

# Mapping Myosin Light Chains by Immunoelectron Microscopy. Use of Anti-Fluorescyl Antibodies as Structural Probes

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**Abstract.** The two classes of light chains in vertebrate fast muscle myosin have been selectively labeled with the thiol specific reagent 5-(iodoacetamido) fluorescein to determine their location in the myosin head. The alkali light chains (A1 and A2) were labeled at a single cysteine residue near the COOH terminus, whereas the regulatory light chain (LC2) was reacted at either cysteine 125 or 154. The two cysteines of LC2 appear to be near each other in the tertiary structure as evidenced by the ease of formation of an intramolecular disulfide bond. Besides having favorable spectral properties, fluorescein is a potent haptenic immunogen for raising high affinity antibodies. When anti-fluorescyl antibodies were added to the fluorescein-labeled light chains, the fluorescence was quenched by >90%, thereby providing a simple method for determining an association constant. The interaction with antibody was the same for light chains exchanged into myosin

as for free light chains. Complexes of antibody bound to light chain could be visualized in the electron microscope by rotary shadowing with platinum. By this approach we have shown that the COOH-terminal regions of the two classes of light chains are widely separated in myosin: the cysteine residues of LC2 lie close to the head/rod junction, whereas the single cysteine of A1 or A2 is located  $\sim 90$  Å distal to the junction. These sites correspond to the positions of the NH<sub>2</sub> termini of the light chains mapped in earlier studies (Winkelmann, D. A., and S. Lowey. 1986. *J. Mol. Biol.* 188:595-612; Tokunaga, M., M. Suzuki, K. Saeki, and T. Wakabayashi. 1987b. *J. Mol. Biol.* 194:245-255). We conclude that the two classes of light chains do not lie in a simple colinear arrangement, but instead have a more complex organization in distinct regions of the myosin head.

**L**IGHT chains first attained prominence in the 1970s when it was suggested that they regulate the ATPase activity of myosin (Dreizen and Gershman, 1970). This hypothesis was reinforced by the advent of SDS-PAGE, which showed that myosin from vertebrate fast skeletal muscles had a different light chain pattern than that from slow muscles (Lowey and Risby, 1971). Subsequent work involving the formation of hybrid myosins from fast light chains and cardiac heavy chains challenged this hypothesis by showing that the heavy chain was the principal determinant of the level of actin-activated ATPase activity in vertebrate striated muscles (Wagner, 1981). The most compelling evidence against a regulatory role for the light chains in the active site of vertebrate skeletal muscle myosin has come from the preparation of light chain free-heavy chains which, nevertheless, are able to hydrolyze ATP at a reasonable rate (Wagner and Giniger, 1981; Sivaramakrishnan and Burke, 1982).

Contrary to their obscure role in vertebrate, striated muscles, the light chains' contribution to calcium regulation in invertebrate molluscan (Szent-Györgyi and Chantler, 1986) and in vertebrate smooth muscles (Adelstein and Eisenberg, 1980) is firmly established. In scallop myosin, the "regula-

tory" or "EDTA-light chain" is essential for inhibiting the actin-activated ATPase in the absence of calcium, and activating ATPase activity upon addition of calcium (Szent-Györgyi et al., 1973). In gizzard myosin, phosphorylation of a 20,000-D light chain by a calcium-calmodulin-dependent kinase is necessary to elevate the actin-activated ATPase activity (Sellers et al., 1981).

Despite the absence of a direct connection between light chains and enzymatic activity in vertebrate, skeletal muscles, it is possible that light chains may play an important structural role in the mechanism of muscle contraction. Current cross-bridge models invoke a substantial conformational change within the myosin molecule when it attaches to actin during the contractile cycle. To account for a power stroke of 40-100 Å, the distance over which an attached cross-bridge develops tension, the existence of an elastic element within the myosin molecule has to be invoked. Two theories are currently in vogue regarding the identity of this extensible element: one considers the change to reside primarily in the subfragment 1 (S1) region of myosin (Huxley and Kress, 1985) and the other proposes a helix-coil transition in the hinge portion of the myosin rod (Harrington, 1979). The recent development of an assay for testing myosin-based movement on actin cables from the alga *Nitella* has made it possible to show that heavy meromyosin which is missing a hinge

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region is still capable of movement (Hynes et al., 1987). This finding supports the hypothesis that the head (S1) is the principal domain for generating tension upon interaction with actin. If that is the case, where in the S1 head is the site(s) for the elusive conformational change?

A variety of spectroscopic approaches have been used to search for conformational changes in the S1 head, but independent of whether the label was introduced at the nucleotide site or at the reactive SH1 thiol (Cooke et al., 1984), none of these experiments provided convincing evidence for head rotation. It has been suggested that the region of the myosin head containing these labels may be attached to actin in a fixed orientation, and the portion of S1 adjacent to the rod may undergo the structural changes associated with the power stroke (Huxley et al., 1983).

Several studies have mapped the light chains to the "neck region" of the myosin head, that portion of the pear-shaped head which tapers to the rod (Flicker et al., 1983; Winkelmann et al., 1983; Waller and Lowey, 1985). This so-called "domain," consisting of the two classes of light chains and the COOH terminus of the S1 heavy chain, has been proposed for a possible regulatory role in mediating interactions between heads in a single molecule, or between heads of neighboring molecules in a filament (Vibert et al., 1986). A plausible extension of this model is to assume that light chain/heavy chain interactions may contribute to the long sought for conformational change in the myosin molecule.

Any mechanism involving the light chains requires that they first be localized along their entire length within the myosin head. Although monoclonal and polyclonal antibodies of well-defined specificities have been used to map the NH<sub>2</sub>-terminal regions of both classes of light chains in myosin (reviewed in Winkelmann and Lowey, 1986), the position of their COOH-terminal regions has received less attention. The single cysteine residue in the COOH terminus of the alkali light chain was localized to the distal region of the S1 head by fluorescence energy transfer (Marsh and Lowey, 1980) and by electron microscopy using an avidin-biotin system (Yamamoto et al., 1985). However, no reports exist on the location of the COOH terminus of the regulatory or LC2 type light chain.

In this study, we describe methods to independently label the two COOH-terminal cysteine residues in the LC2 light chain. A fluorescent reagent (5-[iodoacetamido] fluorescein) was chosen because of the advantages of a spectroscopic probe; not only do such labels permit energy transfer studies, but antibodies against fluorescein haptens have the property of quenching >90% of the emitted signal (Lopatin and Voss, 1971). By titrating the modified light chain with anti-fluorescyl antibodies, it was possible to measure the affinity of antibody for free as well as for bound light chains. Attempts to visualize the antibody/light chain complex by electron microscopy are described. Labeled light chains were also exchanged into myosin, and anti-fluorescyl antibodies were used to localize the fluorescein epitope by immunoelectron microscopy. By this approach we have shown that the COOH-terminal region of LC2 lies close to the head/rod junction, the same location that earlier studies had assigned to the NH<sub>2</sub> terminus of the light chain (Winkelmann et al., 1983; Tokunaga et al., 1987b).

## Materials and Methods

### Protein Preparation

Myosin was prepared from adult White Rock chicken pectoralis muscle according to the method of Margossian and Lowey (1982). Total light chains were dissociated from myosin in 6 M guanidine hydrochloride followed by ethanol precipitation of the heavy chains (Perrie and Perry, 1970). Light chains were separated by ion exchange chromatography (Holt and Lowey, 1975), or by ethanol fractionation (Perrie et al., 1973). The latter procedure was modified by increasing the ethanol concentration to 26% and allowing precipitation to occur during overnight storage at 0°C. The precipitate consisted mainly of LC2, with only slight contamination by alkali light chains. Protein concentration was determined by the method of Bradford (1976) using myosin as the standard curve; or by absorbance using the following extinction coefficients (1%, 280 nm): 5.0, 6.0, 0.86, 1.06, 11, and 14 for myosin, LC2, A1, A2, actin, and IgG, respectively. Molecular weights of 500,000, 19,000, 20,000, 16,000, and 150,000 were used for myosin, LC2, A1, A2, and IgG, respectively.

Polyclonal antibody to the amino-terminal regions of A1 (anti-Δ1) and A2 (anti-Δ2) was prepared according to the procedure of Silberstein and Lowey (1981), and kindly provided by G. Waller. The monoclonal antibody against LC2 (7C10.2) is described in Winkelmann et al. (1983). Polyclonal antibody against fluorescein was prepared according to the method of Lopatin and Voss (1971), using fluorescein I-keyhole limpet hemocyanin as the immunogen.

### Modification of Light Chains

The fluorescent probes N[[[iodoacetyl]amino]ethyl]-5-naphthylamine-1-sulfonic acid (1,5-IAEDANS)<sup>1</sup> and 5-(iodoacetamido)fluorescein (IAF) were purchased from Molecular Probes (Eugene, OR). The thiol reagent 5,5'-dithio bis (2-nitrobenzoic acid)(DTNB) was obtained from Sigma Chemical Co., and 2-nitro-5-thio-cyanobenzoic acid (NTCB) was prepared as described by Degani and Patchornik (1974).

The alkali light chains were labeled at their single cysteine residue by 5-IAF according to the procedure of Marsh and Lowey (1980). Lyophilized LC2 (freeze-dried from 10 mM NaPi, 2 mg sucrose/mg protein) was incubated for 2 h at room temperature in 6 M Gu-HCl, 10 mM DTT, 0.1 M Tris-HCl, pH 8.0, to ensure complete reduction of its sulfhydryl residues, and then dialyzed against 0.1 M NaCl, 5 mM Pipes, pH 6.2, for storage. Before labeling with fluorophore, LC2 at ~1 mg/ml was dialyzed into 0.1 M NaCl, 2 mM EDTA, 20 mM Imidazole, pH 7.0. Reaction was initiated with about a five-fold molar excess of 5-IAF or 1,5-IAEDANS in the dark at 0°C, and terminated by the addition of DTT to a final concentration of 20 mM. Purification of labeled light chains was by ion-exchange chromatography: ~15 mg of LC2 was applied to a DEAE-Sephacel column (1.5 × 10 cm) equilibrated in 0.2 mM DTT, 0.2 mM EDTA, 0.15 M NaPi, pH 7.0. Elution with a linear gradient of NaPi up to 0.4 M (total volume = 200 ml) separated the modified LC2 into three peaks identified as unlabeled, singly labeled, and doubly labeled LC2 light chain. Pooled fractions were dialyzed vs. 1 mM DTT, 30 mM ammonium bicarbonate, and freeze-dried. The degree of labeling was determined by using an extinction of  $5.47 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  at pH 8.0 and 495 nm for fluorescein conjugated to light chain (Marsh and Lowey, 1980), and an extinction of  $6.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at 337 nm for the dansyl group (Hudson and Weber, 1973).

Cyanylation and cleavage of any unmodified cysteine residues were performed as described by Degani and Patchornik (1974). Fully reduced LC2 at 0.5 mg/ml was incubated for 1 h in 0.5 mM NTCB, 5 mM NaCN, 0.1 M NaCl, 2 mM CaCl<sub>2</sub>, and 10 mM morpholine propane sulfonic acid (MOPS), pH 7.0, at 0°C. Cleavage was carried out at room temperature by raising the pH to 9.7 with 0.2 M Na<sub>2</sub>CO<sub>3</sub>. The reaction was terminated by the addition of 1 N HCl to pH 7.

The reaction of LC2 with DTNB was done by adding an equimolar amount of DTNB to 0.2 mg/ml light chain dissolved in 0.1 M NaCl, 2 mM EDTA, 5 mM Pipes, pH 6.5, at 15°C. The release of thionitrobenzoate (TNB) was monitored at 412 nm using a Perkin-Elmer 552A UV/VIS spec-

1. *Abbreviations used in this paper:* DTNB, 5,5'-dithio bis (2-nitrobenzoic acid); IAF, 5-(iodoacetamido) fluorescein; 1,5-IAEDANS, N[[[iodoacetyl]amino]ethyl]-5-naphthylamine-1-sulfonic acid; NTCB, 2-nitro-5-thio-cyanobenzoic acid; TNB, thionitrobenzoate.

trophotometer. The concentration of TNB was determined by using a molar extinction coefficient of  $1.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (Ellman, 1958).

The 5-(2-hydroxyethyl)thioacetamido-fluorescein conjugate (Fl.SCH<sub>2</sub>-CH<sub>2</sub>OH) was prepared by reacting 2 mM 5-IAF in 0.1 M NaCl, 40 mM Tris-HCl, pH 8.0, with 0.14 M 2-mercaptoethanol at room temperature in the dark for 30 min. The compound was purified by passage through a Sephadex G10 column equilibrated with the above buffer.

Light chain labeled with fluorescein at Cys 154 was prepared by reacting DNS-LC2 (light chain singly labeled with the dansyl group, see above) with a 10-fold molar excess of 5-IAF in 80 mM NaCl, 2 mM CaCl<sub>2</sub>, 40 mM Tris-HCl, pH 8, at 0°C in the dark. After 2 h, the reaction was terminated by adding DTT to 20 mM. Excess reagent and iodide were removed by passage through a Sephadex G25 (fine) column.

### Exchange of Light Chains into Myosin

Alkali light chains were incorporated into myosin by a minor modification of the method of Wagner and Weeds (1977); myosin precipitated at low ionic strength ( $\sim 5 \text{ mg/ml}$ ) was added to a solution containing 10- to 20-fold molar excess of labeled A1 or A2 over myosin, 4.7 M NH<sub>4</sub>Cl, 12 mM EDTA, 2 mM DTT, pH 8, adjusted by Tris. Solid NH<sub>4</sub>Cl was added to saturation at 0°C, and the solution was gently stirred for 90 min at 0°C. The reaction was terminated by the addition of 20 mM MgCl<sub>2</sub> and 4 vol of 3 mM MgCl<sub>2</sub>, 2 mM DTT, 0.5 M NaCl, 40 mM Tris-HCl, pH 8.0. Myosin was precipitated by ammonium sulfate at 50% saturation, and redissolved in a small volume of the above buffer. Although little free light chain remained at this stage, the myosin was further purified by gel filtration through a Sepharose 6B column to remove any last traces. In general, 40–50% exchange occurred as determined by gel electrophoresis and absorbance of the labeled species.

Regulatory (LC2) light chain can be exchanged into myosin at elevated temperatures in the presence of EDTA (Wikman-Coffelt et al., 1979). Myosin (2 mg/ml) and 1.5 mg/ml labeled LC2 (20-fold molar excess over myosin) were incubated at varying temperatures in 0.5 M NaCl, 2 mM DTT, 2 mM EDTA, 5 mM ATP, 40 mM Tris-HCl, pH 8.0 (at 25°C) for 15 min with gentle stirring under nitrogen in the dark. The reaction was terminated by the addition of MgCl<sub>2</sub> to a final concentration of 10 mM and cooled on ice. The cold solution was diluted 20-fold with 1 mM MgCl<sub>2</sub>, 5 mM Pipes, pH 6.2, and the precipitate was collected by centrifugation. The myosin was resuspended in salt, and the precipitation step repeated to remove any traces of free light chain.

### ATPase Measurements

The K<sup>+</sup>/EDTA and Ca<sup>2+</sup>-ATPase activity of myosin were measured in 0.5 M KCl, 2 mM ATP, 40 mM Tris-HCl, pH 8.0, with 2 mM EDTA added to the former, and 5 mM CaCl<sub>2</sub> added to the latter ATPase at 25°C. The actin-activated Mg-ATPase activity was determined in 50 mM NaCl, 1 mM DTT, 4 mM MgCl<sub>2</sub>, 2 mM ATP, 20 mM Tris-HCl, pH 8.0, at concentrations of 0.1  $\mu\text{M}$  myosin and 6  $\mu\text{M}$  actin, 25°C. Phosphate release was measured by the colorimetric method of White (1982).

### Gel Electrophoresis

Myosin light chains were analyzed by SDS gel electrophoresis by the method of Laemmli (1970). Labeled light chains were separated on urea/acrylamide gels essentially by the method of Perrie and Perry (1970). The running gel contained 8 M urea, 8% acrylamide, 0.27% bisacrylamide; the acrylamide concentration was reduced to 3.5% in the stacking gel, but the acrylamide/bisacrylamide ratio was kept at 30:1. The running and gel buffers were 20 mM Tris/glycine, pH 8.8. The samples were applied in 8 M urea, 20 mM Tris/glycine, pH 8.8, 0.1% 2-mercaptoethanol.

Protein band intensities were determined by densitometry using an EC Densitometer connected to a Hewlett-Packard Recording Integrator.

### Fluorescence Spectroscopy

Fluorescence measurements were performed with a Perkin-Elmer MPF 44 instrument equipped with a thermostated cell housing. Emission spectra were obtained by excitation at 495 nm. Conditions used were 2–400 nM fluorescein-labeled light chains, 0.1 mg/ml BSA, 0.1 or 0.5 N NaCl, and 40 mM Tris-HCl, pH 8.0. After each addition of anti-fluorescyl IgG, the change in fluorescence intensity was monitored at 520 nm. Degree of binding and concentration of free antibody sites were calculated from the titra-

tion data by assuming a linear relationship between degree of quenching and amount of fluorescein bound to IgG.

### Immunoelectron Microscopy

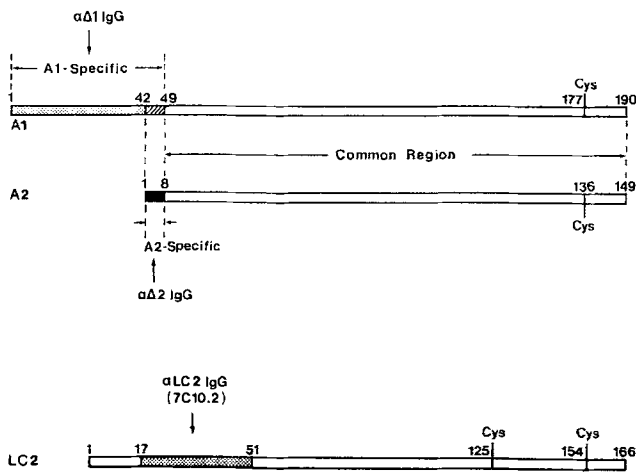
Electron microscopy was performed on a Philips EM301 electron microscope operated at 60 kV. Light chain- (or myosin) antibody complexes were rotary shadowed with platinum as described by Tyler and Branton (1980). Labeled light chains (or myosin) and antibodies were mixed at  $\sim 1:1$  molar ratio in 0.1 M (or 0.5 M) NaCl, 40 mM Tris-HCl, pH 8.0, at a concentration of 3–20  $\mu\text{M}$  light chain (or 1  $\mu\text{M}$  myosin), and left on ice for several hours. Samples were diluted  $\sim 100$ -fold in 70% glycerol, 0.3 M ammonium acetate, pH 7.2, immediately before spraying onto mica, as described in Winkelmann et al. (1983).

### Results

Myosin from fast-twitch skeletal muscles contains two classes of low molecular weight subunits: two moles of alkali (A1/A2) light chains and two moles of regulatory (LC2) light chains (see review by Lowey, 1986). The major difference between the A1 and A2 light chains lies in the additional 41 NH<sub>2</sub>-terminal residues in A1, although there are also five amino acid replacements in the NH<sub>2</sub> terminus of A2 relative to the corresponding sequence in A1 (Fig. 1). The two proteins are identical in sequence over the remaining 141 COOH-terminal residues. Polyclonal antibodies (anti- $\Delta 1$  and anti- $\Delta 2$ ) specific for the NH<sub>2</sub>-terminal regions of the alkali light chains have been isolated (Silberstein and Lowey, 1981) and used to localize the light chains in myosin by electron microscopy (Waller and Lowey, 1985). A monoclonal antibody specific for an epitope in the NH<sub>2</sub>-terminal third of LC2 has been used to localize the regulatory light chain in myosin (Winkelmann et al., 1983) (Fig. 1). No antibodies specific for epitopes in the COOH-terminal regions of the light chains have been prepared to date. As an alternative approach to preparing anti-peptide antibodies against this portion of the sequence, we chose instead to chemically modify cysteine residues near the COOH terminus with a haptenic group against which antibodies could easily be raised.

### Reaction of Fluorescein-labeled Alkali Light Chains with Anti-Fluorescyl Antibodies

The single cysteine residue in A1 (Cys 177) and A2 (Cys 136) was labeled with 5-IAF as described by Marsh and Lowey (1980). The degree of labeling was estimated spectrophotometrically as  $\sim 0.8$  mol fluorescein per mol alkali light chain. The interaction between the labeled light chains and anti-fluorescyl antibodies could be monitored by observing the decrease in extrinsic fluorescence intensity upon the binding of antibody to fluorescein. After each addition of antibody, the emission spectrum was recorded until a maximum quenching of  $\sim 90\%$  was obtained. This procedure is essentially the same as that originally reported by Lopatin and Voss (1971) in their studies of the combining site of antibodies. By plotting the decrease in fluorescence intensity at 520 nm as a function of added antibody, a maximum value of  $\sim 0.9$  mol IgG combining site per mol bound fluorescein was obtained, in agreement with the expected stoichiometry. No fluorescence quenching was observed with nonimmune IgG (Fig. 2A). By using lower concentrations of light chains, the amount of free and bound IgG could be determined at each



**Figure 1.** Schematic diagram of A1, A2, and LC2 light chains. The regions in the sequence containing the epitopes for the polyclonal antibodies, anti- $\Delta 1$  and anti- $\Delta 2$ , are indicated, as well as the epitope in LC2 for the monoclonal antibody (7C10). The position of the single cysteine residue in the alkali light chains, and the two cysteines in the regulatory light chain (LC2) are shown. The numbering system is according to Matsuda (1983), and should be increased by one to account for the blocked  $\text{NH}_2$ -terminal amino acid which has been shown to be  $\alpha$ -*N*-trimethylalanine in A1 and LC2 (Henry et al., 1985).

concentration of added antibody, and the data fitted by a curve with an apparent dissociation constant of 2.5 nM (Fig. 2 B and Table I).

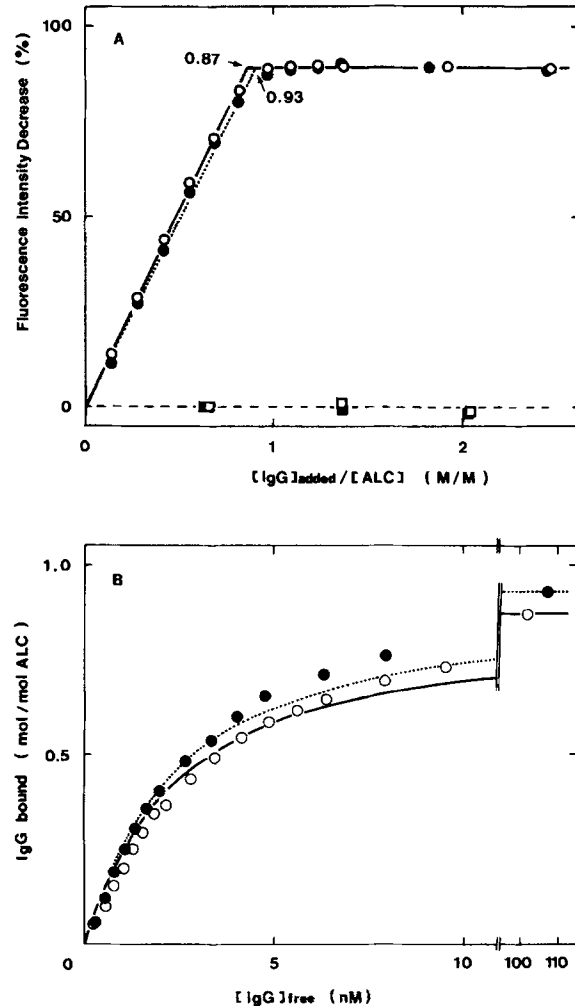
The affinity of anti-fluorescein antibodies for fluorescein was  $\sim 40$ -fold greater than the affinity of anti-light chain antibodies for epitopes in the  $\text{NH}_2$  terminus of the light chains (data not shown). This strong binding has been ascribed to the size, aromaticity and dianionic nature of the fluorophore (Kranz et al., 1982). The light chain to which the fluorescein was covalently bound had little effect on the interaction, as indicated by a similar  $K_d$  for protein-free fluorescein. Moreover, the simultaneous presence of anti-light chain antibodies did not interfere with the binding of anti-fluorescein IgG to the labeled light chain (data not shown). In fact, the fluorescein group interacted freely with the homologous antibodies even when the labeled light chain was associated with the heavy chain in myosin. Apparently, the fluorescein moiety is fully exposed and reactive under all conditions, a property ideally suited for its role in forming a strong antigen/antibody complex for direct visualization in the electron microscope.

### Labeling of Regulatory Light Chains with Fluorescein

The labeling of LC2 with 5-IAF was complicated by the presence of two cysteine residues at positions 125 and 154 in the sequence (Fig. 1). It was not known whether either of the cysteine residues could be labeled selectively. The extent of the reaction with IAF was followed electrophoretically by taking advantage of the two charged groups on fluorescein at  $\text{pH} > 8$ . Native, singly and doubly labeled light chains were readily separated by urea-PAGE (Fig. 3). As the native LC2 band disappeared, two faster migrating (fluorescent) bands appeared with time. By measuring the intensity of each band, the course of the reaction could be plotted as shown

in Fig. 3. The initial rate of appearance of the band of intermediate mobility was  $\sim 10$  times greater than that of the fastest migrating band, suggesting that the two cysteine residues have different reactivities.

To identify the chemical modification in each electrophoretic band, the reaction was terminated at 30 min and the mixture was fractionated into three distinct peaks by DEAE-Sephacel ion-exchange chromatography (see Materials and Methods). Spectroscopic analysis of the peaks confirmed that the bands of intermediate and fastest mobility were singly and doubly labeled LC2, respectively. The question of whether Cys 125 or Cys 154 was the fast reacting thiol was resolved by cyanylation and cleavage at the unlabeled cysteine residue as described by Degani and Patchornik (1974).



**Figure 2.** Titration of fluorescein-labeled alkali light chains by anti-fluorescein antibodies. (A)  $0.44 \mu\text{M}$  FI-A1 ( $\text{FI}/\text{A1} = 0.84$ ) ( $\circ$ ), and  $0.44 \mu\text{M}$  FI-A2 ( $\text{FI}/\text{A2} = 0.80$ ) ( $\bullet$ ) were titrated with specific antibody or nonimmune IgG ( $\square$ ), and the drop in fluorescence intensity monitored at 520 nm. (B) The concentration of light chains was reduced to 4.3 nM FI-A1 ( $\circ$ ), and 3.4 nM FI-A2 ( $\bullet$ ) and the concentration of anti-fluorescein antibody ranged from 0.2 to 100 nM. Solvent conditions were 0.1 mg/ml BSA, 0.5 M NaCl, 40 mM Tris-HCl, pH 8.0, 20°C. The solid and dotted lines represent theoretical binding curves with a dissociation constant of 2.5 nM, and maximum binding of 0.87 and 0.93 mol IgG site/mol FI, respectively.

**Table I. Binding of Anti-Fluorescyl IgG to Fluorescein-labeled Light Chains**

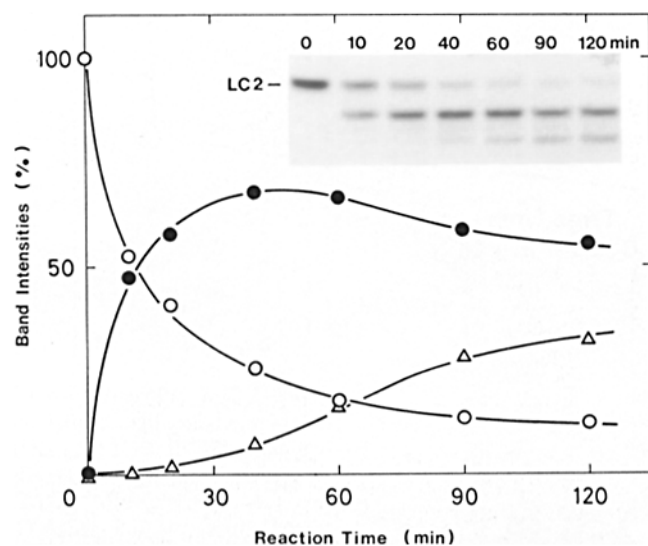
Fluorescein-labeled light chain	Dissociation constant*	Maximal binding‡
	<i>nM</i>	
Free LC		
A1 (cys 177)	3	0.87
A2 (cys 136)	3	0.93
LC2 (cys 125)	8	1.25
LC2 (cys 154)§	3	0.98
Bound to MHC		
A1 (Cys 177)	3	0.82
A2 (Cys 136)	3	0.75
LC2 (Cys 125)	5	1.10
LC2 (Cys 154)§	4	0.98
FL-SCH <sub>2</sub> CH <sub>2</sub> OH	4	1.05

\* Solvent conditions: 0.5 M NaCl, 0.1 mg/ml BSA, 2 mM DTT, 40 mM Tris-HCl, pH 8.0, at 20°C. Dissociation constants were estimated assuming first-order binding. The constants were reduced ~10-fold in 0.1 M NaCl.

‡ Expressed as mole binding site per mole fluorescyl group.

§ Cys 125 of this derivative was blocked by 1,5-IAEDANS.

The singly labeled LC2 light chain was cyanylated by NTCB to an extent of 0.86 mol SH/mol LC2 as compared to a value of 1.9 mol SH/mol LC2 for the unmodified light chain. The reaction can be conveniently followed at 412 nm by measuring the release of TNB which accompanies cyanylation. Cleavage of the cyanylated cysteine is achieved by incubation at alkaline conditions (pH ~10) for 24 to 72 h at room temperature (Fig. 4). SDS polyacrylamide gels showed a 17-kD and a 13-kD doublet for the unlabeled LC2 (Fig. 4, lane 3), and a 17-kD doublet for the singly labeled LC2 (lane



**Figure 3.** Reaction of regulatory light chain with 5-IAF. LC2 (0.6 mg/ml) and 0.33 mM 5-IAF were incubated in 0.1 M NaCl, 20 mM imidazole, pH 7.0, at 0°C. At fixed time intervals, 50  $\mu$ l were withdrawn and mixed with 2  $\mu$ l of 1 M DTT and 0.2 M EDTA to stop the reaction. After adding solid urea to 8 M, the samples were run on 8% acrylamide urea gels as shown in the inset. Intensity is plotted as a function of reaction time for the first (○), second (●), and third (Δ) bands from the top of the gel. The latter two bands were fluorescent.

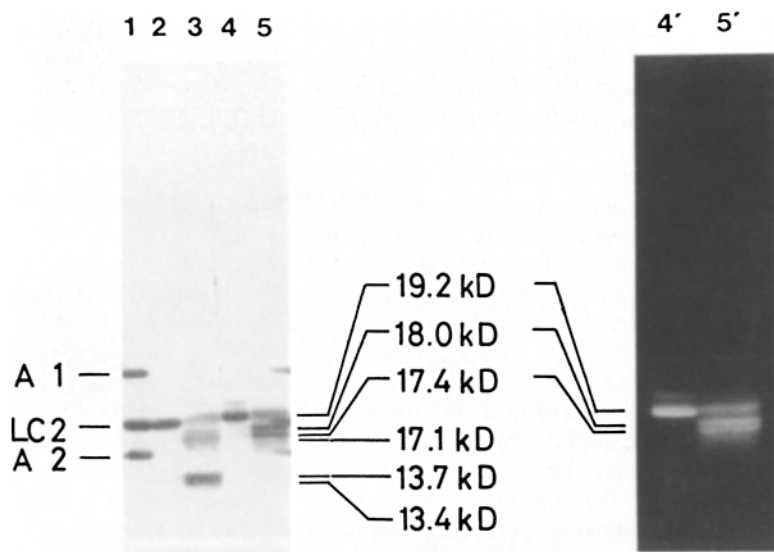
5). On the basis of their apparent molecular masses, the 17-kD band would result from cleavage at Cys 154, and the 13-kD band from cleavage at Cys 125. Since the labeled light chain only gave a band(s) in the 17-kD range, which moreover was fluorescent (lane 5'), we concluded that Cys 125 was the thiol labeled with 5-IAF. When a different thiol-specified fluorophore was used, namely 1,5-IAEDANS, similar rapid labeling of Cys 125 was observed.

To modify Cys 154 selectively, we originally planned to reversibly block the fast reacting Cys 125 with DTNB, react Cys 154 with IAF, and then reduce Cys 125 with DTT. However, when LC2 was reacted with DTNB at the molar ratio of 1 mol DTNB/mol LC2, 1.7 mol TNB/mol LC2 was released instead of the expected 1 mol TNB (Fig. 5). The release of 2 mol of TNB could only occur by intra- or intermolecular disulfide formation (Ellman, 1959). Examination of the reaction products by SDS-PAGE showed a faster migrating species at a time point (15 min) in the plateau region of the reaction, but no evidence of any dimer formation. This fast migrating band could readily be reversed to native LC2 by DTT, suggesting that its anomalous migration was due to an intramolecular disulfide bond. Since it proved to be difficult to block Cys 125 by this approach, we first reacted it with 1,5-IAEDANS, and then modified Cys 154 with 5-IAF. Light chains thus modified at different cysteine residues by two different fluorophores could be separated by ion-exchange chromatography, and were shown to have distinct mobilities on urea-polyacrylamide gels (data not shown).

#### Visualization of Antibody/Light Chain Complexes by Electron Microscopy

LC2 light chains labeled at cysteine residue 125 or 154, as described above, were titrated with anti-fluorescyl antibodies by the procedure described for labeled alkali light chains. Apparent dissociation constants of 3–8 nM were obtained, which is in a range comparable to those observed for the fluorescein-labeled alkali light chains (Table I).

The strong interaction observed between both types of light chains and anti-fluorescyl antibodies suggested that it might be feasible to visualize this complex by electron microscopy. Light chains alone are too small to show any distinct features by metal shadowing (data not shown); however, it was thought that by fixing one end with the larger antibody molecule, more structural information might be obtained. Unfortunately, this did not prove to be the case; antibody/light chain complexes usually showed only small wispy structures emanating from the F<sub>ab</sub> arms of the triangular-shaped IgG (Fig. 6, b, c, and e). Occasionally, a more globular structure could be seen at the corners of the antibody molecule (Fig. 6f). Aggregates of antibodies were rarely seen, except for the anti- $\Delta$ 1/A1 complex (Fig. 6a). This polyclonal antibody is specific for a region of the A1 light chain which probably includes several epitopes by virtue of spanning 49 residues (Fig. 1). Multivalency of antigens (e.g., A1) is responsible for antibody/antigen aggregates in that each antigen is bound to more than one antibody molecule. A similar effect is obtained by adding two different antibodies simultaneously to the fluorescein-labeled light chain; one specific for the NH<sub>2</sub> terminus (anti-LC2 or anti- $\Delta$ 2) and one for the COOH-terminus (anti-fluorescyl IgG) (Fig. 6, d and g). Thin connecting links between antibodies were seen in some of these images, but no consistent lengths could be measured.



**Figure 4.** SDS gels (15% acrylamide) showing products of cyanation followed by cleavage of regulatory light chain. Lane 1, total light chains; lane 2, unmodified LC2; lane 3, cleavage products of native LC2 after 24 h reaction show primarily a doublet at  $\sim 13$  kD and a minor doublet at  $\sim 17$  kD (see Materials and Methods); lane 4, fluorescein-labeled LC2 ( $\sim 1.3$  FI/LC2); lane 5, cleavage products of modified LC2 after 72 h show only the higher molecular mass doublet. Lanes 4' and 5' show the bands visualized in UV light before staining.

Although these observations favor a more compact structure for the light chains than the 100-Å-long ellipsoid suggested by hydrodynamic studies (Stafford and Szent-Györgyi, 1978; Alexis and Gratzer, 1978), the molecules are too small in size to permit any firm conclusions.

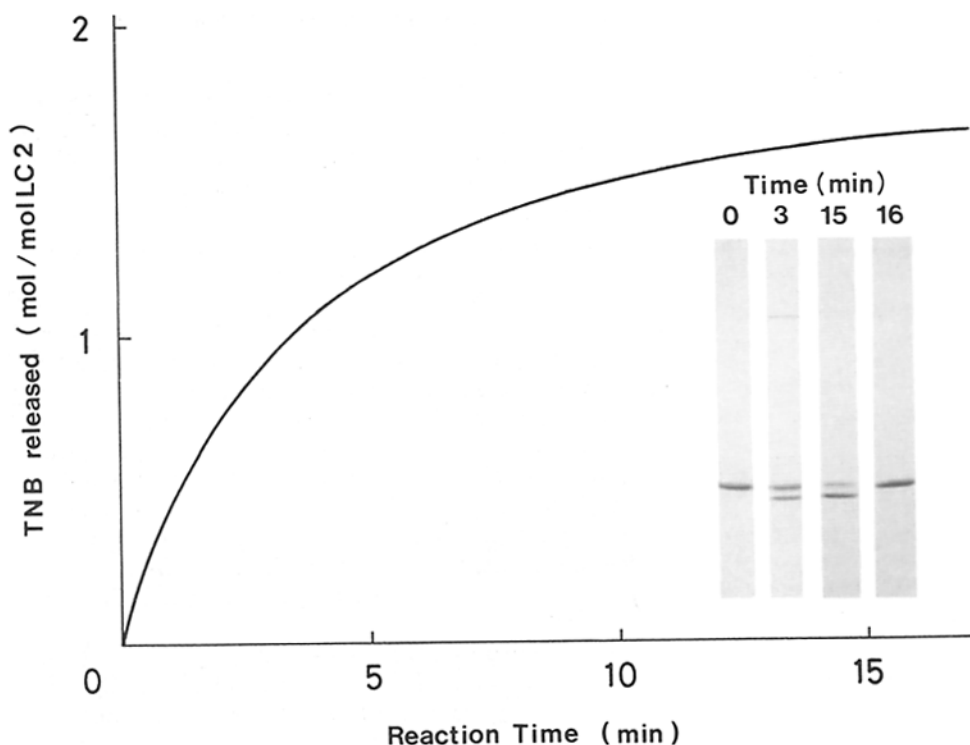
#### Exchange of Labeled Light Chains into Myosin

L2 light chain that had been singly or doubly labeled with fluorophore was exchanged into myosin at increasing temperatures in the presence of EDTA (Table II). The extent of exchange was estimated by comparing the intensities of the labeled and unlabeled LC2 bands on urea gels. As much as 80% exchange could be attained for the singly labeled LC2

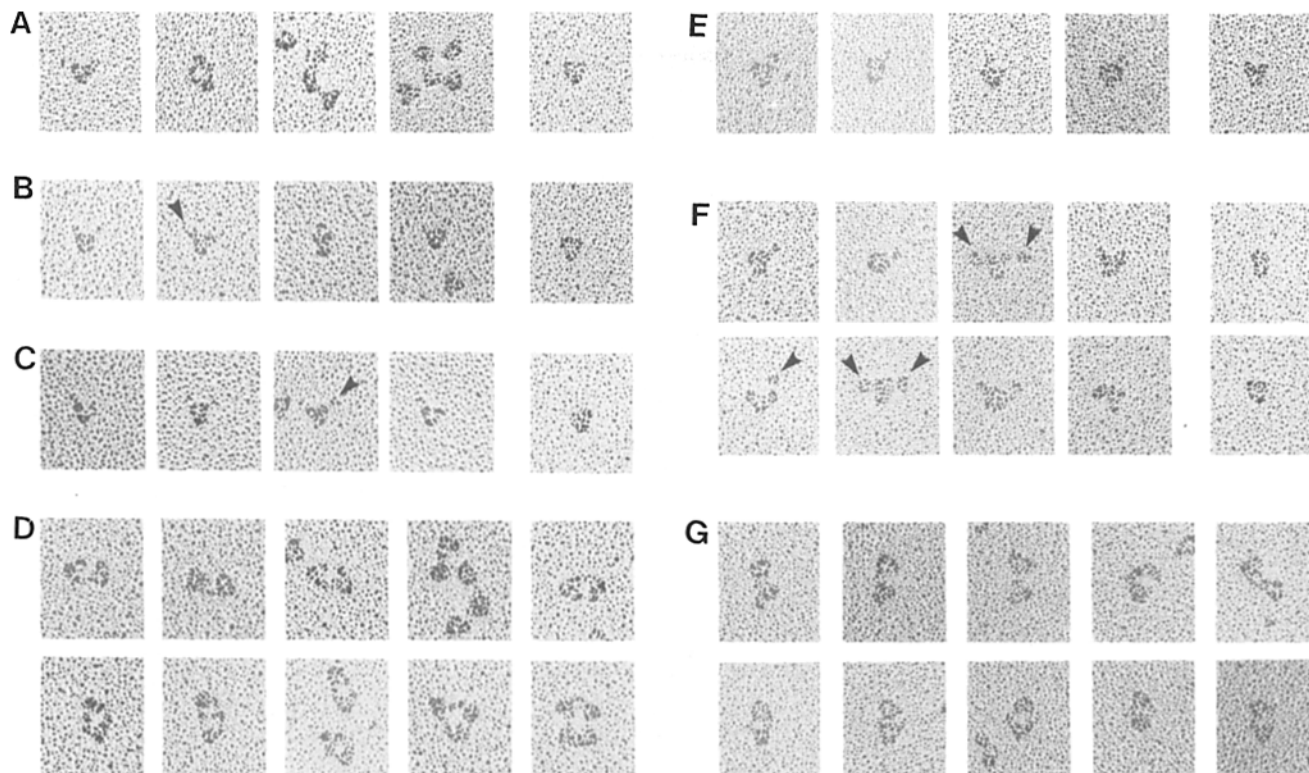
by 40°C; higher temperatures were not feasible because of a loss of alkali light chains with accompanying denaturation. The native state of the exchanged myosin was demonstrated by the relative constancy of the different ATPase activities up to 40°C (Table II).

The singly labeled alkali light chains were exchanged into myosin in 4.7 M ammonium chloride according to established procedures (Wagner and Weeds, 1977). About 80% of the  $\text{Ca}^{2+}$ - and  $\text{K}^+$ /EDTA-activated ATPase activities were retained.

The affinity of anti-fluorescyl antibodies for the bound light chains was measured by fluorescence quenching as described for the free light chains (see Fig. 2). The apparent dissociation constants obtained by this method were approxi-



**Figure 5.** Time course of the reaction of regulatory light chain with equimolar DTNB. LC2 ( $12.1 \mu\text{M}$ ) was reacted with  $12.1 \mu\text{M}$  DTNB in 0.1 M NaCl, 2 mM EDTA, 5 mM Pipes, pH 6.5, at 15°C. The reaction was monitored by the absorbance at 412 nm. The inset shows the SDS gel (17.5% acrylamide) pattern of the reaction products at the indicated times. The reaction was terminated by the addition of NEM and Tris-HCl, except for the 16-min time point where DTT was added.



**Figure 6.** Gallery of electron micrographs of fluorescein-labeled light chains complexed with antibodies specific for the hapten or for the amino-terminal region of the light chain. *A*, FI-A1 and anti- $\Delta$ 1 IgG. The presence of antibody aggregates implies more than one epitope on A1. *B*, FI-A2 and anti- $\Delta$ 2 IgG. The existence of only monomeric antibody suggests that A2 has a single epitope. *C*, FI-A2 and anti-fluorescyl IgG. *D*, FI-A2 reacted with both anti- $\Delta$ 2 and anti-fluorescyl IgG. The aggregates here are a consequence of two classes of antibodies reacting with two different sites on the light chain: the epitope at the NH<sub>2</sub> terminus and the fluorescein on Cys 136 (see Fig. 1). *E*, FI-LC2 and anti-LC2 (7C10). *F*, FI-LC2 and anti-fluorescyl IgG. *G*, FI-LC2 reacted with both anti-LC2 (7C10) and anti-fluorescyl IgG. The last column on the right in *A-C* and *E-F* shows antibody in the absence of added light chain. The arrows point to the bound light chain. Bar, 50 nm.

mately the same as those determined for the free light chains, suggesting that the accessibility of the fluorescyl group was not altered by the presence of the heavy chains (see Table I).

**Table II.** Exchange of Fluorescyl-labeled LC2 into Myosin

Residue labeled*	Temperature (°C)	Percent exchange	ATPase activities (s <sup>-1</sup> )		
			AM	Ca <sup>2+</sup>	K <sup>+</sup> /EDTA
Myosin control Cys 125	25	0	4.4	3.8	16.5
	25	7	4.8	4.0	16.8
	30	26	4.6	4.3	17.8
	35	49	3.1	3.0	13.3
	40	84	3.6	3.9	17.1
Myosin control Cys 125/154	25	0	5.4	4.2	16.5
	35	51	4.6	4.0	15.5
	40	72	4.4	3.9	15.1
	45	89	1.1	1.5	6.5
Myosin control Cys 154‡	25	0	4.2	3.2	13.7
	40	56	3.2	3.0	14.1

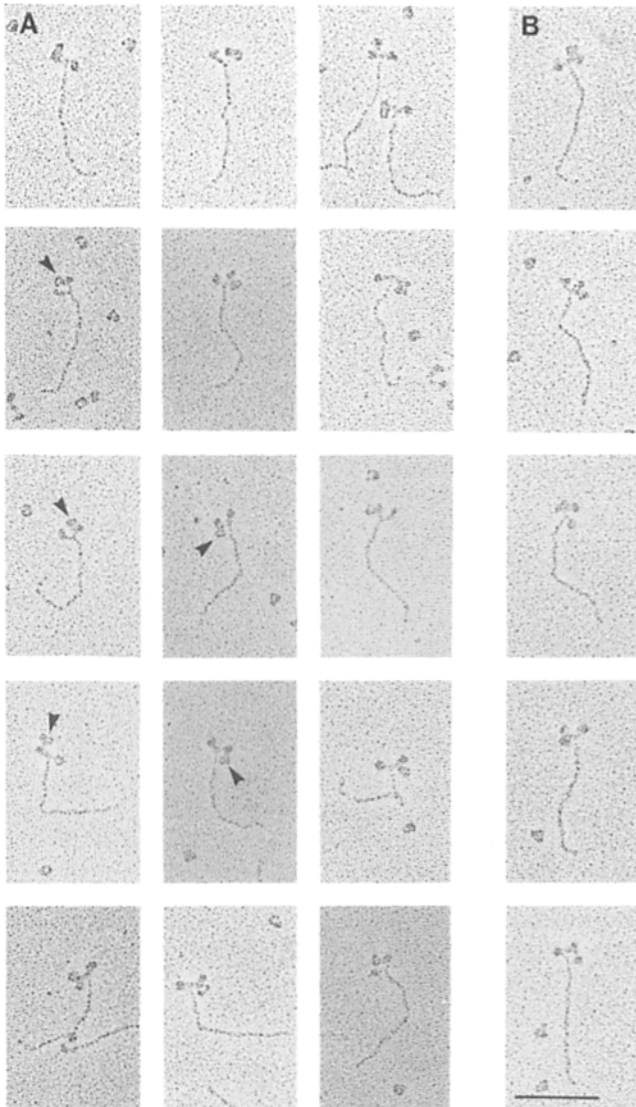
\* Exchange was done using a 20-fold molar excess of labeled LC2 over myosin; solvent conditions: 0.5 M NaCl, 2 mM EDTA, 2 mM DTT, 5 mM ATP, 40 mM Tris-HCl, pH 8.0.

‡ Cys 125 of this derivative was blocked by 1,5-IAEDANS.

### Visualization of Myosin/Antibody Complexes by Electron Microscopy

Anti-fluorescyl antibodies complexed to myosin were rotary shadowed with platinum and examined in the electron microscope. A high proportion of myosin molecules showed antibody attached to the head, compared to controls with non-immune IgG, or anti-fluorescyl antibodies incubated with unmodified myosin. Unambiguous localization of antigenic sites in the myosin head is not easy, however, because of the similarity in size between the head region and antibody, and the difficulty in defining the point of attachment of the antibody to the head. In favorable complexes, the trinodular appearance of the antibody helps to distinguish it from the rounder, slightly smaller myosin head. Moreover, when the antibody is bound directly at the junction of the head with the rod, its attachment site is more readily visualized than when it binds away from the junction. Nevertheless, only by inspecting a large number of such complexes is it possible to determine where a particular antibody is binding in the myosin head. A montage of some typical images is shown in Figs. 7 and 8.

When anti-fluorescyl IgG was added to myosin which was fluorescein-labeled at either Cys 177 of alkali 1 or Cys 136 of alkali 2 light chain, the bound antibody was most frequently seen at some distance from the head/rod junction



**Figure 7.** Gallery of electron micrographs of antibody-myosin complexes. (A) Anti-fluorescyl antibody complexed with myosin whose alkali light chain was exchanged with F1-A2. (B) Anti- $\Delta 2$  antibody complexed with native myosin. The arrows point to the attached antibody. Note that the antibody binds to a site removed from the head-rod junction in most of the images. Bar, 100 nm.

(Fig. 7 *a*). In contrast, myosin which was labeled at Cys 125 or Cys 154 of the LC2 light chain showed predominantly complexes in which the antibody was bound at or near the head/rod junction (Fig. 8, *a* and *b*). Localization experiments using antibodies specific for the NH<sub>2</sub>-terminal regions of the two classes of light chains were also performed, even though sites mapped by these antibodies had been published previously (Winkelmann et al., 1983; Waller and Lowey, 1985). In agreement with the earlier work, antibody specific for the NH<sub>2</sub>-terminal portion of A2 was bound distal to the junction (Fig. 7 *b*), whereas monoclonal antibody specific for the NH<sub>2</sub> terminus of LC2 bound at the junction (Fig. 8 *c*). Given the large uncertainty in mapping specific sites in the myosin head by immunoelectron microscopy, it was considered important to use the amino-specific antibodies as controls for the COOH-terminal localizations. After mea-

suring the point of attachment of the different types of antibodies to the myosin head, it became clear that two locations were favored: Both the COOH- and the NH<sub>2</sub>-terminal regions of the alkali 2 light chain were 80–90 Å from the head/rod junction (Fig. 9, *a* and *b*), whereas the ends of the LC2 chain were mapped predominantly to the junction (Fig. 9, *c–e*). Although the spread of data in the individual histograms is broad, particularly when the antibody binds to the middle of the head, it is possible to conclude that the light chains are located in distinct regions of the myosin head, and do not lie parallel to each other.

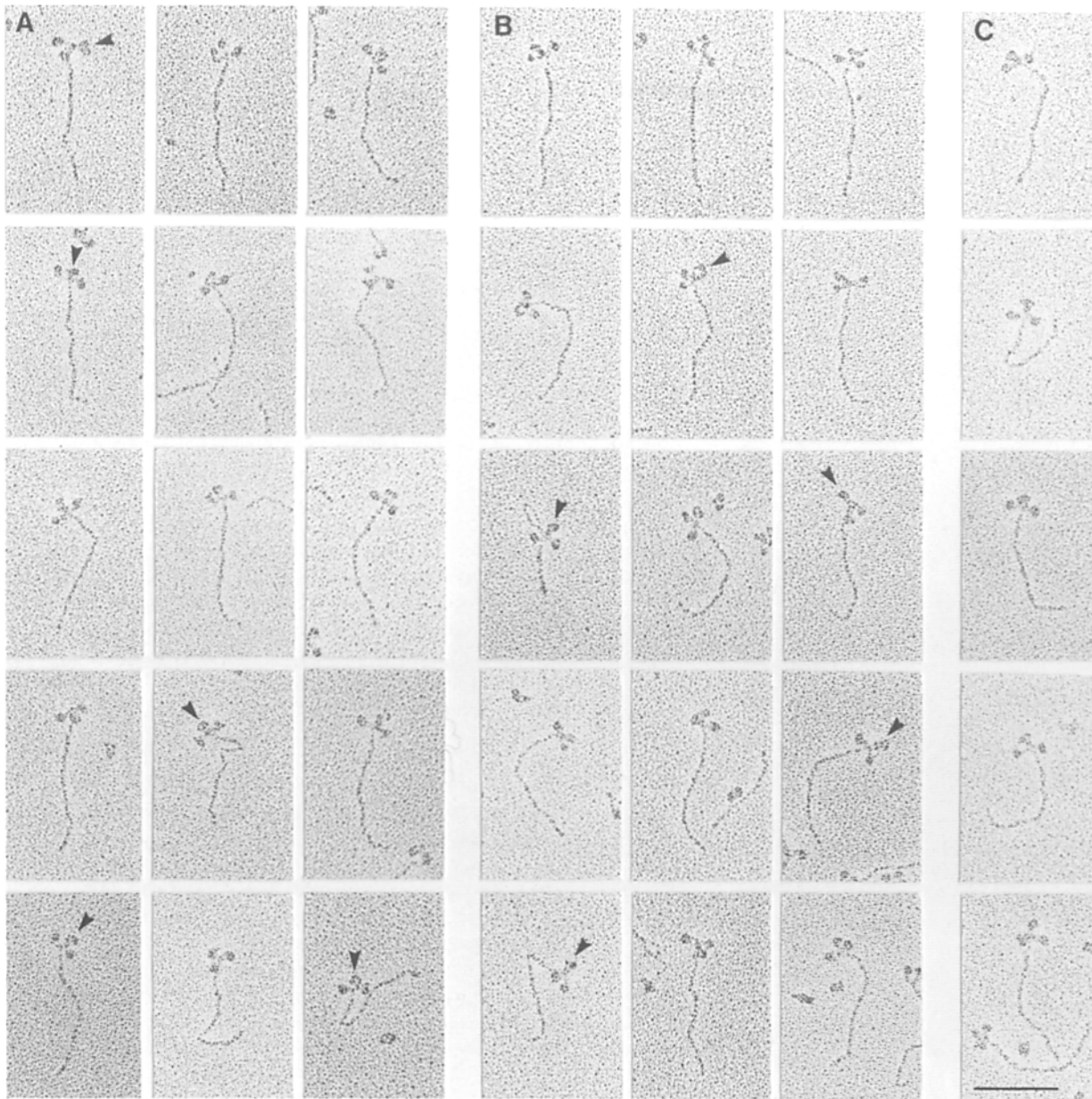
## Discussion

Electron microscope images of myosin contrasted by metal shadowing have shown a pear-shaped head  $\sim 200$  Å in length connected to a rod-like tail (Lowey et al., 1969; Elliott and Offer, 1978). By negative staining it has been possible to see some substructure in the head; in particular, a large domain of  $\sim 100$  Å which accounts for more than half of the mass of the head, and two smaller domains in the neck region (Walker and Trinick, 1988). Ever since the light chains were shown by hydrodynamic methods to be highly elongated structures, it has been assumed that they interact with the heavy chain and with each other along the length of the neck region (Wallimann et al., 1982). Our present findings suggest that the light chains may be more compact and more separated in their locations than previously envisaged. One might even speculate that the two domains in the neck region of the negatively stained molecules may include portions of the regulatory (LC2) and essential (alkali) light chains (Fig. 10).

Both classes of light chains are considered highly asymmetric structures, with a length of  $\sim 100$  Å based on a prolate ellipsoid model (Alexis and Gratzer, 1978; Stafford and Szent-Györgyi, 1978). It is significant that the light chains, troponin C, and calmodulin all have a similar Stokes radius ( $\sim 24$  Å), sedimentation coefficient ( $\sim 1.8$  S), and molecular weight ( $\sim 18,000$ ) (Dedman et al., 1977). Moreover, the radius of gyration, a sensitive measure of the distribution of mass, is similar ( $\sim 23$  Å) for calmodulin (Seaton et al., 1985), troponin C (Hubbard et al., 1988), and the light chains (Mendelson, R., personal communication). Since these proteins also have a high degree of sequence homology (Weeds and McLachlan, 1974; Collins, 1976), it follows that their three-dimensional structures are probably closely related. From recent crystallographic determinations of the structure of calmodulin (Babu et al., 1985) and troponin C (Herzberg and James, 1985; Sundaralingam et al., 1985*a*) we know that these molecules have a dumbbell shape with an overall length of  $\sim 70$  Å. If we assume that the light chains have a similar shape and dimension, the problem arises of how to arrange the light chains in the myosin head so that their location is compatible with the available chemical and electron microscopic data.

Cysteine residues 125 and 154 of the LC2 light chain, and Cys 177(136) of the alkali light chains, were labeled selectively with iodoacetamido fluorescein to map their positions in the myosin head. Anti-fluorescyl antibodies localized the thiols of LC2 at the head/rod junction, whereas the single thiol of alkali 2 was observed  $\sim 90$  Å distal to the junction. Despite the large standard deviation in the measurement, the position of the COOH-terminal cysteine of alkali 2 found



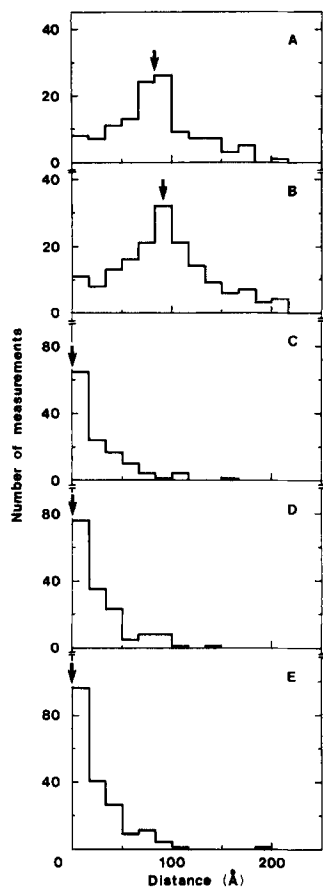


**Figure 8.** Gallery of electron micrographs of antibody-myosin complexes. (A) Anti-fluorescyl antibody complexed with myosin whose LC2 light chain was exchanged with Fl-LC2 and (B) with DNS-LC2-Fl. (C) Anti-LC2 (7C10) antibody complexed with native myosin. The arrows point to the attached antibody. Note that the antibody binds at the head-rod junction in most of the images. Bar, 100 nm.

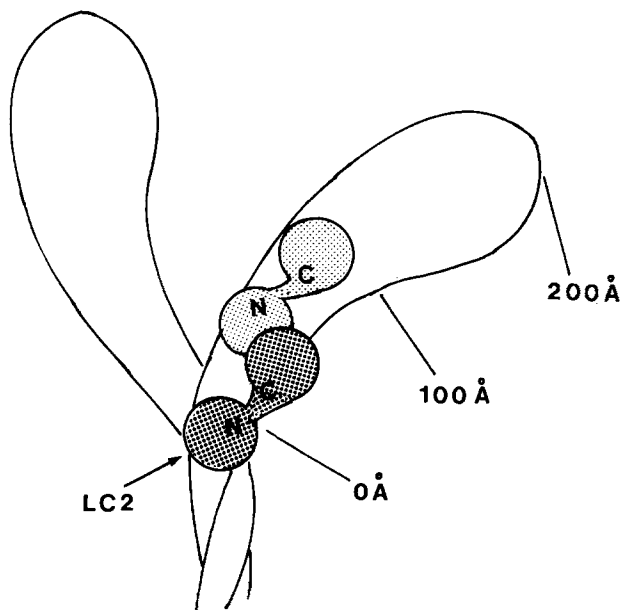
here agrees well with an earlier mapping study using avidin-biotin as a probe (Yamamoto et al., 1985) and is consistent with distances estimated by fluorescence energy transfer (Marsh and Lowey, 1980). The wide separation between the COOH-terminal regions of the two types of light chains was unexpected since one had usually imagined them to lie in an extended, if somewhat staggered, colinear arrangement (Waller and Lowey, 1985). Instead, the COOH-terminal regions of the chains were found in the same locations as the NH<sub>2</sub>-terminal regions mapped here and in previous studies (Winkelman et al., 1983; Winkelman and Lowey, 1986; Tokunaga et al., 1987b).

Can these locations be reconciled with a calmodulin-like structure for the light chains? Although the length of calmodulin is  $\sim 65$  Å, the COOH and NH<sub>2</sub> termini of the chain

are only separated by  $\sim 40$  Å in the crystal structure. Under conditions where the molecule is bent at the central helix, the terminal regions of the chain can approach to within 20 Å or less of each other (Persechini and Kretsinger, 1988; Heidorn and Trewella, 1988). It is therefore reasonable to suggest that the structure of the light chains may resemble two globular domains connected by a flexible central helix (Sundaralingam et al., 1985b). When the LC2 light chain is associated with the myosin heavy chain, it may assume a more compact configuration than when it exists free in solution in the absence of divalent cations (Alexis and Gratzner, 1978). An attempt was made to visualize the light chains directly by complexing them with antibodies specific for the amino and carboxyl ends of the chain (Fig. 6). Antibody molecules were linked together by bivalent light chains in the



**Figure 9.** Histograms showing the location of antibody-binding sites in the NH<sub>2</sub>- and COOH-terminal regions of the A2 and LC2 light chains on the myosin head. Measurements were made on enlarged images of the antibody-myosin complex from the center of the head-rod junction along the length of the head to the midpoint of the attached antibody. Arrows indicate mean values normalized to a length of 20 nm for the myosin head. *A*, amino-terminal region 1-8 of A2,  $8 \pm 4$  nm ( $n = 120$ ); *B*, Cys 136 of A2,  $9 \pm 5$  nm ( $n = 165$ ); *C*, amino-terminal region 17-51 of LC2,  $0 \pm 2.5$  nm ( $n = 128$ ); *D*, Cys 125 of LC2,  $0 \pm 2.5$  nm ( $n = 157$ ); *E*, Cys 154 of LC2,  $0 \pm 2.5$  nm ( $n = 188$ ).



**Figure 10.** Schematic diagram of the myosin head showing the approximate positions of the epitopes for the NH<sub>2</sub>- and COOH-terminal regions of the alkali and regulatory (LC2) light chains. The shape of the light chains is assumed to resemble the dumbbell structure found for homologous Ca<sup>2+</sup>-binding proteins (see Discussion), but this is purely speculative. The large experimental error inherent in the mapping technique precludes a definitive assignment for the orientation of the light chains, but based on the available data, we favor the NH<sub>2</sub> termini pointing toward the head-rod junction.

form of cyclic dimers, trimers, and tetramers, similar to the images observed by Valentine and Green (1967) for their hapten/antibody complexes. Although the resolution is too poor to assign a definitive value, the results are more compatible with a dimension of  $\sim 70$  Å for the light chains than one  $>100$  Å.

A globular structure for the COOH-terminal region of LC2 is consistent with the observation that a disulfide bond is readily formed between Cys 125 and Cys 154 (Minowa et al., 1983). Moreover, a compact structure for the NH<sub>2</sub>-terminal portion of the chain is suggested by the localization of several monoclonal antibodies, whose specificities range from the NH<sub>2</sub>-terminal trimethylalanine (Tokunaga et al., 1987*b*) to an epitope including residue 35 (Reinach and Fischman, 1985) at the head/rod junction. Globular domains are also indicated by an  $\alpha$ -helix content of  $\sim 35\%$  for LC2 (Holt and Lowey, 1975) compared to a value between 40% and 50% for troponin C and calmodulin (Dedman et al., 1977).

If we adopt a calmodulin-like structure as a reasonable approximation for both classes of light chains (see Carboni et al., 1988 for a myosin-like 100-kD subunit that binds several molecules of calmodulin), then two such subunits, with some bending and overlap of domains, could be accommodated in the neck region of myosin and still be consistent with the electron microscopic mapping studies (Fig. 10). The difficulty with such a model is that some of the chemical cross-linking data are difficult to reconcile with light chains that don't span a greater portion of the head. Using photolabile bifunctional cross-linkers attached to the sulfhydryl residue of a number of regulatory light chains, it was concluded that the essential (alkali) and chemically modified regulatory light chains overlap by more than half of their length and lie in close proximity to each other in the myosin molecule (Walimann et al., 1982). Although there is enough uncertainty in the mapping data to accommodate these studies, it is difficult to see how one can also fit in photolabeling experiments which implicate the essential light chain in the active site of smooth muscle myosin (Okamoto et al., 1986). The ATPase site has been localized  $\sim 50$  Å from the tip of the myosin head (Tokunaga et al., 1987*a*), or  $\sim 140$  Å from the COOH-terminal end of S1. The finding that the essential light chain of scallop myosin can be cross-linked to Cys 125/154 of chicken LC2 implies that some portion of the essential light chain must extend to the vicinity of the head/rod junction. But the light chain would then have to be at least 150 Å long to bridge that great a distance.

These disparate data lead one to conclude that either the light chains of different muscle types occupy unique locations in the myosin head, despite the high degree of sequence homology, or that there may be sufficient motion of the light chains to permit cross-linking between subunits that are on average spatially distant (Chantler and Bower, 1988). Some evidence for a relative movement between the essential and regulatory light chains has been obtained for molluscan muscles (Hardwicke et al., 1983). There is also an extensive literature on cross-linking of different regions of the heavy chain, the principal conclusion of which is that so-called heavy chain domains may undergo substantial movements under different experimental conditions (see review by Vibert and Cohen, 1988).

The advantage of using a fluorescent probe to label sulf-

hydriyl residues is that a combined approach of spectroscopy and electron microscopy can be applied to the analysis of the myosin head. Until a crystallographic structure of S1 is available (Rayment and Winkelmann, 1984), electron microscopy is the only direct means to visualize the sites of interest, although one must recognize that the resolution of rotary-shadowed myosin/antibody complexes is low. By averaging the images using correlation methods, it may be possible to raise the resolution to a level where more structural information is available (Vibert, 1988). Avidin has been a very useful probe in mapping studies of the myosin head, because of the high affinity of avidin for biotinylated myosin ( $\sim 10^{15} \text{ M}^{-1}$ ). However, anti-fluorescein antibodies of affinities  $>10^{10} \text{ M}^{-1}$  can be prepared (Kranz et al., 1982), and their ability to quench the fluorescence of fluorescein offers a unique opportunity to characterize the reactivity of the labeled sites.

Fluorescein is most widely used as a probe in spectroscopic studies. Techniques such as fluorescence energy transfer have the advantage of being able to measure distances in the 10–60-Å range with an error of no greater than 20%, but only relative distances between two sites can be obtained. However, the spectroscopic approach does have the ability to detect changes accompanying dynamic processes such as ATP hydrolysis and actin activation. If, in fact, rearrangement of the light chains and/or heavy chain domains takes place during contraction, it may indeed be necessary to use such a combination of optical and microscopic techniques to localize the reactive sites in myosin as the molecule undergoes its different conformational transitions.

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