Site-directed mutagenesis has been used to insert cysteine residues at specific locations in the myosin light chain 2 (LC2) sequence. The aim was to modify these cysteines with one or more spectroscopic probes and to reconstitute myosin with labeled light chains for structural studies. Native LC2 has two endogenous cysteine residues at positions 126 and 155; a third sulfhydryl was added by replacing either Pro', Ser173, or Pro154 with cysteine. By oxidizing the endogenous cysteines to an intramolecular disulfide bond (Katoh, T., and Lowey, S., (1989) J. Cell Biol. 109, 1549), it was expected that the new cysteine could be selectively labeled with a fluorescent probe. This proved more difficult to accomplish than anticipated due to the formation of secondary disulfide bonds between the newly engineered cysteines and the native ones. Nevertheless, the unpaired cysteines were labeled with 5-(iodoacetamido)fluorescein, and singly labeled species were purified by ion-exchange chromatography. Chymotryptic digestion of the light chains, followed by high performance liquid chromatography separation of the peptides, led to the identification of the fluorescein-labeled cysteines. After light chain exchange into myosin, the position of the thiols was mapped by anti- fluorescein antibodies in the electron microscope. Rotary-shadowed images showed the antibody bound at the head/rod junction of myosin for all the mutants. These mapping studies, together with the finding that widely separated cysteines can form multiple disulfide bonds, support a model for LC2 as a flexible, globular molecule that resembles other Ca/Mg-binding proteins in tertiary structure.

Ever since the sliding filament mechanism for muscle contraction was first proposed in the 1950s, the myosin cross-bridge has been the focus of efforts to explain how force generation is coupled to ATP hydrolysis. Current cross-bridge models invoke a substantial conformational change within the myosin head (Sl), the most widely accepted model has the force-generating mechanism contained in the myosin head (Sl), thus since this proteolytic fragment can move actin in the presence of ATP by an in vitro motility assay (Toyoshima et al., 1987). An alternative helix-coil model proposes that melting within the S2 hinge region of the myosin rod triggers force generation (Harrington, 1979). One way to reconcile these two theories is to consider S1 alone sufficient to move actin under no-load conditions, but the contribution of the rod may be needed to generate force in an actively working muscle (Harrington et al., 1990).

A variety of spectroscopic probes have been used to search for conformational changes in the S1 head, but independent of whether the label was introduced at the nucleotide binding site or at the reactive thiol (SH1) on the myosin heavy chain (MHC) (Cook et al., 1984), no rotation or tilting of the attached head was observed. One possible explanation is that the region of the 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 long sought for structural changes (Huxley et al., 1983). We have therefore chosen to focus our efforts on the regulatory light chain (LC2), which is a 19,000-Da subunit located in the "neck region" of myosin at its head/rod junction. If a change in orientation occurs in this region, it will most likely be reflected by a change in the light chain. The ability to exchange LC2 into myosin makes it an attractive means to introduce specific probes into the head of isolated myosin molecules, as well as into more complex systems such as muscle fibers and motile cells.

Without a crystallographic structure of S1 available (Winkelmann et al., 1985), the topography of the myosin head has been largely determined by immunoelectron microscopy. Monodonal and polyclonal antibodies have been used to map the NH2-terminal (reviewed in Winkelmann and Lowey, 1986) and COOH-terminal (Katoh and Lowey, 1989) regions of both classes of light chains. By this approach it was concluded that the ends of light chain-2 are located at the head/rod junction. This still left open the possibility that portions of the intervening sequence might extend to more distal regions of the head, closer to the ATP-binding site. It is otherwise difficult to understand how phosphorylation of the smooth muscle light chain, which is highly homologous to skeletal LC2, can regulate the ATPase from such a large distance.

In order to map more of the sequence of light chain-2, and to have additional reactive sites available for spectroscopic labeling, we decided to insert several new cysteine residues into LC2 by site-directed mutagenesis. Native LC2 has 2 endogenous cysteines at positions 126 and 155; a third cysteine was added by replacement of Pro1, Ser173, or Pro154. The

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†The abbreviations used are: MHC, myosin heavy chain; LC2, regulatory light chain-2; LC, light chain(s); S1, subfragment 1; SH1, reactive heavy chain thiol; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; 5-IAP, 5-(iodoacetamido)fluorescein; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoate; NEM, N-ethylmaleimide; DTT, dithiothreitol; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
choice of these residues arose from the assumption that the tertiary structure of LC2 would resemble that of calmodulin (Babu et al., 1985) and troponin C (Herzberg and James, 1985). Myosin light chains and calcium-binding proteins show considerable homology in primary structure (Collins, 1976), and hydrodynamic studies indicate that these proteins are all asymmetric molecules with globular and elongated regions (reviewed in Katoh and Lowey (1989)). On the basis of this structural homology, Pro2 and Ser13 would reside in the NH2-terminal globular domains, and Pro84 would lie at the end of a putative linker region that connects the NH2-terminal domains with the COOH-terminal domains where the endogeneous Cys14 and Cys15 are located.

The initial plan was to retain the endogenous cysteines in the mutated LCs, because the stability of the LCs might be modified by their removal, but more importantly, because it had been shown that Cys14 and Cys15 readily oxidize to form a reversible disulfide bond (Katoh and Lowey, 1989; Huber et al., 1989). By blocking 2 of the 3 cysteines, it was thought possible to selectively label the engineered thiol. In experiments where introduction of a second Pro residue was disastrous, such as resonance energy transfer, the LC could be readily reduced and labeled with another fluorophore. This strategy worked to only a limited extent, because, unexpectedly, disulfide bond formation occurred between the engineered thiols and the native ones. In the case of the Pro2→Cys mutant we were able to isolate a LC singly labeled at position 2 with fluorescein. The other mutants were mixtures of singly labeled LCs, with the label at more than one site. The sites were identified by chymotryptic digestion of the labeled light chains and analysis of the peptides by HPLC chromatography.

Myosin, stripped of its native LC2 light chain by affinity chromatography, was reconstituted with the mutant light chains, and the position of the fluorescein-labeled cysteines was mapped by anti-fluoresceyl antibodies in the electron microscope. We conclude from the mapping studies, and from the formation of multiple disulfide bonds, that the light chain can assume a relatively compact structure that is localized at the head/rod junction of myosin.

**MATERIALS AND METHODS**

**Plasmids, Bacterial Strains, and Reagents**—The cDNA clone L10 for the chicken skeletal muscle myosin regulatory light chain was kindly provided by Donald A. Fischman (Reinach and Fischman, 1985). The expression vector pT7-7 and Escherichia coli strains K38, JG5601, and UT4400 containing plasmid pGP1-2 were a gift from Stanley Tabor (Tabor and Richardson, 1985). Plasmid pKK223-3 was purchased from Pharmacia LKB Biotechnology Inc. Restriction enzymes were obtained from Bethesda Research Laboratories, endopeptidase Arg-C was obtained from Boehringer Mannheim, 5-iodoacetamido)fluorescein (5-IAF) was from Molecular Probes (Eugene, OR), and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), and a-chymotrypsin were from Sigma. The DNA manipulations were carried out as described (Maniatis et al., 1982).

**Construction of Expression Plasmids**—The cDNA for LC2 was isolated from clone L10 by EcoRI digestion, and treated with Bal31 nuclease in order to delete the 5' and 3' noncoding regions. After the ends were repaired with Klenow fragment of DNA polymerase, the cDNA was ligated to the pKK223-3 expression vector, which had been linearized with SmaI and treated with calf intestinal phosphatase. Ampicillin-resistant colonies (E. coli strain K38) containing the helper plasmid pGP1-2 (provides T7 RNA polymerase and kanamycin resistance) were transformed, and the colonies resistant to ampicillin and kanamycin were selected. Crude extracts (pelleted cells from 1-ml cultures dissolved directly in 100 μl of Lysenin's gel buffer) from these clones were analyzed by SDS-PAGE.

**Site-Directed Mutagenesis**—The oligonucleotides 5'-CCCCATGGCTCGAAGAAGGCGG-3' and 5'-CAAGAGGCGCAGGCCC-3', and 5'-GGCGGCTGACTGCGAGCGTC-3', used to replace Pro2, Ser13, and Pro84, respectively, were synthesized on a 200 ng oligonucleotide DNA synthesizer and band-purified on a 20% polyacrylamide denaturing gel. The entire cDNA (EcoRI/HindIII fragments) was ligated into group K9. The in vitro transcription/translation setup was used for site-directed mutagenesis. Plasmid plaques were selected by 32P-labeled oligonucleotide hybridization as described in the manual. The mutant CDNs were sequenced by the dyeoxy method (Sequenase DNA sequencing kit from U.S. Biochemical Corp.) and transferred to the pT7-7 vector.

**Purification of the Expressed Protein**—One-liter culture of E. coli UT4400 containing plasmida pT7-7/LC2 and pGP1-2 was grown in erbridged medium (2% Bacto-tryptone, 1% yeast extract, 6.5% NaCl, 0.2% glycerol, 50 mM potassium phosphate, pH 7.2) with kanamycin and ampicillin (50 μg/ml each) at 30 °C for 18-18 h. The cultures were pelleted at 8,000 × g for 10 min and washed once with 50 ml of Tris-EDTA buffer (25 mM Tris, pH 8.0, 1 mM EDTA). Cells were resuspended in 50 ml of lyso buffer (25 mM Tris, pH 8.0, 50 mM glucose, 1 mM EDTA, 0.2 mg/ml lysozyme) and incubated on ice for 10 min. The mixture was then centrifuged at 15,000 × g for 30 min on ice. Lysed cells were centrifuged at 15,000 × g for 10 min, the pellet washed with 50 ml of the lyso buffer without lysozyme, and centrifuged as before. This pellet was dissolved in 10 ml of 6 M guanidine HCl in PBS (10 mM sodium phosphate, pH 7.0, 150 mM NaCl, 3 mg/ml BSA) containing 10 mM DTT. The solution was stirred at 25 °C for 1 h, and centrifuged at 100,000 × g for 30 min. The supernatant was dialyzed against PBS, 0.5 mM DTT, 4 °C, with several changes of buffer, and the denatured proteins were removed by centrifugation at 100,000 × g for 30 min. The resulting solution of protein at 8 °C was frozen in liquid nitrogen and thawed. Magnesium chloride (10 mM) was added before treating the cell suspension with DNase (10 μg/ml) for 30 min on ice. Lysed cells were centrifuged at 15,000 × g for 10 min, the pellet washed with 50 ml of the lyso buffer without lysozyme, and centrifuged as before. This pellet was dissolved in 10 ml of PBS, pH 7.0, and freeze-dried in the presence of an equal weight of sucrose. Lyophilized LC2 was stored at −20 °C under desiccant.

**DTNB Titration of the Light Chains**—In order to determine the number of thiol groups in the recombinant protein, the lyophilized protein was dissolved in 6 M guanidine HCl in PBS, 10 mM DTT, and stirred gently at room temperature for 2 h before dialysis against PBS in the cold overnight, with at least two changes of buffer. The clarified light chains (10 μM) were reacted with 2.5-80 μM DTNB in 0.2 M Tris, pH 8.0, 50 mM EDTA, and after a 10-min incubation the absorbance was measured at 412 nm. The number of moles of TNB released per mol of LC2, as a function of the ratio of DTNB to LC2 is shown in Fig. 4, using a molar extinction coefficient of 1.36 × 103 M−1 cm−1 for free TNB. Samples were treated with NEM (3-5 mM) at room temperature before addition of Laemmli gel buffer (minus the reducing agent, 2-mercaptoethanol) for SDS-PAGE analysis.

**Oxidation of Light Chain Cysteines**—The lyophilized protein was dissolved and dialyzed against PBS as described above. Light chain (1 mg/ml) was incubated at room temperature or on ice (if incubated overnight) for air oxidation of the cysteines. In order to facilitate oxidation, 10 μM cupric sulfate was added in later experiments. At various time intervals, 10 μM light chains were reacted with 30-40 μM DTNB in order to determine the remaining number of free thiol groups. An aliquot from the reaction mixture was taken at the same time, reacted with excess CM-cellulose to block the unoxidized thiols, and analyzed by nonreducing SDS-PAGE.

**IAF Labeling of Light Chains**—Lyophilized wild-type LC2 was reduced in 6 M guanidine HCl, 10 mM DTT as described above. After dialysis against PBS to remove DTT, the protein was reacted with 4-μmol excess of 5-iodoacetamide in the dark. The reaction was stopped by adding DTT, and the sample dialyzed against PBS. FPLC purification was performed as described above with slight modifications. The buffers contained 1 mM DTT, and a salt gradient...
of 15–70% B was applied in 13 min. In the case of IAF labeling of mutant light chains, the protein was first oxidized in the presence of copper until approximately one free -SH group remained. After chelation of the metal ions with 1 mM EDTA, the light chains were reacted with 4-fold molar excess of IAF for 1 h on ice. The reaction was terminated with DTT, and the sample was chromatographed as described for wild-type LC2. Fractions from FPLC were analyzed by urea-PAGE in the presence of a reducing agent (Fig. 6).

**Solution Competition ELISA**—Varying amounts (0.002–100 μg/ml) of light chains (dialyzed against PBS and 1 mM DTT) were reacted with a fixed amount of monoclonal anti-LC2 antibody 7C10.2 (0.1 μg/ml in PBS, 1% BSA) in the presence or absence of magnesium ions (1 mM magnesium or EDTA) for 1 h at room temperature. Antibody not complexed by the light chains in solution was bound to myosin adsorbed on microtiter plates at 50 μg/ml (25 μl/well) in PBS. The solution-phase antibody was detected with biotinylated second-ary antibody, followed by streptavidin-b-galactosidase, and the substrate p-nitrophenyl-b-galactopyranoside (Bethesda Research Laboratories). Absorbance at 405 nm was measured on a plate reader (model EL308; Bio-Tek Instruments). The amount of bound antibody is expressed as a percent of the antibody bound at the lowest concentration tested.

**HPLC Analysis of Labeled Light Chain Peptides**—Singly labeled light chains were prepared as described above, and the unreacted cysteines were blocked with NEM. After dialysis against 50 mM ammonium bicarbonate, 1 mM DTT, the modified proteins (0.2–0.3 mg) were digested with chymotrypsin (10 μg/ml) for 12–16 h at 37 °C and dried under vacuum. The dried samples were resuspended in 0.1% trifluoroacetic acid in water (buffer A), clarified, and applied to a reverse phase column (Altex Ultrasphere ODS; 4.6 mm, inner diameter, × 25 cm) equilibrated with buffer A. An anion exchange gradient (buffer B: 0.1% trifluoroacetic acid in CH₃CN) was applied (0–50% B in 60 min, followed by 50–100% B in 5 min, and then 100% for 10 min) at a flow rate of 1 ml/min. Absorbance of the eluate was monitored at 214 and 280 nm. The fluorescence of individual fractions was measured on a Perkin Elmer MFP-44 fluorospectrophotometer at excitation and emission wavelengths of 495 and 520 nm, respectively.

**Electron Microscopy**—Singly labeled light chains were introduced into myosin by a slight modification of the procedure of Pastrana-Landis and Lowey (1986). Briefly, LC2-deficient myosin at 0.2–0.3 mg/ml was incubated with 2–2.5-fold molar excess of labeled light chain in 10 mM MgCl₂ and 10 mM DTT in phosphate buffer for 1 h on ice, and then dialyzed against low salt buffer (10 mM sodium phosphate, pH 6.5, 20 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 10% glycerol, 0.02% Na₂SO₄) for 12–16 h in the cold. The myosin precipitate was collected by centrifugation, washed twice with low salt buffer, and gently resuspended in 1 M ammonium acetate. Clarified reconstituted myosin at 0.5–1 mg/ml was incubated with anti-fluorescein antibody (0.3–0.5 mol/head) at room temperature for 1 h, diluted into 0.5 M ammonium acetate, 66% glycerol, and sprayed on mica at a concentration of 50% B in 60 min, followed by 50–100% B in 5 min, and then 100% for 10 min at a flow rate of 1 ml/min. Absorbance of the eluate was monitored at 214 and 280 nm. The fluorescence of individual fractions was measured on a Perkin Elmer MFP-44 fluorospectrophotometer at excitation and emission wavelengths of 495 and 520 nm, respectively.

**RESULTS**

**Expression and Purification of Recombinant Light Chains**—The cDNA for chicken skeletal muscle myosin light chain-2 (LC2; Reinach and Fischman (1985)) was originally cloned into the plasmid pKK223-3 for expression in E. coli (see "Materials and Methods"). The amount of light chain obtained from this construct was low, and the protein showed proteolytic degradation during its isolation. To improve the yield, the cDNA was cloned into the expression vector pT7-7 under the transcriptional control of the T7 RNA polymerase promoter (Fig. 1A). The pT7-7 construct was used to transform an E. coli strain K38 that contained a plasmid to overproduce T7 RNA polymerase (Tabor and Richardson, 1985). The yield was considerably improved in this coupled T7 RNA polymerase/promoter system, but soluble protein was still degraded at the NH₂ terminus when the cells were lysed. It had been observed by Tabor and Richardson (1985) that T7 RNA polymerase is cleaved by an endoprotease during the purification procedure. The protease was later identified as the product of the ompT gene which encodes an outer membrane protein of E. coli (Grodberg and Dunn, 1988). This protease was shown to be specific for Lys/Lys and Lys/Arg bonds, sequences which occur in RNA polymerase as well as in the NH₂-terminal region of the light chain, where the myosin LC kinase-binding site is located. By using OmpT protease-deficient strains of E. coli, JG6601, or UT4400 (Earthart et al., 1979), we were able to eliminate proteolysis and obtain intact LC in high yield (30–40 mg of purified LC/liter of culture) (Fig. 1B). The mobility of the expressed light chain is reduced relative to the chicken myosin light chain due to 5 additional amino-terminal residues, Ala-Arg-Ile-Pro-Met arising from the vector construct. Three mutant light chains were made by site-directed mutagenesis in which Pro³, Ser⁵, or Pro⁸ was replaced by cysteine (Fig. 2A). The mutants had the same mobility and high level of expression as wild type (Fig. 2B).

Most of the LC was found in inclusion bodies when the cells were grown in an enriched medium at 30 °C. These insoluble aggregates were readily solubilized in 6 M guanidine

![Fig. 1. A, structure of the plasmid construct for the expression of myosin light chain-2. The fusion protein synthesized by the plasmid consists of 5 amino-terminal residues prior to the alanine at position 6 which marks the beginning of the light chain sequence. The origin of replication (ori), ampicillin-resistance gene (Amp⁰), and T7 RNA polymerase promoter (6) are indicated. B, isolation and purification of the light chains. Samples at various stages were analyzed by SDS-PAGE. Lane 1, total cell extract; lane 2, supernatant after lysis and centrifugation; lane 3, supernatant after guanidine treatment of pellet and renaturation of LC2; lane 4, FPLC-purified light chain.](image-url)
Cysteine Mutants of Myosin Light Chain

Fig. 2. A, diagram of chicken skeletal myosin light chain-2 indicating the positions of the residues changed to cysteine and the 2 native cysteines. The 5 amino-terminal residues arising from the vector construct are not included in the numbering. B, crude extracts from E. coli strains JG5601 (lanes 1, 3, 5, 7, and 9) and UT4400 (lanes 2, 4, 6, 8, and 10) containing the plasmids with inserts for wild-type (WT) and mutant proteins were analyzed on 15% polyacrylamide SDS-gels for expression of LC2. The plasmid pT7-7 (lanes 1 and 2) without insert, and total light chains (TLC) from skeletal muscle myosin (lane 11) are included for comparison. All recombinant light chains have a slightly slower mobility compared with native LC2.

hydrochloride, a procedure which irreversibly denatures many of the E. coli proteins, but which does not affect the ability of the LC to refold in a benign solvent. The resistance to denaturation is a well known property of light chains, since they are routinely prepared by dissolving muscle myosin in guanidine hydrochloride, a solvent which irreversibly denatures the myosin heavy chain. The viability of the renatured LC was evident from its ability to restore the native properties of myosin after reconstitution with the heavy chain (Trybus and Lowey, 1988). Although this simple denaturation of the inclusion bodies resulted in a protein preparation that was greatly enriched in light chains, residual traces of denatured E. coli proteins were removed by ion-exchange chromatography on a FPLC system (see “Materials and Methods”). Light chains purified by this procedure showed predominantly a single band by polyacrylamide gel electrophoresis (Fig. 1B).

Immunological Characterization—The wild-type and mutant light chains differ from chicken light chain isolated from muscle tissue in having five extra amino acids before the NH2-terminal alanine, a third cysteine residue in the case of the mutants, and no posttranslational trimethylation of the alanine. What criteria can be used to establish whether the light chain is folded in a native configuration? As we shall show in a later section, all the recombinant light chains were able to bind to the heavy chains. A more sensitive criterion was to test the conformation of the LC with a monoclonal antibody capable of detecting magnesium-induced conformational changes. We had observed previously with chicken LC2 that magnesium was required to preserve the epitope for the anti-LC2 antibody (7C10), the location of which is near the divalent cation-binding site (Winkelmann and Lowey, 1986). LC2 showed no reaction with the monoclonal antibody in the presence of EDTA, irrespective of whether binding was measured by solution-competition ELISA or by direct solid-phase ELISA. Antibody binding to myosin was less dependent on divalent cations, although binding in EDTA was somewhat reduced relative to magnesium. This unusual antibody/antigen interaction suggested a sensitive assay to determine whether the conformation of the metal-binding site was preserved in the engineered proteins. Increasing concentrations of the mutant light chains were incubated with a fixed concentration of antibody, and the solution was transferred to a myosin-coated microtiter plate. Antibody not complexed by the light chains was bound to the plate and detected by a labeled secondary antibody in a colorimetric assay. As shown by solution competition ELISA in Fig. 3, wild-type and mutant light chains bound with the same high apparent affinity to anti-LC2 antibody as chicken LC2 in the presence of magnesium, but failed to react in EDTA (the data shown for one sample in EDTA are representative of all the LCs). These binding curves provide evidence that recombinant light chains, in which residues have been replaced by cysteines, retain structural similarities to LC2 isolated from chicken myosin.

DTNB Titration—The presence of 3 cysteine residues in the mutant light chains was confirmed by DNA sequencing and by sulfhydryl analysis with the DTNB reagent. Incubation of light chains (10 μM) with varying concentrations of DTNB (2.5-80 μM) leads to the formation of TNB-mixed disulfides, as well as protein disulfide bonds. When the reaction of wild-type LC2 with DTNB reached a molar ratio of 1 mol of DTNB/mol of LC2, about 2 mol of TNB were released (Fig. 4, top). The mutant light chains required 2 mol of DTNB to reach a plateau of about 3 mol of TNB released, confirming that the mutant LCs have 3 cysteine residues/mol. Usually the number of moles of TNB released are equivalent to the moles of DTNB added at sub-saturating levels of reagent, unless inter- or intramolecular disulfide bonds are formed (Ellman, 1959). After reaction with NEM to irreversibly block any free thiols and thereby prevent disulfide interchange, these same samples were analyzed by nonreducing SDS-PAGE (Fig. 4, bottom).

The presence of a fast migrating electrophoretic species, which could be reduced by addition of DTT, confirmed that disulfide formation had occurred upon exposure of the light

\[ \text{Antibody binding to myosin was less dependent on divalent cations, although binding in EDTA was somewhat reduced relative to magnesium.} \]

\[ ^3 \text{L. D. Saraswat and S. Lowey, unpublished data.} \]
Cysteine Mutants of Myosin Light Chain

FIG. 4. DTNB titration of the recombinant light chains. Light chains (10 µM) were reacted with varying amounts of DTNB in 0.2 M Tris, pH 8.0, 2 mM EDTA at room temperature for 10 min, and the absorbance measured at 412 nm (top). The ratio of DTNB to light chain ranged from 0.22 to 6.0 for wild type (WT, □); 0.26 to 7.2 for Cys7 (○); 0.24 to 5.4 for Cys73 (○, △). Samples were blocked with NEM (3-5 mM) before addition of Laemmli gel buffer (minus β-mercaptoethanol), and analyzed on 15% SDS gels (bottom). Each lane corresponds to a point on the graph starting from left to right with increasing amounts of DTNB. The oxidized LC2 has a greater mobility compared with the reduced light chain. Some dimerized LC2 is visible near the top of the gels.

chains to DTNB. The major oxidized band in wild-type LC2 can be accounted for by a disulfide bond between Cys126 and Cys156, as shown for chicken LC2 by Katoh and Lowey (1989) and for rabbit LC2 by Huber et al. (1989). (The origin of the relatively minor band of intermediate mobility is not known.) The cysteine mutants all showed more than one fast migrating band, and below 1 mol of DTNB, interchain disulfides formed to varying degrees. As the concentration of DTNB increased, the higher molecular weight bands disappeared and the mobility of the fast migrating species changed. The chemical modification of unpaired thiols by bound TNB can account for the shift in band mobility with increasing DTNB, as well as the decrease in interchain disulfide formation. Although we had originally expected only the endogenous LC cysteines to form a disulfide bond, it became clear from these data that additional disulfide bonds could form between the engineered and native cysteines. In order to have a free thiol available for subsequent reaction with fluorescent probes, we turned to air oxidation as a means of forming disulfide bonds.

Air Oxidation—In the absence of a reducing agent, the light chain cysteines slowly oxidize to disulfide bonds. Addition of trace amounts of copper increased the rate of disulfide bond formation, which could be monitored by measuring the free thiols with DTNB. Analysis by nonreducing SDS-PAGE showed a pattern which was remarkably similar to that obtained by reaction with lower concentrations of DTNB (Fig. 5). In the case of the Cys7 mutant, a faster migrating doublet was observed as the free sulfhydryl concentration approached one; the slower of the two bands co-migrated with the single oxidized wild-type band. Multiple fast migrating bands, that were more difficult to resolve, appeared for the Cys73 mutant, and the major oxidized band for Cys94 did not co-migrate with the wild-type species. With increasing air oxidation, more interchain disulfide bonding took place, presumably via any unpaired cysteine residues. By reacting the light chains with a fluorescent sulfhydryl reagent at a time when approximately 1 mol of free thiol remained, we hoped to determine which disulfide bonds were formed in these oxidized species.

FIG. 5. Air oxidation of light chain cysteines. Fully reduced light chains (1 mg/ml) were oxidized at room temperature in PBS buffer for various lengths of time, and the extent of oxidation determined by DTNB titration of the remaining free thiols as indicated on the abscissa. Addition of 10 µM copper reduced the time to reach <1 mol of SH/mol of LC to less than 4 h. Samples were treated with NEM and analyzed by urea-PAGE under nonreducing conditions as described in Fig. 4. WT, wild type.
Cysteine Mutants of Myosin Light Chain

FIG. 6. Separation of IAF-labeled light chains by ion-exchange chromatography. Light chains (1 mg/ml) were treated with 210 μM 5-IAF in a completely reduced state for wild type (WT) or after partial oxidation for mutants (see details under “Materials and Methods”). The various IAF-labeled species (2–3 mg) were separated by FPLC using a Mono Q ion-exchange column, and the absorbance was monitored at 280 nm. Samples of unchromatographed starting material and peaks from the chromatograms were adjusted with a salt gradient to 1 M NaCl. The fluorescent gels were photographed before staining with Coomassie Blue. (An explanation of this anomalous elution position is given below.) Analysis of the labeled Cys' fractions by urea-PAGE showed that the first peak migrated as a relatively homogeneous single band with a slightly lower mobility than singly labeled wild type; the front of the second peak contained a band that co-migrated with singly labeled wild type, whereas the back of the peak contained primarily the doubly labeled species; the third peak contained small amounts of triply labeled light chain (Fig. 6, middle). The Coomassie Blue-stained gels are more indicative of relative protein concentrations than the fluorescent gels or the absorbance spectra, since the more highly labeled fractions will contribute greater fluorescence and absorbance signals per mol of protein. The Cys' and Cys' fractions (data not shown) each showed one major peak on the chromatogram (Fig. 6, bottom panel). Analysis of the Cys' and Cys' fractions (data not shown) by urea-PAGE showed unlabeled (peak 1), singly labeled (peak 2), and doubly labeled (peak 3) bands (Fig. 6, bottom), which co-migrated with the analogous bands from the wild-type fractionation.

A direct comparison of wild-type and mutant light chains, singly labeled with the fluorescein, is shown in Fig. 7A. It is clear that all light chains have the same electrophoretic mobility on urea gels with the exception of Cys'. The reduced mobility of Cys' is most likely due to a stretch of highly basic residues, Lys-Lys-Ala-Lys-Arg-Arg, adjacent to the NH2-terminal-labeled cysteine, which has the effect of neutralizing the negative charge on the fluorescein, and thereby slowing the migration of the light chain. (The charge effect can also account for the elution profile of Cys' in Fig. 6.) This sequence of basic residues is the binding site for myosin light chain kinase and therefore represents a unique region in the light chain. Besides enabling us to isolate a homogeneous-labeled light chain, this Lys-Arg-rich region is also readily cleaved by endoproteinase Arg-C, a proteolytic enzyme whose specificity is for basic sequences (Bertrand et al., 1989). Digestion of singly labeled Cys' with Arg-C released a small NH2-terminal peptide containing the fluorescent group, and left the remainder of the light chain intact and nonfluorescent, demonstrating that the label is restricted to a single site at the amino terminus (Fig. 7C). As a control, chicken LC2 labeled at Cys' showed a truncated LC2 band that retained the original fluorescence upon digestion with Arg-C (Fig. 7B).

Identification of Labeled Cysteines by Peptide Analysis—
Although it was possible to isolate Cys\(^2\) mutant labeled at a single site, we had no way of separating light chains which were mixtures of singly labeled light chains. For instance, if the Cys\(^23\) mutant formed two disulfide bonds between cysteines 126 and 155, and between cysteines 73 and 155, unpaired cysteines at positions 73 and 126, respectively, would be free to react with 5-IAF (see Fig. 9). We have seen that neither ion-exchange chromatography (Fig. 6) nor electrophoresis (Fig. 7) would be able to distinguish between light chains labeled at cysteines 73, 94, 126, or 155. We therefore digested the singly labeled proteins to peptides with chymotrypsin and separated the peptides by HPLC chromatography (Fig. 8). Each fluorescein-labeled recombinant LC gave a distinct chromatogram; for instance, wild-type LC labeled on cysteine 126 showed a unique peak with a retention time of 68 min, whereas digested Cys\(^2\) had a unique peak with a retention time of 40 min. It is not possible to prepare wild-type LC singly labeled at position 155, but doubly labeled wild-type LC showed a unique peak at about 53 min and a sharp peak at 50 min, in addition to the fluorescent peak previously assigned to cysteine 126. The mutant Cys\(^7\) had a unique peak at 60 min, as well as a peak ascribed to cysteine 126. Surprisingly, the Cys\(^94\) mutant showed mainly peaks previously assigned to cysteine 155, and no unique peak was observed. Other peaks in the chromatograms, many of them common to all the light chains, were not fluorescent when screened in a fluorometer. When the fluorescent peaks were run on urea gels, they gave fluorescent bands (Fig. 8C), which could be accounted for in the starting material before chromatography (Fig. 8B). On the basis of the analysis of fluorescent peptides described above, we deduced the principal disulfide bonds formed in the different mutant light chains (Fig. 9).

**DISCUSSION**

The high level expression of myosin regulatory light chain (LC2) in the T7 RNA polymerase/promoter system (Tabor et al., 1991) has provided a simple method to prepare cysteine mutants of the light chain for structural studies. Labeling with spectroscopic probes has in the past been largely restricted to the reactive thiol, SH1, on the heavy chain of the myosin head (reviewed by Cooke (1986)). More recently, however, the thiol of isolated LC2 have been reacted with spectroscopic probes, and the labeled light chains were exchanged into skinned muscle fibers (Shrimpton et al., 1990; Hambly et al., 1991). To date these spectroscopic studies have revealed no new conformational states beyond those seen in myosin.
rigor and relaxation. However, the labeling of LC2 has been limited to the native cysteines in the COOH terminus of the light chain, and additional sites need to be created along the length of the light chain before any firm conclusions can be drawn regarding the orientation of the myosin cross-bridge during force generation.

In order to provide new sites for spectroscopic experiments, we have prepared a series of light chain mutants in which cysteine residues have been inserted at several positions in the light chain sequence. The native light chain of skeletal muscle myosin has 2 endogenous cysteines located at positions 126 and 155, the latter being 12 amino acids removed from the COOH terminus. A third cysteine residue was added by mutating Pro, Ser, or Pro. Our initial strategy was to retain the endogenous cysteines, because we wished to have the option of introducing a second probe into LC2 for the purpose of measuring energy transfer between sites. Previous studies had shown that a disulfide bond can form between cysteines located at positions 126 and 155 (Katoh and Lowey, 1989; Huber et al., 1989), and therefore we believed it might be possible to specifically label the newly engineered thiol by first oxidizing the native ones. It is difficult to preferentially label a single thiol in the presence of others, unless it happens to be unusually reactive, such as the SH1 in the myosin heavy chain. This strategy proved to have limited success since we found that intramolecular disulfide bonds readily formed between the endogenous and new sulphydryls, even for cysteines as far apart in the primary structure as residues 2 and 155.

The first evidence that disulfide bonds could form between engineered and native cysteines came from the presence of several fast migrating electrophoretic bands in nonreducing SDS gels of oxidized light chains. In the case of the mutant Cys, the major band co-migrated with that of the oxidized wild-type LC, and a minor second band migrated with somewhat faster mobility. The two oxidized species could be separated from each other by ion-exchange chromatography, and labeling with IAF confirmed that the minor band was due to a disulfide bond between cysteine 2 and one of the COOH-terminal cysteines. Determination of disulfide bonding in the Cys and Cys mutants was accomplished by labeling the oxidized light chains with IAF and identifying the fluoro-

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**Fig. 9.** Schematic diagram showing intramolecular disulfide bond formation possible in wild-type and mutant light chains based on labeling studies. WT, wild type.

**Fig. 10.** A gallery of electron micrographs of antibody-myosin complexes. Native LC2 in myosin was replaced by wild-type and mutant light chains labeled with 1 fluorescein/mol, and the reconstituted myosin was incubated with anti-fluorescyl antibodies. The complex was shadowed with platinum, and the metal replica observed by electron microscopy. A, wild type labeled on cysteine 126; B, Cys mutant labeled at cysteine 2; C, Cys mutant is a mixture of light chains singly labeled at cysteine 73 or cysteine 126; D, Cys mutant singly labeled primarily at cysteine 155 (see text). Note that the antibody binds at the head/rod junction in all images regardless of the position of the label. The arrowhead points to the antibody. Bar, 50 nm.
The ease of disulfide bond formation in LC2 was unexpected, since a cysteine bond is constrained by steric considerations, and cysteine residues engineered into proteins are usually based on prior knowledge of the three-dimensional crystallographic structure of a protein. We do not believe that the recombinant light chains necessarily are more unfolded or denatured than the native LC2: the mutant light chains retain the valient metal-binding site as evidenced by their reactivity with conformation-dependent monoclonal antibodies (Fig. 3). Moreover, the light chains bind strongly to LC-deficient myosin (Fig. 10), and the mutants can compete with native light chains for heavy chain-binding sites in exchange experiments. A more likely explanation for multiple disulfide combinations, and cysteine residues engineered into proteins are restricted to epitopes located near the ends of different regions of the molecule.

There is a precedent for forming a disulfide bond between the NH₂- and COOH-terminal regions of a polypeptide chain in calcium-binding proteins. Site-directed mutagenesis of calmodulin was used to replace residues at positions 3 and 146 with cystein (Persechini and Kretsinger, 1988). Even though the α-carbons are 4 mm apart in the crystal structure, the cysteines were able to oxidize to a disulfide bond in solution, emphasizing the flexibility of the central helix in this protein. The extensive sequence homology between calmodulin, troponin C, and light chains (Béchet and Houadji, 1989; Collins, 1991), and similarities in hydrodynamic parameters (reviewed in Katoh and Lowey (1989)) provide a rationale for making structural comparisons between the light chains and calmodulin.

There exist no biochemical data at present to suggest that disulfide bond formation can occur when the light chain is bound to the myosin heavy chain (Huber et al., 1989). Electron microscopy mapping of the NH₂- and COOH-terminal regions of the bound light chain has shown, however, that these regions of LC2 probably lie in close proximity to each other (Katoh and Lowey, 1989). The ability to form a disulfide bond between residues 2 and 155 in the free light chain is therefore at least compatible with the microscopic observations. Previous mapping studies using monoclonal antibodies (Winkelmann and Lowey, 1986; Tokunaga et al., 1987) or polyclonal anti-fluorescein antibodies (Katoh and Lowey, 1989) have been restricted to epitopes located near the ends of LC2. Whereas this portion of the light chain has been localized to the head/rod junction of myosin, it remains to be shown where the intervening sequences of LC2 reside. A hairpin type structure has been proposed to reconcile the elongated hydrodynamic shape of light chains with the close proximity of their NH₂ and COOH termini (Tokunaga et al., 1987). In view of our finding that the fluorescein-labeled residues 2, 73, 126, and 155 are all located at the head/rod junction, it appears more likely that the light chain has a globular type structure that interacts with the heavy chain sequence joining the S1 head to the rod.

Recombinant DNA methods have been used to assign a light chain-2 binding site to a 30-amino acid sequence NH₂-terminal to a proline residue considered to be the junction between S1 and the rod (Mitchell et al., 1989). This approach complements earlier biochemical studies that showed S1 prepared by chymotryptic cleavage is missing ~30 COOH-terminal residues and cannot bind LC2 light chain. If indeed LC2 binds to this highly hydrophobic region, then our electron microscope mapping of LC2 would tend to place this sequence of the heavy chain at the head/rod junction. Recent electron microscopic and molecular biological studies on Acanthamoeba myosin led to the novel proposal that the conserved proline (~847) may be located in the head region of myosin rather than at the head/rod junction, which has always been the assumed location (Rimm et al., 1989). Although it is entirely conceivable that Acanthamoeba may have a somewhat different organization from vertebrate myosin, we do not favor extrapolating these conclusions to muscle myosin, but prefer to retain the conventional view of the myosin structure.

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REFERENCES

Baba, Y. S., Sack, J. S., Grodberg, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1986) Nature 319, 37–40
Béchet, J. J., and Houadji, M. (1989) Biochim. Biophys. Acta 966, 199–206
Bertrand, R., Derencourt, J., and Kassab, R. (1988) FEBS Lett. 246, 171–176
Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
Collins, J. H. (1976) Nature 269, 699–700
Collins, J. H. (1991) J. Mus. Res. Cell Motil. 12, 3–25
Cooke, R. (1986) CRC Crit. Rev. Biochem. 21, 53–118
Cooke, R., Crowder, M. S., Wendt, C. H., Barnett, V. A., and Thomas, D. D. (1984) in Contractile Mechanisms in Muscle (Pollack, G. H. and Sugih, H., eds) pp. 413–427, Plenum Publishing Corp., New York
Earhart, C. F., Lundrigan, M. Pickett, C. E., and Pierce, J. R. (1979) FEBS Microbiol. Lett. 6, 277–280
Elman, G. L. (1939) Arch. Biochem. Biophys. 20, 70–77
Grodberg, T. J., and Dunc, J. J. (1988) J. Bacteriol. 170, 1245–1253
Hambly, B., Franka, K., and Cocke, R. (1991) Biochem. J. 59, 127–138
Harrington, W. F. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5066–5070
Harrington, W. F., Karr, T., Buss, B. W., and Lovell, S. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7452–7456
Hersberg, D., and James, M. N. G. (1985) Nature 313, 653–659
Huber, P. A. J., Brunner, U. T., and Schaub, M. C. (1989) Biochemistry 28, 9116–9123
Huxley, H. E., and Kress, M. (1985) J. Muscle Res. Cell Motil. 6, 153–161
Huxley, H. E., Simmons, R. M., Farago, A. R., Kress, M., Bordas, J., and Koch, M. H. J. (1985) J. Mol. Biol. 189, 499–506
Katoh, T., and Lowey, S. J. (1989) J. Cell Biol. 109, 1549–1600
Laemmli, U. K. (1970) Nature 227, 680–685
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
March, D., and Lowey, S. (1989) Biochemistry 19, 774–784
Mitchell, E. J., Karn, J., Brown, D. M., Newman, A. J., and Kendrick-Jones, J. (1989) J. Mol. Biol. 208, 190–205
Pastra-Landis, S. C., and Lowey, S. (1986) J. Biol. Chem. 261, 14811–14816
Perrie, W. T., and Perry, S. V. (1979) Biochem. J. 119, 31–38
Persechini, A., and Kretsinger, R. H. (1988) J. Biol. Chem. 263, 12175–12178
Reinach, F. C., and Fischman, D. A. (1986) J. Biol. Chem. 261, 411–422
Rimm, D. L., Sinard, J. H., and Pollard, T. D. (1989) J. Cell Biol. 108, 1783–1789
Shrimpton, C., Sleep, J., and Irving, M. (1990) Biophys. J. 57, 545 (abstr.)
Tabor, S., and Richardson, C. C. (1983) Proc. Natl. Acad. Sci. U. S. A. 82, 1074–1078
Tokunaga, M., Suzuki, M., Saeki, K., and Wakabayashi, T. (1987) J. Mol. Biol. 194, 245–255
Toyoshima, Y. K., Yon, J. S., McNally, E. M., Mieblang, K. R., Toyoshima, C., and Spadich, J. A. (1987) Nature 326, 530–539
Trybus, K. M., and Lowey, S. (1988) J. Biol. Chem. 263, 16485–16492
Winkelmann, D. A., and Lowey, S. (1986) J. Mol. Biol. 186, 595–612
Winkelmann, D. A., Lowey, S., and Press, J. L. (1983) Cell 34, 295–306
Winkelmann, D. A., Mekel, H., and Raymond, I. (1985) J. Mol. Biol. 181, 487–501
Wolfe, V., Saraswat, L. D., and Lowey, S. (1991) Biophys. J. 59, 410 (abstr.)