

Two 10-mg samples of SH-LC were reduced with 5 mM-dithiothreitol in 1 mM-EDTA, 4 M-urea, 50 mM-sodium borate (pH 8·2), under N_2 at 22°C for 0·5 h. While one sample was kept on ice under N_2, iodoacetic acid was added to the other in a 1·7 molar ratio to the thiol groups. After incubation at 22°C for 1 h in the dark under N_2, the sample was cooled on ice and a 2-fold molar ratio of dithiothreitol was added to both samples. The samples were dialyzed against 0·1 mM-dithiothreitol, 30 mM-sodium phosphate (pH 6·7), and dansyl chloride, equimolar to the total thiol in the reduced non-alkylated sample, was added under N_2 to each sample. The samples were stirred for 7 h at 4°C in the dark, centrifuged at

100,000 *g* for 15 min to remove particles of dansyl chloride, and then dialyzed extensively against 30 mM-sodium phosphate (pH 6·7).

(e) *Determination of the extent of dansylation of the SH-LCs of intact, desensitized and resensitized myosin*

The relative labeling of the SH-LCs of the myosin samples was estimated in two ways: 10% polyacrylamide/urea gels of the dansylated samples were fixed and urea was removed first in 50% (v/v) methanol/10% (v/v) acetic acid and then in 5% (v/v) methanol/7·5% (v/v) acetic acid. The gels were illuminated at 302 nm and the fluorescent bands were recorded photographically on Polaroid type 55 film. Negatives with non-saturated grain densities were chosen for quantitative analysis from an exposure time series. Microdensitometry of the SH-LC bands on the transparent negatives was then carried out in a Photoscan P-1000 system (Optronics) linked to a Grinell graphic screen, which gave the integrated absorbancies across the SH-LC band. The relative absorbancies so determined were then related to the relative original concentrations of fluorescent emitters (dansyl groups) in the gels by the relation $C_2/C_1 = 10^{(A_2-A_1)/a}$ using a value for a (the slope of the characteristic curve of Polaroid type 55 film) of 0·65. The gels were then scanned at 335 nm in a Gilford linear scanner and the integrated relative absorbancies across the peaks, i.e. the areas of the peaks as determined by planimetry, taken as proportional to the dansyl content of the bands obtained. Finally, the relative protein contents of the bands were estimated by staining the same gels with Acid fast green, scanning them at 660 nm in the Gilford linear scanner, and using the areas of the peaks determined by planimetry as measures of the relative amounts of protein (Szent-Györgyi *et al.*, 1973). The dansyl content in relation to the protein content for a given SH-LC band was in arbitrary units, normalized to 1 for the dansyl content of the SH-LC of intact myosin.

The fluorescence emission spectra of alkylated SH-LC and reduced, non-alkylated SH-LC after dansylation were recorded on a Perkin-Elmer MPF-44 spectrophotofluorimeter using an excitation wavelength of 320 nm.

(f) *Labeling with IAEDANS*

IAEDANS (2·5 mM in 0·1 M-sodium phosphate, pH 7·0) was added in molar ratios of 4 and 8 mol/mol to intact or desensitized scallop myosin (1 to 2 mg ml^{-1}) in Mg^{2+}-wash (this corresponds to 0·087 and 0·174 molar ratios of IAEDANS to the total thiol content, 46 mol thiol/470,000 g intact myosin and 46 mol thiol/435,000 g desensitized myosin). Care was taken to adjust the final pH of the reaction mixture to 7·2. Control samples of intact and desensitized myosin were taken through exactly the same procedure, except that no IAEDANS was added to them. After agitating gently on a rotary mixer for 18 h at 4°C in the dark, a 100-fold molar ratio of 2-mercaptoethanol to IAEDANS was added, and the preparation was centrifuged in the Sorvall centrifuge at 3000 *g* for 5 min. The supernatants were discarded and the myosin washed 3 times with Mg^{2+}-wash. After separating the light and heavy-chain fractions, the IAEDANS contents were determined in a Perkin Elmer MPF-44 spectrophotofluorimeter by measuring the fluorescence emission at 500 nm (excitation at 337 nm) relative to a standard solution of purified SH-LC conjugated with a known amount of IAEDANS (determined from $\epsilon_{336} = 6{\cdot}1 \times 10^3$ M^{-1} cm^{-1}; Hudson & Weber, 1972). All fluorescence spectra were determined in 6·4 M-guanidine·HCl, 2 mM-EDTA, 25 mM-Tris·HCl (pH 8·0) to normalize the environment of the fluorophore. The light-chain fractions from intact and desensitized myosins treated with IAEDANS were run on 10% polyacrylamide/8 M-urea gels to determine the proportion of the total protein represented by the SH-LC (Kendrick-Jones *et al.*, 1976). Note that all IAEDANS in the light-chain fractions is bound to the SH-LC, since the R-LC of scallop myosin has no thiol groups.

(g) *Estimation of thiol content*

Intact and desensitized scallop myosins were incubated for 1 h with 10 mM-dithiothreitol in Mg^{2+}-wash at pH 7·5 on ice under N_2. The reduced preparations were then centrifuged briefly in the Sorvall RC-2B centrifuge, the supernatant was discarded and the myosin washed 4 times with Mg^{2+}-wash, before resuspending in Mg^{2+}-wash and adding NaCl to a final concentration of 0·5 M. The solubilized myosins were then centrifuged at 100,000 *g* for 0·5 h and the supernatants immediately assayed for thiol by the method of Ellman (1959), either in 6·0 M-guanidine·HCl, 1·1 mM-EDTA, 54 mM-Tris·HCl (pH 8·0) or in 0·6 M-NaCl, 2 mM-$MgCl_2$, 54 mM-Tris·HCl (pH 8·0), in a total volume of 1 ml using 0·5 mM-*N*-acetyl-L-cysteine as a standard.

(h) *Other methods*

Amino acid analysis was carried out on a Durrum D-500 analyzer. Lyophilized protein samples were hydrolyzed with constant-boiling 6 M-HCl plus 10 mM-phenol *in vacuo* at 110°C for 24 h. Butanedithiol was added before hydrolysis when the carboxymethylcysteine constant was to be determined. Protein was determined either by the method of Lowry *et al.* (1951) or by the Biuret method (Gornall *et al.*, 1949) using an A_{550} of 0·07 cm^{-1} $(mg/ml)^{-1}$ using bovine serum albumin as the standard. Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out in tube gels according to Weber & Osborn (1969) and in slab gels according to Laemmli (1970). Gel electrophoresis in the presence of urea was carried out in 10% polyacrylamide/8 M-urea gels at pH 8·6 as described by Kendrick-Jones *et al.* (1976). Gels were stained with 0·25% (w/v) Coomassie blue or 0·25% (w/v) Acid fast green in 50% (v/v) methanol/10% (v/v) acetic acid. ATPase assays were carried out on a pH-stat as described previously (Szent-Györgyi *et al.*, 1973; Chantler & Szent-Györgyi, 1980).

3. Results

A total of 46 thiol groups were found in intact and desensitized scallop myosins by 5,5′-dithiobis-2-nitrobenzoic acid assay in the presence of guanidine·HCl (Table 1). In the absence of guanidine·HCl, slightly lower values were obtained; the results, however, were variable. Since there are no cysteine residues in scallop R-LC, and there are three cysteine residues in the SH-LC (Kendrick-Jones *et al.*, 1976), each scallop heavy-chain contains 20 thiol groups.

TABLE 1

Thiol content of intact and desensitized scallop myosin

	Mol thiol/mol myosin	
	Non-denaturing	Denaturing
Preparation 1		
Intact myosin	46·5 (3)	44.6 (2)
Desensitized myosin	46·5 (2)	45·3 (2)
Preparation 2		
Intact myosin	41·3 (3)	44·7 (2)
Desensitized myosin	44·7 (3)	46·4 (2)

Thiol contents of intact and desensitized scallop myosin were determined in 0·6 M-NaCl, 2 mM-$MgCl_2$, 56 mM-Tris·HCl (pH 8·0), and 6·0 M-guanidine·HCl, 1·1 mM-EDTA, 54 mM-Tris·HCl (pH 8·0). The values are averages of the numbers in parentheses.

TABLE 2

Protection of SH-LC thiol groups by R-LC

Reagent	Ionic strength	pH	Moles reagent/ total SH	Method	Mol reagent detected				
					Heavy-chain (mol^{-1})		SH-LC (mol^{-1})		
					Intact	Des.	Intact	Des.	Res.
IAA	0·06	7·2	13·2	Amino acid analysis	6·6	6·48	0	2·86	—
IAA	0·06	7·5	5·0	Amino acid analysis	—	—	0	1·78	—
IAA	0·06	7·24	12·0	Amino acid analysis	—	—	0	1·66	—
IAA	0·06	7·2	13·2	Amino acid analysis	—	—	0	1·45	—
IAA	0·06	7·5	2·5	^{14}C incorporation	1·29	1·56	0·04	1·31	–
IAA	0·53	7·5	5·0	^{14}C incorporation	—	—	0·19	3·6†	0·26
IAA	0·06	6·8	5·0	^{14}C incorporation	0·62	1·0	0·01	0·56	—
IAEDANS	0·06	7·24	0·17	Fluorescence	0·83	1·15	0·02	1·2	–
DANS	0·06	6·7	1·9	Gel scan†	—	—	1·0‡	3·13‡	1·06‡
				Relative fluorescence	—	—	1·0‡	3·1‡	1·22‡

All the samples were incubated with the reagents for 18 h at 4°C in the dark as described in Experimental Procedures. Light and heavy-chains were separated by treatment with guanidine. IAA, iodoacetic acid; DANS, dansyl chloride; Des, desensitized; Res, resensitized.

†SH-LC separated on polyacrylamide/urea gels.

‡Values normalized to 1 for intact myosin.

The presence of R-LC in scallop myosin protected the thiol groups of the SH-LC from reaction with iodoacetic acid (Table 2, Fig. 1). The cysteine residues of the SH-LCs were carboxymethylated only if the R-LCs had been removed from myosin. The SH-LCs were not alkylated in intact or resensitized myosins. The extent of carboxymethylation in desensitized myosins depended on pH and the concentration of iodoacetic acid. Below pH 7·0, alkylation was limited (Table 2). At pH values from 7·2 to 7·5, between one and two of the thiol groups of the SH-LC were carboxymethylated after an overnight incubation with an excess of iodoacetic acid. Occasionally, all three SH-LC thiol groups reacted (Table 2). Alkylation appeared to be more effective at high ionic strength; however, a portion of the myosin denatured in these conditions, therefore treatment with iodoacetic acid was usually performed under conditions of low ionic strength. Carboxymethylation of desensitized myosin led to a splitting of the SH-LC band on polyacrylamide/urea gels, a new band appearing closer to the anode than native SH-LC (Fig. 2). R-LCs were not labeled by [^{14}C]iodoacetic acid (Fig. 1).

Nearly one-third of the cysteine residues of the heavy-chains can react at the higher concentrations of iodoacetic acid. At lower iodoacetic acid ratios, fewer than two of the heavy-chain thiols were carboxymethylated. The reactivity of the thiol groups of the heavy-chain was not significantly dependent on the presence of the R-LC, and the heavy-chains of the intact and desensitized myosin were labeled to a similar extent.

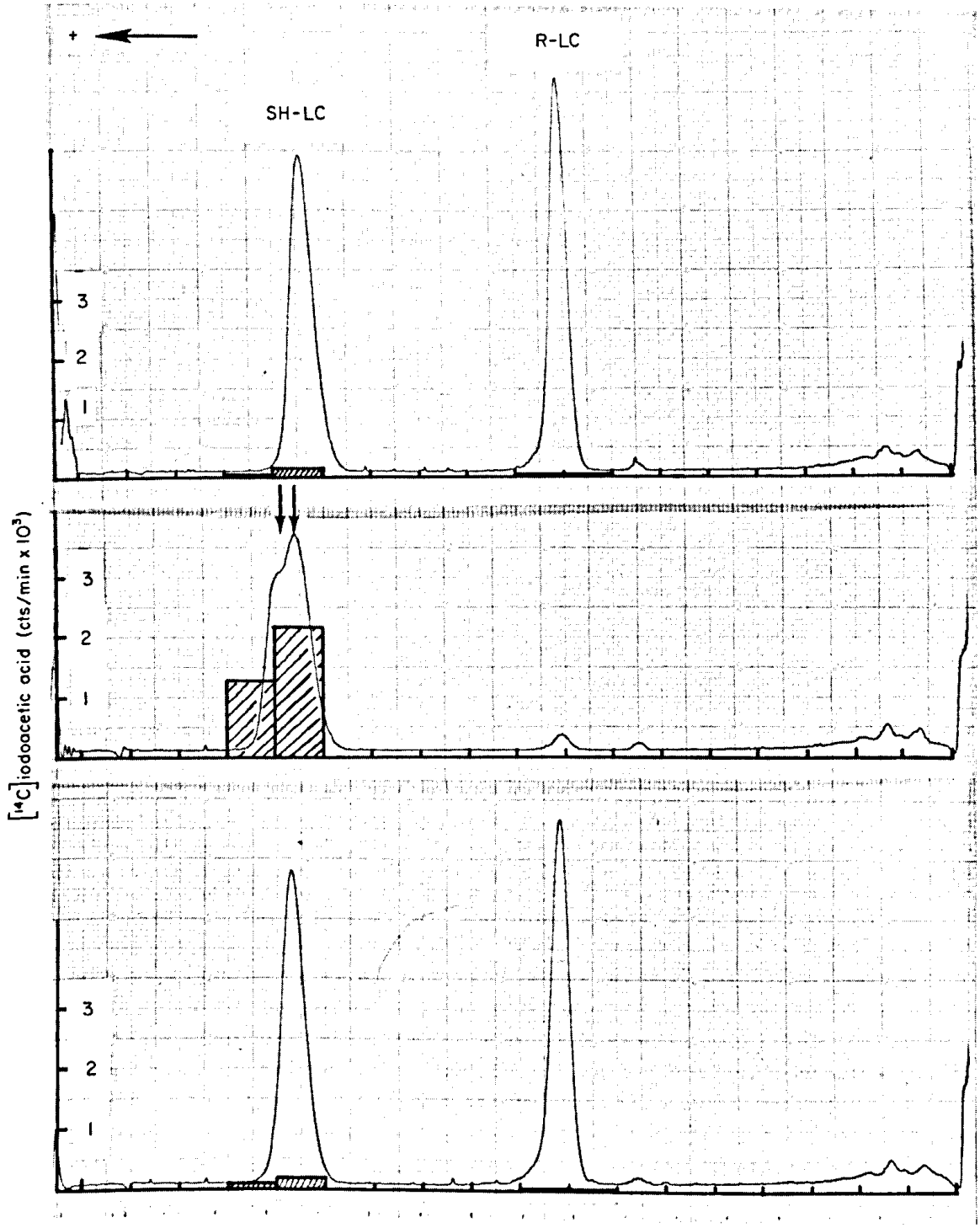

FIG. 1. Selective incorporation of [^{14}C]iodoacetic acid into the SH-LC of desensitized myosin. Densitometry tracings of intact (top), desensitized (middle) and resensitized (bottom) scallop myosin after polyacrylamide/urea gel electrophoresis and staining with Acid fast green. Bars represent [^{14}C]iodoacetic acid content (cts/min $\times 10^3$) of the corresponding 4-mm slices of parallel gels.

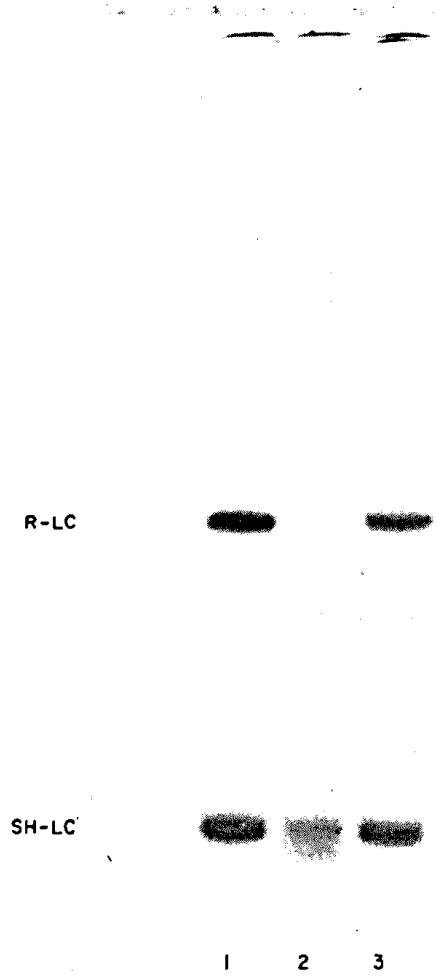

FIG. 2. Effect of the presence of R-LC on the reaction of the SH-LC of scallop myosin with iodoacetic acid. Gel electrophoresis in 10% polyacrylamide/8 M-urea gels. Intact, fully desensitized and resensitized myosins were reacted with a 5-fold molar ratio of iodoacetic acid to the total protein thiol content for 18 h at 4°C in the dark in 0·4 M-NaCl, 2 mM-$MgCl_2$, 25 mM-sodium phosphate (pH 7·5). Myosin was freed of reagent as described in Experimental Procedures. Note that myosin heavy-chain does not enter the 10% gels. (1) Intact myosin; (2) 23°C-desensitized myosin; (3) resensitized myosin. The SH-LC band splits into 2 components only in the case of the desensitized myosin.

Carboxymethylation of the cysteine residues of the heavy-chains and of the SH-LCs by iodoacetic acid did not reduce ATPase activities. The high Ca^{2+} and the EDTA/K-activated ATPase activities of intact and desensitized myosins remained essentially unaltered by reaction with iodoacetic acid. The actin-activated Mg-ATPase activity of intact and resensitized myosin was not changed by treatment with iodoacetic acid, but the activity of desensitized myosin was elevated by about 50%. R-LC was not released by treatment with iodoacetic acid, and the ratio of R-LC to SH-LC of intact myosin remained the same (Table 3). Intact myosin after reaction with iodoacetic acid could still be desensitized with EDTA, and R-LC rebound in the presence of magnesium; however, rebinding of the R-LC to iodoacetic acid-treated desensitized myosin was only partial, and variable.

The presence of R-LC on scallop myosin during treatment with IAEDANS led to

TABLE 3

Stability of scallop myosin to treatment with iodoacetic acid and IAEDANS

	ATPase (μmol min^{-1} mg^{-1})						
			Actin-activated			R-LC	Mol reagent bound/
	EDTA	Ca^{2+}	$-Ca^{2+}$	$+Ca^{2+}$	Ratio	SH-LC	mol myosin
A.							
Intact myosin	0·69	1·84	0·025	0·42	0·06	0·85	—
Intact myosin, IAA-treated	0·7	2·4	0·06	0·67	0·09	0·92	2·66
Desensitized myosin	0·65	1·69	0·24	0·18	1·33	0·03	—
Desensitized myosin, IAA-treated	0·57	1·6	0·31	0·31	1·0	0·02	5·74
B.							
Intact myosin	—	—	0·03	0·53	0·06	0·98	—
Intact myosin, IAA-treated	—	—	0·09	0·58	0·16	0·88	8·4
Desensitized myosin	—	—	0·25	0·18	1·39	0·09	—
Desensitized myosin, IAA-treated	—	—	0·39	0·31	1·26	0·04	14·0
Resensitized myosin	—	—	0·02	0·44	0·05	0·96	—
Resensitized myosin, IAA-treated	—	—	0·06	0·4	0·15	0·87	8·55
C.							
Intact myosin, IAEDANS-treated	—	—	0·08	0·1	0·8	0·85	1·68
Desensitized myosin, IAEDANS-treated	—	—	0·07	0·087	0·8	—	4·70

A. Myosins treated with a 2·5-fold ratio of iodoacetic acid (IAA) to total thiol at pH 7·5, 4°C, 0·06 ionic strength for 18 h.

B. Myosins treated with 5-fold ratio of iodoacetic acid (IAA) to total thiol at pH 7·5, 4°C, 0·53 ionic strength for 18 h.

C. Myosins treated with 0·174-fold ratio of IAEDANS to total thiol at pH 7·24, 4°C, 0·06 ionic strength for 18 h.

a very significant degree of protection of the thiol groups of the SH-LC (Table 2). However, scallop myosin is not as stable to treatment with IAEDANS as it is to iodoacetic acid. Reaction of low molar ratios of IAEDANS (0·17 molar ratio to total thiol) with intact and desensitized myosins impaired both the ATPase activity and calcium sensitivity of intact myosin, and the ATPase activity of desensitized myosin. Treatment with IAEDANS did not cause any loss of R-LC from intact myosin at the levels used (Table 3).

At pH 6·7, dansyl chloride reacts mainly with thiol groups, the protonated ϵ-amino groups of lysine not reacting (Gray, 1972). To show that dansyl chloride reacts preferentially with thiol groups of the isolated SH-LC at pH 6·7, both reduced and alkylated scallop SH-LCs were treated with dansyl chloride at pH 6·7, when it was found that the dansyl content of the reduced SH-LC was 14-fold higher than that of the alkylated material. When intact, desensitized and resensitized myosins were reacted with dansyl chloride at pH 6·7, both the methods used to quantitate the relative extent of dansylation of the SH-LCs of the myosins indicated that the labeling of the SH-LC of desensitized myosin was approximately threefold greater than that seen in the SH-LCs of intact and resensitized myosin

(Table 2). Dansylation of intact and resensitized myosin at pH 6·7 did not cause loss of R-LC, but the ATPase activities of all three types of myosin were lost completely.

4. Discussion

The R-LC of scallop myosin protects the SH-LC from reaction with the two relatively specific thiol reagents, iodoacetic acid and IAEDANS: and from dansyl chloride at a pH value where it reacts mainly with thiol groups. In the case of the iodoacetic acid at least, this protection is very substantial, extending to all three thiol groups of the SH-LC. Iodoacetic acid was selected because Lundsgaard (1930) has shown in his classical studies that it does not impair tension development of frog muscle. Scallop myosin is also remarkably stable to treatment with iodoacetic acid in the cold, and up to one-third of the heavy-chain thiols can be carboxymethylated without deleterious effect on the activity and sensitivity (6 to 7 out of the 20 thiols of the heavy-chain). Thus, these heavy-chain thiols are not important in a catalytic sense, and they are also very unlikely to be involved in the binding of the R-LC, as R-LC is not lost from intact or resensitized myosin on treatment with iodoacetic acid. Any impairment in R-LC uptake seen in iodoacetic acid-treated desensitized myosin is thus likely to be due to carboxymethylation of SH-LC thiols. R-LC selectively protects the thiol groups on the SH-LC. The lack of large conformational effects on the heavy-chain by the removal of R-LC agrees with previous spectroscopic studies (Chantler & Szent-Györgyi, 1978).

In contrast to the stability of scallop myosin to iodoacetic acid, reaction of low molar ratios of IAEDANS with intact and desensitized scallop myosin impairs both the activity and Ca^{2+} sensitivity. Since the thiol groups of the SH-LC of intact myosin are well-protected from IAEDANS at the levels used, the lower activity and sensitivity is likely to be due to modification of heavy-chain thiols. The overall level of IAEDANS labeling at which extensive inactivation and loss of sensitivity occur is about one mole per mole of heavy-chain, which may imply that there is one thiol per heavy-chain essential for enzyme activity that is highly reactive towards IAEDANS. Previous studies showed a similar inactivation of the ATPase of scallop myosin at low concentrations of *N*-ethylmaleimide (Chantler, P. D., unpublished results). The R-LC to SH-LC ratio of intact myosin did not change on reaction with IAEDANS, so that the loss of sensitivity is not simply due to loss of the R-LC. Thus, a heavy-chain thiol that does not react with iodoacetic acid may react with IAEDANS. Iodoacetic acid reactions are sensitive to electrostatic effects (e.g. see Gurd, 1972), and a negatively charged group may be present near this thiol that repels the approach of the carboxylate group of iodoacetic acid. The reactivity of the uncharged iodoacetamide moiety of IAEDANS is consistent with such an explanation. It is also possible that the large aromatic grouping of the IAEDANS inserts into a hydrophobic region inacessible to iodoacetic acid or that substitution on the same thiol by the bulky IAEDANS group instead of the carboxymethyl group interferes with the enzymatic mechanism and its regulation.

Dithiobis-nitrobenzoic acid assays in non-denaturing medium failed to establish clearly that some of the thiol groups are protected in intact myosin. The closeness

of the thiol contents in denaturing and non-denaturing media probably indicates that the cumulative substitution of thionitrobenzoate groups leads to an unfolding of the protein structure. In fact, dithiobis-nitrobenzoic acid in conjunction with EDTA removes R-LC selectively and fully from scallop myosin and, at the same time, irreversibly inactivates its ATPase (Kendrick-Jones *et al.*, 1976).

We explain the protection of the SH-LC by the R-LC in terms of a direct interaction between R-LCs and SH-LCs. It is possible that the reactivity of iodoacetic acid towards the SH-LC is attenuated by an electrostatic effect of the R-LC nearby. One notes, however, that the SH-LC thiols are also protected from IAEDANS and dansylation by the presence of R-LC.

At least two, possibly all three, of the thiols of the SH-LC can be protected by the R-LC. The interaction between R-LC and SH-LC may thus involve a substantial stretch of these peptides, depending on the separation of the cysteine residues in the SH-LC sequence. One notes that the cross-linking studies reported in the accompanying paper (Wallimann *et al.*, 1982) suggest that the overlap between the two light-chain types is substantial. The lower protection by R-LCs when dansyl chloride is used to that seen with iodoacetic acid and IAEDANS may be due to the lesser selectivity of this reagent towards thiol groups even at pH 6·7.

Re-uptake of R-LCs by the thiol-modified myosin and its resensitization was only partial, and the results were variable. It is possible that alkylation of thiols of the SH-LC interferes with R-LC binding. However, modification of other groups, e.g. amino groups on the SH-LC, may also impair rebinding of R-LCs. Differences between the reactivity of the three thiol groups of the SH-LC has not been studied in detail. The reactivity of at least one is fairly great in desensitized myosin, since at the lower concentrations of IAEDANS or iodoacetic acid about one mole of SH-LC thiol reacts when only about one out of 20 thiols of the heavy-chain has reacted. The role of the thiols of the SH-LC in the interaction between R-LC and SH-LC evidently requires further study.

The importance of this particular problem lies in the feasibility of distance measurements between specific residues on the two light-chains by energy transfer, in particular, in studying the effect of ATP and calcium. Protection of the SH-LC by the R-LC from reaction with chemical reagents is consistent with its protection by the R-LC from digestion by papain (Stafford *et al.*, 1979) and with the electron microscopic investigations indicating a similar location of the light-chains on myosin (Flicker *et al.*, 1981). The results agree with immune studies that suggested a regulatory role for the SH-LC and indicated proximity between SH-LCs and R-LCs (Wallimann & Szent-Györgyi, 1981). These experiments also complement the cross-linking studies between R-LC and SH-LC (Wallimann *et al.*, 1982).

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