Cysteine Mutants of Light Chain-2 Form Disulfide Bonds in Skeletal Muscle Myosin*

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Multiple disulfide bonds form in recombinant myosin light chain-2 mutants that contain an engineered cysteine at positions 2, 73, or 94, in addition to the endogenous cysteines at residues 126 and 155 (Saraswat, L. D., and Lowey, S. (1991) J. Biol. Chem. 266, 19777). By replacing one of the native cysteines with an alanine, mutants with a single pair of thiols were created: Cys<sup>2</sup>/Cys<sup>126</sup>, Cys<sup>73</sup>/Cys<sup>155</sup>, Cys<sup>126</sup>/Cys<sup>155</sup> and Cys<sup>2</sup>/Cys<sup>155</sup>. Oxidation of these mutants resulted in a fast migrating band on nonreducing SDS gels, which was attributed to an intramolecular disulfide bond. To determine if disulfide formation could also occur when the light chains (LC) are bound to the myosin heavy chains, LCs were added to myosin which had been depleted of its native LC2 by an immunoadsorbent. When the reconstituted myosin was reacted with 5,5'-dithiobis(2-nitrobenzoic acid) in the absence of divalent cations, intramolecular disulfide bonds formed in the mutant and wild type LCs, but the LCs did not remain bound to the myosin heavy chains. Addition of magnesium ions prevented LC dissociation, but intramolecular disulfide bonds no longer formed. Instead, mutants containing cysteines in the NH<sub>2</sub>-terminal domain formed intermolecular disulfide bonds between the two heads of myosin. The ability to cross-link the heads demonstrates the existence of close head/head interactions in the myosin molecule, a feature that may be essential for regulation.

All myosin IIs contain two classes of light chains: the so-called essential light chains (LC1/3) and the regulatory light chains (LC2) (reviewed in Lowey (1986)). A combination of biochemical data and immunoelectron microscopy has localized the light chains to the "neck region" of the myosin head (reviewed in Katoh and Lowey (1989)). More recently, cysteine residues have been engineered into chicken muscle myosin LC2 for the purpose of introducing specific probes into the myosin molecule (Saraswat and Lowey, 1991). By an electron microscopic approach it could be shown that the LC2 light chain is wrapped around the heavy chain at the head/rod junction (Saraswat et al., 1992), where it interacts with about 30 residues amino-terminal to the invariant proline (Mitchel et al., 1989). A flexible globular conformation for LC2 was inferred from the ability of cysteine residues widely separated in the primary structure to form multiple disulfide bonds under oxidizing conditions; specifically, cysteines introduced at positions 2, 73, or 94 by site-directed mutagenesis formed intramolecular bonds with native cysteines at residues 126 and 155 (Saraswat and Lowey, 1991).

Although a surprisingly large number of disulfides could be accommodated in the free light chain, it was not clear whether any disulfide bonds would form when the light chain was bound to the myosin heavy chain. Earlier studies by Huber et al. (1989) showed that the two endogenous cysteines in the carboxyl-terminal region of native LC2 were unable to form a disulfide bond on the myosin molecule in the presence of magnesium, even though the cysteines readily oxidized to an intramolecular bond in the isolated light chain under similar ionic conditions (see also Katoh and Lowey (1989)). We therefore wished to determine whether light chains with cysteines at other positions in the sequence could form disulfide bonds when bound to the heavy chain. This experimental approach is of importance insofar as it provides a means to assess the conformational flexibility of the light chain in the bound state. The functional role of LC2 light chain, particularly in the case of myosins that are regulated by phosphorylation or calcium binding (Adelstein and Eisenberg, 1980; Szent-Gyorgyi and Chantler, 1986) is generally assumed to involve a change in interactions between the light and heavy chains. There is little direct evidence at present, however, to support a model for conformational changes within the neck region of the myosin molecule. Here we show that cysteine mutants of LC2 can form intramolecular disulfide bonds in the presence of the heavy chain, provided EDTA is present. However, these disulfide-bonded light chains dissociate from the heavy chains. The addition of magnesium ions prevents intramolecular disulfide bond formation, but instead, intermolecular disulfide bonds now form between the engineered cysteines in the amino-terminal region of LC2. The resulting dimeric light chains remain tightly bound to myosin. The finding that the two heads of myosin can approach closely enough to form a disulfide cross-link is consistent with current hypotheses that head/head interactions are required for regulation of myosin activity.

MATERIALS AND METHODS

Construction of Expression Plasmids—The expression vector pT7-7 containing wild type (WT) and mutant cDNAs for the regulatory light chain (LC2) of chicken skeletal muscle myosin has been described previously. Mutagenesis was used to replace Pro<sup>2</sup>, Ser<sup>73</sup>, or Pro<sup>94</sup> with cysteine and thereby create mutant light chains with a third cysteine in addition to the two endogenous cysteines at positions 126 and 155 (Saraswat and Lowey, 1991). These cDNAs were used as starting material to generate light chains containing either a pair of cysteines, or a single cysteine residue, Table I. The oligonucleotides 5'-CTGAACACCAAAAGGGACACACCC-3' and 5'-GAACATTCGCTAGCTCATCACC-3' were used to replace cysteines 126 and 155, respectively, with alanine. An EcoRI/HindIII fragment containing the full length cDNA was ligated into Mi3 mp19, and site-directed mutagenesis was carried out using the in vitro Mutagenesis Kit from Amersham Corp. Mutant cDNAs were
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sequenced by the dideoxy method (Sequenase DNA Kit from United States Biochemical Corp.) and ligated into pT7-7 for expression. Procedures followed for DNA manipulations are described in Sambrook et al. (1989).

**Purification of Expressed Protein**—One liter cultures of BL21(DE3) containing a pT7-7/LC2 construct were grown 14–16 h at 37°C in enriched buffered media (2% Bacto-tryptone, 1% yeast extract, 0.2% glycerol, 50 mM potassium phosphate, pH 7.2, 0.5 mM ampicillin). Cells were pelleted at 8,000 × g for 10 min and washed once with 25 mM Tris, pH 8.0, 5 mM EDTA. After resuspension in lysis buffer (25 mM Tris, pH 8.0, 5 mM EDTA, 50 mM glucose, 0.2 mM MgSO4) and incubation on ice for 1 h, the cells were frozen in liquid nitrogen and allowed to thaw slowly (3 h). Magnesium chloride was added to 10 mM for DNAse treatment (5 μg/ml, 60 min on ice). Triton X-100 was added to 0.1%, and inclusion bodies which contained expressed LC2 were pelleted (15,000 × g, 15 min). The pellet was washed three times in lysis buffer/Triton excluding the lysozyme, after which a final wash was performed in the absence of detergent. The pellet was solubilized in 6 mM guanidine HCl in PBS (20 mM sodium phosphate, pH 7.0, 150 mM NaCl, 3 mM sodium azide) containing the lysozyme, after which a final wash was performed in the absence of detergent. The sample was applied to a hydroxylapatite column (1.5 × 10 cm) equilibrated in the same buffer lacking DTT as a cryoprotectant. Freeze-dried LC2 powder was stored at −20°C under desiccant.

**Protein Preparations**—Lyophilized purified recombinant LC2 was dissolved in phosphate-buffered, pH 7.2, 6 mM guanidine hydrochloride, incubated at room temperature for 1 h in the presence of 20 mM DTT, and dialyzed into denaturing conditions, 2-mercaptoethanol was omitted from the gel sample conditions. DTT was added to 10 mM as the fractions were collected. The LC2-deficient myosin, eluted in the void volume (4 ml of 0.2–0.4 mg/ml), was mixed with a 5-fold molar excess of purified recombinant LC2 in the presence of 10 mM MgCl2. After incubation on ice for 1 h, the reconstituted myosin was dialyzed against low salt buffer, centrifuged to collect the precipitate, and washed several times to remove unbound LC2. Reconstituted myosin pellets were then resuspended in 0.7 mM NaCl, 25 mM sodium phosphate, pH 7.5, 10 mM DTT and incubated on ice for 1 h. Aggregates were removed by centrifugation of the myosin in the Beckman TL-A at 45,000 × g for 15 min.

**Western Blotting**—Proteins were electrophoresed into nitrocellulose according to the method of Towbin and Staehelin (1979). The LCs were probed with specific antibodies and visualized by the IgG-horse radish peroxidase immunoblot assay kit from Bio-Rad.

**RESULTS**

**Characterization of LC2 Mutants**—A number of light chain mutants with 1 or 2 cysteine residues/chains were expressed in high yield in *Escherichia coli*, Table I. From a 1-liter culture, about 50 mg of protein was obtained after hydroxylapatite chromatography (see "Materials and Methods"). All light chains moved as single bands by SDS-PAGE, examples of which are shown in Figs. 1 and 2, indicating a high level of purity. The properties of single cysteine mutants were described previously in Saraswat et al. (1992). The double cysteine mutants prepared here, Cys5-Cys126, Cys2-Cys155, Cys73-Cys155, and Cys84/Cys155 readily formed intramolecular disulfide bonds in the absence of a reducing agent. Titration of the light chain with DTNB showed increasing amounts of a single

**TABLE I**

<table>
<thead>
<tr>
<th>Recombinant light chains</th>
<th>Position of cysteines</th>
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<tbody>
<tr>
<td>Wild type</td>
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</tr>
<tr>
<td>Cys2/Cys155</td>
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<tr>
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<tr>
<td>Cys126</td>
<td>P126C, C155A</td>
</tr>
<tr>
<td>Cys155</td>
<td>C126A, Cys155</td>
</tr>
</tbody>
</table>
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faster migrating species which plateaued at about 1 mol of DTNB/mol of light chain (Fig. 1). The liberation of 2 mol of TNB/mol of DTNB confirmed the presence of a disulfide bond (Ellman, 1959). No intermolecular disulfides were formed, as evidenced by the absence of higher molecular weight bands. An excess of NEM was added to each sample to irreversibly block any free thiols before analysis by nonreducing SDS gels. The reaction was unaffected by the substitution of magnesium for EDTA, and addition of DTT reverted the mobility of the faster migrating band to that of the reduced light chain.

In order to compare the relative rates of disulfide formation between the wild type light chain and the four double cysteine mutants, the reduced light chains were subjected to air oxidation, blocked with NEM at various time points, and the distribution of the reduced and oxidized species was analyzed by densitometry of nonreducing SDS gels (data not shown). We found that the rate of disulfide formation was similar for all the LC2 species. The unexpected ease of disulfide formation between cysteines widely separated in the primary structure raised the question of the structural integrity of the recombinant protein. In addition to replacing certain residues with cysteine or alanine (Table I) there are 5 additional residues (Ala, Arg, Ile, Pro, and Met) at the amino terminus of LC2 from myosin which had been depleted of its native LC2 by immunoaffinity chromatography (Fig. 2A). The ratio of the recombinant LC2 to the sum of LC1 plus LC3 was generally 1 as determined by gel densitometry. When occasionally less LC2 bound to the myosin heavy chain, it could be ascribed to a tendency of the LC2-deficient MHC to aggregate irreversibly (Pastra-Landis and Lowey, 1986). The double cysteine mutants were also able to compete with endogenous LC2 in exchange experiments at 0°C (to ensure that myosin pellets were washed free of unbound LC) and 40°C in the presence of EDTA and ATP. Recombinant LC2 has a slower mobility than native LC2 due to the presence of 5 additional residues at the NH2 terminus. All recombinant LCs exchanged to about 50% with the endogenous LC under these conditions.

It appears that the affinity of the mutants for the native myosin by Huber et al. (1989). Between 1- and 2-fold molar excess of DTNB over myosin was used in these experiments, since higher ratios of the thiol reagent led to extensive cross-linking of the heavy chain. In the presence of EDTA, intramolecular disulfide bonds were formed by wild type and all mutant LCs (Fig. 3A, lane 2, in each set of three). The oxidized light chain was released into the supernatant upon centrifugation and does not appear in the pelleted myosin (lane 3). Addition of Mg2+ prevented intramolecular disulfide bond formation in all the samples, but instead, a higher molecular weight band now appeared for the mutant light chains (Fig. 3B). This species migrated with a mobility indicative of a light chain dimer formed by intermolecular disulfide bonding. Unlike the intramolecularly cross-linked LC2, the dimer pelleted with the myosin (lane 3 of each set). Myosin reconstituted with wild type light chain did not show any disulfide bonding in magnesium, in agreement with the studies of native myosin by Huber et al. (1989).

The light chain dimer observed for myosin reconstituted with mutant LC2s could in principle be formed by disulfide bonding with the single cysteine of the essential light chain. In that case cross-linking could take place within a head rather than between heads. In order to determine the composition of the LC2-dimer, the gel samples were transferred to nitrocellulose and reacted with antibodies specific for LC2 and the essential light chains, LC1 and LC3 (Silverstein and Lowey, 1981). The immunoblots in Fig. 4 clearly show that the LC2 dimer is composed solely of LC2, and no heterodimers with essential light chains are formed.

**Effect of Salt**—The DTNB titrations were initially done in low salt (0.15 M NaCl) in order to compare our results on recombinant light chains with those of Huber et al. (1989) on native myosin. Furthermore, it was convenient to precipitate the DTNB-reacted myosin by centrifugation and determine whether the oxidized light chains remained bound to the heavy chain or were released into the supernatant. When we found that an LC2-dimer formed in the presence of Mg2+ however, the question arose as to whether intermolecular disulfide bonds might be forming between light chains on adjacent molecules in

**DTNB Titration of Reconstituted Myosin—Myosin reconstituted with wild type and recombinant LCs was reacted with DTNB in the presence or absence of divalent cations in 0.15 M NaCl (Fig. 3). Between 1- and 2-fold molar excess of DTNB over myosin was used in these experiments, since higher ratios of the thiol reagent led to extensive cross-linking of the heavy chain. In the presence of EDTA, intramolecular disulfide bonds were formed by wild type and all mutant LCs (Fig. 3A, lane 2, in each set of three). The oxidized light chain was released into the supernatant upon centrifugation and does not appear in the pelleted myosin (lane 3). Addition of Mg2+ prevented intramolecular disulfide bond formation in all the samples, but instead, a higher molecular weight band now appeared for the mutant light chains (Fig. 3B). This species migrated with a mobility indicative of a light chain dimer formed by intermolecular disulfide bonding. Unlike the intramolecularly cross-linked LC2, the dimer pelleted with the myosin (lane 3 of each set). Myosin reconstituted with wild type light chain did not show any disulfide bonding in magnesium, in agreement with the studies of native myosin by Huber et al. (1989).**

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the filamentous myosin. We repeated the DTNB reaction at higher salt concentrations (0.5 M NaCl), where myosin is monomeric, and obtained similar results (Fig. 5). In the absence of EDTA, wild type and mutant light chains formed predominantly intramolecular disulfide bonds. In the presence of Mg\(^{2+}\), a new band appeared at the LC2-dimer position for all the double cysteine recombinants, except wild type LC2. The light chains also underwent limited intramolecular oxidation in the presence of DTNB, probably due to a higher equilibrium concentration of free light chain in 0.5 M NaCl. Nevertheless, there was a clear difference in intramolecular disulfide bonding between the engineered cysteine LCs and wild type.

Identification of Cysteines in Disulfide Cross-link—In forming a cross-link between myosin heads containing light chains with 2 cysteines, the disulfide bond could involve 2 different cysteine residues; for example, Cys\(^2\) on one light chain could interact with Cys\(^{155}\) on the other regulatory light chain. In order to determine which residues comprised the bond, LC2 mutants containing a single cysteine residue were used to prepare reconstituted myosin. Reaction of these myosins with DTNB in the presence of Mg\(^{2+}\) resulted in the formation of a dimer band by Cys\(^2\) and Cys\(^{155}\). No higher molecular weight bands were formed by C\(^{126}\) or C\(^{155}\), consistent with the finding that wild type does not form an intermolecular disulfide bond (Fig. 6). It is therefore likely that the LC2-dimer observed for C\(^{94}\)/Cys\(^{155}\), C\(^{73}\)/Cys\(^{155}\), and C\(^{94}\)/Cys\(^{155}\) is due to bonding between homologous NH\(_2\)-terminal cysteines. The only questionable dimeric bond is that formed by C\(^{94}\)/Cys\(^{155}\), since the single Cys\(^{94}\) mutant did not form a light chain dimer (data not shown). Conceivably, Cys\(^{94}\) may bond to Cys\(^{155}\), but that re-
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**Fig. 6. Myosin reconstituted with single cysteine LCs.** Myosin containing LCs with a single cysteine at position 2, 73, 94, 126, or 155 was reacted with a 2-fold molar excess of DTNB in 0.5 M NaCl and magnesium. Each set of two gels shows myosin before (-) and after (+) addition of DTNB. Samples were analyzed on 12.5% nonreducing SDS gels. Only Cys\(^2\) and Cys\(^73\) formed intermolecular disulfide bonds (arrowheads); Cys\(^{126}\), Cys\(^{155}\), and Cys\(^{94}\) (data not shown) did not form higher molecular weight species.

mains to be shown. The position of cysteine 94 is probably near the region linking the NH\(_2\)- and COOH-terminal domains of the light chain, and any slight perturbations in the structure caused by mutagenesis, such as deletion of Cys\(^{155}\), might affect the interaction.

**DISCUSSION**

**Free Light Chains**—The thiol reagent, DTNB, has been used to probe the reactivity of cysteine residues introduced into myosin light chain-2 (LC2) by site-directed mutagenesis. The unusual conformational flexibility of the free light chain was evidenced by the formation of disulfide bonds in the Cys\(^2\)/Cys\(^{155}\), Cys\(^9\)/Cys\(^{126}\), Cys\(^{73}\)/Cys\(^{155}\), and Cys\(^{94}\)/Cys\(^{155}\) mutants, in which the thiols are distant in the primary structure. The ability of the endogenous cysteines, Cys\(^{126}\) and Cys\(^{155}\), in native LC2 to form a disulfide bond had been noted earlier (Huber et al., 1989; Katoh and Lowey, 1989). The unexpected reactivity of the engineered thiols at the extreme ends of the NH\(_2\)- and COOH-terminal domains of the light chain is reminiscent of the disulfide bridge formed between the lobes of a calmodulin molecule containing engineered cysteines at positions 3 and 146 (Persechini and Kretsinger, 1988). The homology in sequence and similarity in hydrodynamic properties to calmodulin has long been noted (Collins, 1991). The close relationship has been emphasized by the recent finding that multiple calmodulin molecules are substituted for light chains in the so-called "neck" region of unconventional myosins (Cheney and Mooseker, 1992).

**Nativ Myosin**—Thiol-disulfide exchange reactions offer a relatively simple, direct means to map which regions of a protein are in close proximity. This approach has been used to ask whether the light chain, when bound to the heavy chain, will display the same flexibility as observed in the free state. Huber et al. (1989) first showed that in native myosin at physiological salt concentrations, DTNB could induce an intramolecular disulfide bond in the regulatory light chain, provided EDTA was present. We confirmed this observation and showed, in addition, that the cross-linked light chain no longer remained associated with the heavy chain. When light chains were first isolated from myosin in the 1970s, DTNB was shown to dissociate one class of light chains without significant loss of ATPase activity; hence the name DTNB-light chain (Gazith et al., 1970; Lowey and Risby, 1971). It is now clear that one of the causes for subunit dissociation was probably the oxidation of the 2 cysteines in the light chain. When the DTNB reaction with myosin was carried out in excess magnesium ions, however, DTNB formed a mixed disulfide with the protein thiols to yield a thiophenylated light chain, and no intramolecular disulfide bond was formed. It was concluded that the binding of divalent metal cations in the NH\(_2\)-terminus induces a conformational change in the COOH-terminal region of the light chain that affected the reactivity of its thiol residues (Huber et al., 1989).

The other reactive cysteine in the myosin head is the well characterized SH1 on the heavy chain; SH1 and the light chain thiols are among the most reactive cysteines in myosin. If the remaining thiols are not blocked with NEM, or if an excessive amount of DTNB is used, extensive disulfide interchange can occur between both the heavy chains and the light chains leading to highly cross-linked species which will no longer enter SDS-gels (Huber et al., 1989).

**Reconstituted Myosin**—Probing the recombiant light chains in myosin becomes more difficult insofar as it is necessary to prepare a myosin reconstituted with the desired light chain. This can be accomplished by either exchanging myosin with a molar excess of the recombinants or by removing the native light chain before addition of the mutants. The first method suffers from the disadvantage of requiring large amounts of mutant light chain, but more important, due to the reduced mobility of the recombinant light chain, its oxidized form comigrates with the native light chain on SDS gels. To avoid these ambiguities, it was decided to remove the endogenous light chains by affinity chromatography. The latter procedure is not without drawbacks, however, since antibody columns have a relatively low capacity, which diminishes even further with usage. Nevertheless, we were able to prepare small amounts of myosin containing cysteine residues at various positions in the structure.

What can these myosins reconstituted from the double cysteine mutants: Cys\(^2\)/Cys\(^{155}\), Cys\(^9\)/Cys\(^{126}\), Cys\(^{73}\)/Cys\(^{155}\), and Cys\(^{94}\)/Cys\(^{155}\) tell us about light chain interactions in the myosin molecule? It would appear that in the absence of divalent cations the two domains of the light chain can come into sufficiently close contact to form a number of intramolecular disulfide bonds. It is not clear, however, whether these bonds are formed in the free or bound state of the light chain. We know that appreciable exchange of excess light chains with myosin does not occur until about 37–46 °C, but assuming that a finite concentration of free light chains is at equilibrium with myosin at room temperature, it is quite likely that some intramolecular cross-linking occurs in the free state. But irrespective of whether these disulfide bonds are formed in the free or bound state, the cross-linked monomeric LC2 cannot remain bound to the heavy chain and dissociates.

In the presence of divalent cations such as magnesium or calcium, the regulatory light chain is more tightly bound to the heavy chain, and no exchange of LCs occurs, even at elevated temperatures. The absence of intramolecular disulfide bonds at physiological salt concentrations among LC2 mutants and wild type can be most readily explained by postulating a reduction in the concentration of free light chain in equilibrium with bound LC2. Alternatively, divalent cations may promote the movement of LC2 domains away from each other in the bound state and thereby prevent intramolecular disulfide bond formation.

The most surprising result was the ease with which disulfide bonds formed between the regulatory light chains of the two heads of myosin. By using light chains containing a single cysteine at either position 2 or 73 in the sequence, it could be shown that the NH\(_2\)-terminal domains of LC2 were in close enough proximity to form intramolecular disulfide bonds. In contrast, cysteines in the COOH-terminal region at positions...
126 and 155 were unable to form intermolecular disulfide bonds. Presumably this region of the light chain contains the major binding site for the heavy chain, whereas the NH2-terminal portion of LC2 has a wider range of potential interactions. The absence of intermolecular disulfide bonding in wild type and in the mutants, Cys126 and Cys155, also serves as an important control to show that the LC2 dimers did not arise adventitiously through any thiol/disulfide interchange in SDS, despite the addition of NEM.

Functional Importance—Calcium regulation of contraction in vertebrate striated muscle is primarily associated with the thin filament, whereas in invertebrate striated and vertebrate smooth muscles, regulation is inherent in the myosin molecule of the thick filament. Both myosin-regulated systems involve the LC2 light chain, although activation is achieved by direct binding of Ca2+ to myosin in the case of invertebrates, and by calcium activation of a kinase that phosphorylates LC2 in vertebrate striated muscle myosin. Although the regulatory light chains on the two heads of scallop myosin are less than 9 Å apart (Hardwicke and Szent-Gyorgyi, 1985), further evidence for the proximity of the regulatory LCs in scallop myosin was obtained by cross-linking the heads through thiol-specific probes attached to Cys55 of the two Mercenaria light chains (Chantler and Bower, 1988; Bower et al., 1992).

Here we show that proximity of regulatory light chains is not restricted to thick-filament-regulated myosins, but also occurs in vertebrate striated muscle myosin. Although the regulatory role of LC2 in the latter case is less well understood, there is evidence that the rate of force development in skeletal muscles is mediated in part by LC2 (Metzger and Moss, 1992). Moreover, by means of LC2 mutants containing single cysteines, we are able to demonstrate for the first time that the NH2-terminal regions can approach each other closely enough to form a disulfide bond between the light chains. We have shown previously by immunoelectron microscopy that the cysteines in these LC2 mutants are localized at the head/rod junction (Saraswat et al., 1992), but the resolution of this technique was not sufficient to determine whether the LC2 light chains on the two heads can actually interact. The fact that S1 is not regulated, and that unconventional vertebrate myosins consisting of a single head use calmodulin instead of light chains, leads to the speculation that light chains may have evolved to mediate head/head interactions that are perhaps a necessary component of the mechanism that regulates the activity of conventional myosin.

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