Resonance energy transfer was used to measure the distances between pairs of cysteines, Cys2 and Cys155, and Cys73 and Cys155, in recombinant chicken skeletal myosin regulatory light chains in the free and bound states. The fluorescent and nonfluorescent probes N-io-doacetyl-N'-(5-sulfo-1-naphthyl)ethylenediamine and N-(4-dimethylamino-3,5-dinitrophenyl)maleimide were used as the donor and the acceptor, respectively. The distance between Cys2 and Cys155 was measured to be 35 and 30 Å in the absence and presence of myosin heavy chain, respectively, suggesting a slightly more compact structure for the light chain in the bound state. The distance between Cys73 and Cys155 measured in a similar manner was 31 and 30 Å in the free and bound states, respectively; this latter value is in good agreement with that derived from crystallographic structures. For heavy chain-bound light chains, no measurable distance changes were detected with the binding of ATP or actin. These results show that no gross structural changes occur within the regulatory light chain during the contraction cycle, but that resonance energy transfer between other sites could be used to monitor potential changes in the myosin head upon the binding of nucleotides and actin.

Force generation in muscle results from a cyclic interaction of the myosin head (S1) with actin. It is currently believed that S1 may change its orientation relative to the actin filament during this process and thereby produce a displacement of ~50–100 Å, the so-called "working stroke" (Huxley, 1969; Huxley and Simmons, 1971). A persistent problem with the "rotating cross-bridge model" has been the failure to detect any large conformational change in the head by most biophysical methods. It has been suggested that the reactive Cys155 on the heavy chain, the site of labeling for most spectroscopic probes, may not be particularly sensitive to angular changes, and the portion of S1 adjacent to the rod might undergo more pronounced structural changes during hydrolysis (Huxley and Kress, 1985). We have therefore focused our efforts over the last few years on preparing Cys mutants of the regulatory light chain (RLC), a subunit which is readily exchanged into myosin, and can therefore provide a means to introduce probes into the COOH-terminal region of the myosin head (Saraswat and Lowey, 1991; Saraswat et al., 1992). Moreover, this approach avoids modification of the reactive heavy chain Cys residues, SH1 and SH2, which can have adverse effects on the enzymatic activity and motor properties of myosin (Root and Reisler, 1992).

Several earlier studies have used labeled light chains to detect structural changes in the myosin head. By incorporating a 1,5-IAEDANS-labeled RLC into smooth muscle myosin, Morita et al. (1991) were able to show by fluorescence anisotropy that a change in conformation at the head/rod junction can be correlated with the transition from a folded to an extended myosin molecule. Hamblpy et al. (1991) exchanged a spin-labeled light chain into skinned skeletal muscle fibers and showed, using electron paramagnetic resonance, that the region of the head/rod junction in thick filaments is ordered in rigor and disordered in relaxation. In a related study, chicken gizzard light chains were labeled with acetamido-tetramethylrhodamine and exchanged into skeletal muscle fibers for the determination of fluorescence polarization ratios, an approach that may reveal the orientation of the myosin head in contracting fibers (Allen et al., 1994).

Fluorescence resonance energy transfer (RET) has been used to determine distances between sites that can readily be labeled, sites such as single Cys residues on the light chains, the active site in the myosin head, and the fast-reacting thiols on the heavy chain and actin (reviewed in dos Remedios et al., 1987)). The advent of genetic engineering has made it possible to introduce reactive residues at any position in the sequence, and one need only know the three-dimensional structure to determine which sites will be most informative for spectroscopic studies.
scopic studies. The work described here was begun before the crystal structure of S1 was reported (Raymont et al., 1993) and, therefore, the selection of sites for labeling was influenced by the homology between RLC and calmodulin (reviewed in Kato and Lowey (1989)). It was reasoned that probes widely spaced in the structure would offer the best opportunity for observing putative conformational changes in the neck region of S1 upon the binding of ligands (Saraswat and Lowey, 1991). Although such structural changes have yet to be found, we have shown the feasibility of this approach: the distance between Cys$^2$ and Cys$^{155}$ measured by RET in this study agrees remarkably well with the distance obtained from the crystal structure. Unexpectedly, Cys$^2$ cannot be seen in the S1 crystal structure, since the first 18 amino acids of RLC are missing in the electron density map. The RET-determined distance between Cys$^2$ and Cys$^{155}$, therefore, offers the only means at present to predict the approximate location of the NH$_2$ terminus of the RLC.

Since the atomic coordinates of S1 are now available, it will be possible in future studies to select the most favorable sites for probing the orientation and conformation of the light chain region of the myosin molecule.

MATERIALS AND METHODS

Protein Preparations—Wild type chicken skeletal RLC contains two endogenous Cys residues at positions 126 and 155. By the selective removal of endogenous Cys residues and introduction of exogenous Cys residues we have prepared the following mutant RLCs: C2, C73, C155, and C2/C155, and C73/C155, where the first three contain a single Cys at positions 2, 73 and 155, respectively, and the last two contain a pair of Cys residues at positions 2 and 155 and 73 and 155, respectively (Wolff-Leg et al., 1993). Lyophilized purified RLCs were routinely prepared from adult chicken muscle as described in Margossian and Lowey (1992). The protein concentrations were determined using a conventional RLC exchange at elevated temperatures, the procedure described above ensures that all myosin molecules contained a full complement of recombinant RLCs.

Light Chain Exchange—Myosin (0.5 mg/ml) and labeled RLC (0.2 mg/ml) in 0.6 M NaCl, 20 mM sodium phosphate, pH 7.5, 3 mM sodium azide, 5 mM EDTA, 1 mM EGTA, 5 mM ATP, and 5 mM DTT were incubated at 40°C for 30 min. MgCl$_2$ was added to 20 mM and incubation was continued for 90 min. Myosin samples were prepared by dialysis against 40 mM NaCl, 5 mM Pipes, pH 6.5, 5 mM MgCl$_2$, 3 mM sodium azide, 1 mM DTT and pelleted in a Beckman TLA-100 centrifuge. Pellets were washed, resuspended in 0.7 M NaCl, 20 mM sodium phosphate, pH 7.5, 3 mM sodium azide, 5 mM DTT and incubated on ice for 1 h. The reconstituted myosin was dialyzed into an appropriate buffer, and any aggregates were removed by centrifugation at 45,000 $\times$ g for 20 min at 4°C prior to fluorescence measurements.

Fluorescence Measurements—Fluorescence decay measurements were carried out on a modified ORTEC 9200 nanosecond fluorimeter following the procedure described in Tao and Cho (1979). Briefly, the time profiles of the excitation source and of the fluorescence decay were collected from a Lodox colloidal silica suspension and from the labeled RLC samples, respectively. The curves were analyzed by the method-of-moments (Isenberg and Dyson, 1969) utilizing 1, 2, or 3 exponentials, from which the fluorescence lifetimes were obtained. Polarization anisotropy decay measurements were carried out as described in Tao (1978). Steady-state polarization ratios ($R_P = I/I_L$) were obtained on a Perkin Elmer MFP-4 spectrofluorimeter.

Proteolysis of Light Chain and Myosin—Arg-C (Boehringer Mannheim, 10 units/mg RLC) or PMSF (phenylmethanesulfonyl fluoride, Boehringer Mannheim) or PMSF (phenylmethanesulfonyl fluoride, Boehringer Mannheim) were then added to terminate enzymatic activity.

In Vitro Motility Assay—This was performed essentially as described in Warshaw et al. (1990). Myosin free of all light chains was prepared as in Lowey et al. (1993). The heavy chain was reconstituted with purified alkali light chains and labeled or unlabeled recombinant RLCs. Unlike conventional RLC exchange at elevated temperatures, the procedure described above ensures that all myosin molecules contained a full complement of recombinant RLCs.

RESULTS

Reaction of RLC Mutants with the 1,5-IAEDANS Donor and the DDPM Acceptor—From the time course of the reaction between the single Cys mutants C2, C73, and C155 and 1,5-IAEDANS, it is evident that Cys$^2$ is more reactive than either Cys$^73$ or Cys$^{155}$ (Fig. 1). A fluorescent band was not observed until 5 min of incubation for C73 and C155, whereas C2 and C2/C155 showed fluorescence as early as 1 min. We took advantage of this preferential reactivity in the preparation of C2/C155 by selecting reaction conditions which favored completion of the DDPM donor and DDPM acceptor over the DDPM donor and 1,5-IAEDANS acceptor.

All reactions were performed in the dark and terminated by the addition of DTT to a final concentration of 50 mM. All of the DDPM-labeled proteins were dialyzed for 15 min in a microcentrifuge before dialysis. 1,5-IAEDANS (40 mM stock in 50 mM sodium phosphate, pH 7.8) and DDPM (40 mM stock in N,N-dimethylformamide) were freshly prepared and filtered before each reaction.

Determination of Extinction Coefficients—The single Cys mutant, C155, was labeled to completion with either 1,5-IAEDANS or DDPM. Excess label was removed by exhaustive dialysis followed by passage through a 5-mL Sephadex G-50 column. The extinction coefficient was determined using the Bradford assay (Bradford, 1976). Since the light chain was totally labeled, as shown by urea-PAGE, the concentration of label equaled that of the protein. The absorbance of 1,5-IAEDANS or DDPM bound to RLC was determined spectrophotometrically at 337 and 442 nm. The extinction coefficient determined for DAN at 337 nm was 14,500 M$^{-1}$ cm$^{-1}$, similar to the value reported in Hudson and Weber (1973). 1,5-IAEDANS does not absorb at 442 nm and therefore does not interfere with optical density measurements at that wavelength. The extinction coefficient determined for DDPM was 2930 M$^{-1}$ cm$^{-1}$ at 442 nm, in good agreement with the value reported by Gold and Segal (1964), and 3100 M$^{-1}$ cm$^{-1}$ at 337 nm. Using these extinction coefficients and the Bradford protein determination assay, the stoichiometry of labeling was determined for each of the singly and doubly labeled RLC mutants.

Light Chain Exchange—Myosin (0.5 mg/ml) and labeled RLC (0.2 mg/ml) in 0.6 M NaCl, 20 mM sodium phosphate, pH 7.5, 3 mM sodium azide, 5 mM EDTA, 1 mM EGTA, 5 mM ATP, and 5 mM DTT were incubated at 40°C for 30 min. MgCl$_2$ was added to 20 mM and incubation was continued for 90 min. Myosin samples were prepared by dialysis against 40 mM NaCl, 5 mM Pipes, pH 6.5, 5 mM MgCl$_2$, 3 mM sodium azide, 1 mM DTT and pelleted in a Beckman TLA-100 centrifuge. Pellets were washed, resuspended in 0.7 M NaCl, 20 mM sodium phosphate, pH 7.5, 3 mM sodium azide, 5 mM DTT and incubated on ice for 1 h. The reconstituted myosin was dialyzed into an appropriate buffer, and any aggregates were removed by centrifugation at 45,000 $\times$ g for 20 min at 4°C prior to fluorescence measurements.

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In Vitro Motility Assay—This was performed essentially as described in Warshaw et al. (1990). Myosin free of all light chains was prepared as in Lowey et al. (1993). The heavy chain was reconstituted with purified alkali light chains and labeled or unlabeled recombinant RLCs. Unlike conventional RLC exchange at elevated temperatures, the procedure described above ensures that all myosin molecules contained a full complement of recombinant RLCs.
Cys2, thereby producing a small amino-terminal peptide, and Arg-C, which cleaves in the highly basic region adjacent to Cys155. Analysis by urea-PAGE indicated that peak 1 contained exclusively C2DAN/C155 and Arg-C cleaved C2DAN/C155DDP, with the stoichiometry of labeling, DAN:RLC, was 2.0, compared with the value of 0.95 obtained for peak 1. The mixture of singly labeled species, designated as [C73/C155]DAN, was subsequently reacted with the acceptor DDPM, yielding [C73/C155]DAN,DDP. The DDP:RLC ratio of this species was ∼1.2.

Effect of Probes on In Vitro Motility—Myosin reconstituted with labeled mutant RLCs (C2DAN, C73DAN, C155DAN and C2DAN/C155DDP) translocated actin filaments at approximately the same velocity as myosin reconstituted with wild type RLC (∼3 μm/s). Therefore, probes introduced into myosin by means of the mutant light chain do not affect the functional properties of this mechano-enzyme. It should be noted that for the motility assays, light chains were added to myosin heavy chains devoid of any endogenous light chains. This is in contrast to the fluorescence measurements, for which the labeled light chains were exchanged into native myosin at elevated temperatures (see "Materials and Methods"). Since the actin filaments are translocated by a large number of myosin molecules in the motility assay, it is important to have a homogeneous myosin population with regard to the light chain content.

RET from Cys2 to Cys155 in Free and Heavy Chain-bound Mutant RLC—The fluorescence decay of free C2DAN/C155 was measured and found to be essentially mono-exponential; bi-exponential method-of-moments analysis yielded a major decay component with a fractional amplitude of 0.92 and a lifetime of 13.7 ns (Fig. 4A, Table I). Similarly the fluorescence decay of free C2DAN/C155DDP has a major decay component of fractional amplitude 0.95 with a quenched lifetime of 11.3 ns owing to RET from the Cys2 to Cys155 in Free and Heavy Chain-bound Mutant RLC.

When bound to MHC the fluorescence lifetime of C2DAN/C155 increased from 13.7 to 15.5 ns, indicating that the environment surrounding Cys2 becomes more hydrophobic in the presence of heavy chain (Fig. 4B, Table I). In spite of this increase in τb, the lifetime of MHC-bound C2DAN/C155DDP was quenched to 9.4 ns, ∼2 ns shorter than the lifetime of free C2DAN/C155DDP, indicating a small increase in interprobe energy transfer upon binding to MHC. Correspondingly, the calculated transfer efficiency increased from 17 to 39%, and the
separation distance decreased from 35 to 30 Å for free versus MHC-bound C2DA/DAN/155DDP (Table II). A repetition of these measurements using a different preparation of light chains yielded distances of 34 and 30 Å for free and MHC-bound light chains, respectively (Table II). Experiments performed to measure this distance for MHC-bound C2DA/DAN/155DDP in the presence of 5 or 10 mM ATP yielded distances of ~31 Å for the two preparations of light chains. Measurements of this distance in the presence of equimolar actin (absence of ATP) likewise yielded values (30–31 Å), that are indistinguishable from those for unbound myosin (Table II).

RET from Cys155 to Cys2 in Free and Heavy Chain-bound Mutant RLC—Owing to the low reactivity of Cys155 relative to Cys2, it was not possible to prepare and isolate C2/C155DDP. Instead, the fluorescence characteristics of the DAN moiety attached at Cys155 were determined using the labeled single Cys mutant C155DDP. The fluorescence decay characteristics of free and MHC-bound C155DDP and C2DDP/C155DDP are qualitatively very similar to those of free and bound C2/C155 and C2/C155DDP. Quantitatively, the major component lifetimes of free C155DDP and C2DDP/C155DDP are, respectively, 16.2 and 10.1 ns (Table I), yielding a transfer efficiency of 38% and a separation distance of 30 Å (Table II). The major component lifetime of C155DDP increased from 16.2 to 18.8 ns while that of C2DDP/C155DDP decreased from 10.1 to 8.4 ns upon binding to MHC. The transfer efficiency and interprobe distance for MHC-bound C2DDP/C155DDP were calculated from these lifetimes to be 55% and 27 Å, respectively. These results show that the RET-measured distance between residues 2 and 155 does not vary significantly when the donor-acceptor locations are switched. In both cases, a small decrease in this distance was observed upon binding of the RLC to MHC. An experiment performed to measure the distance between Cys155 and Cys2 in MHC-bound C2DDP/C155DDP in the presence of actin yielded a value of 28 Å (data not shown), a value that is virtually identical to that obtained in the absence of actin.

RET between Cys73 and Cys155 in Free and Heavy Chain-bound Mutant RLC—In spite of the fact that the location of the DAN-donor is randomly distributed between residues 73 and 155 in [C73/C155]DAN, its fluorescence decay is mono-exponential with a lifetime of 16.9 ns (Table I), indicating that the environments around each of the residues, 73 and 155, in free RLC is similar. The distance between the two residues can be estimated by assuming that the interprobe distance and the energy transfer efficiency is invariant with donor-acceptor location. The fluorescence decay of free [C73/C155]DAN/DDP is nearly mono-exponential, with a major component fractional amplitudes of 0.91 and a lifetime of 11.2 ns. There is no indication of the presence of additional quenched components that might correspond to heterogeneous transfer rates arising from the two donor-acceptor locations. Taking 16.9 and 11.2 ns as τD and τAR, respectively, a transfer efficiency of 34% and a separation distance of 31 Å were obtained from these lifetimes (Table II).
The fluorescence lifetime of [C73/C155]DAN increased from 16.9 to 18.7 ns upon binding to MHC, indicating a decrease in polarity around these sites (Table I). The fluorescence decay of MHC-bound [C73/C155]DAN/DDP is also nearly mono-exponential with a major component lifetime of 10.6 ns (Table I). Taking 18.7 and 10.6 ns as \( \tau_{\text{d}} \) and \( \tau_{\text{da}} \), respectively, a transfer efficiency of 43% and a separation distance of 30 Å were obtained (Table II), representing a negligible difference in the distance for free versus MHC-bound light chain. Measurements were again made in the presence of 5 and 10 mM ATP, and in the presence of actin, yielding values (29–30 Å) that are indistinguishable within experimental error to those for ligand-free myosin (Table II).

Fluorescence Polarization Measurements of DAN-labeled Mutant RLCs—The fluorescence polarization anisotropy of free C2DAN/C155 decays rapidly (Fig. 5) with a rotational correlation time of \(~5\) ns, consistent with the relatively small molecular volume of RLC. In the presence of MHC the anisotropy

### Table I

| Sample                      | \( A_1 \) | \( \tau_1 \) | \( A_2 \) | \( \tau_2 \) | \( \chi^2/n \) |
|-----------------------------|-----------|-------------|-----------|-------------|---------------|
| C2DAN/C155, free            | 1.0       | 14.72       | 0.078     | 22.07       | 1.9           |
| C2DAN/C155, free            | 0.922     | 13.72       | 0.051     | 21.14       | 1.5           |
| C2DAN/C155 + MHC            | 1.0       | 16.72       | 0.073     | 26.19       | 1.1           |
| C2DAN/C155 + MHC            | 0.927     | 15.48       | 0.340     | 15.58       | 7.2           |
| C15DAN, free                | 1.0       | 12.24       | 0.006     | 43.24       | 1.6           |
| C2DAN/C155, free            | 0.994     | 16.20       | 0.002     | 45.95       | 2.1           |
| C2DAN/C155 + MHC            | 1.0       | 18.92       | 0.002     | 45.95       | 2.1           |
| C2DAN/C155 + MHC            | 0.998     | 18.81       | 0.002     | 45.95       | 2.1           |
| C2DAN/C155 + MHC            | 1.0       | 14.15       | 0.458     | 17.43       | 1.6           |
| [C73/C155]DAN, free         | 1.0       | 13.44       | 0.281     | 18.23       | 1.2           |
| [C73/C155]DAN + MHC         | 1.0       | 16.92       | 0.002     | 45.95       | 2.1           |
| [C73/C155]DAN + MHC         | 0.954     | 18.81       | 0.002     | 45.95       | 2.1           |
| [C73/C155]DAN + MHC         | 1.0       | 14.15       | 0.458     | 17.43       | 1.6           |
| [C73/C155]DAN + MHC         | 0.915     | 11.21       | 0.005     | 21.15       | 4.1           |
| [C73/C155]DAN + MHC         | 1.0       | 18.69       | 0.005     | 21.15       | 4.1           |
| [C73/C155]DAN + MHC         | 1.0       | 18.69       | 0.005     | 21.15       | 4.1           |
| [C73/C155]DAN + MHC         | 1.0       | 12.46       | 0.005     | 21.15       | 4.1           |

### Table II

Energy transfer parameters

\( \tau_{\text{d}} \) and \( \tau_{\text{da}} \) are donor fluorescence lifetimes in the absence and presence of acceptor, respectively, \( E \) is the transfer efficiency, \( E = (1 - \tau_{\text{d}}/\tau_{\text{da}}) \), \( R_g \) is the critical transfer distance, calculated based on the orientation factor \( k^2 \) taking on the isotropically averaged value of 2/3. \( R_g \) was corrected for variations in donor quantum yields using the equation \( R_g = R_g^0 (\tau_{\text{d}}/\tau_{\text{d}})^{1/6} \), where the values of \( R_g^0 \) and \( \tau_{\text{d}} \) were reported to be 29.0 Å and 20.6 ns, respectively, by Dalbey et al. (1983). \( R \) is the donor-acceptor separation distance, \( R = R_g (E^{-1} - 1)^{1/6} \).

| Sample                       | \( \tau_d \) | \( \tau_{\text{da}} \) | \( E \) | \( R_g \) | \( R \) |
|------------------------------|-------------|-----------------|------|----------|------|
| C2DAN/C155, free (1)         | 13.72       | 11.34           | 0.173| 27.1     | 35.2 |
| C2DAN/C155, free (2)         | 15.13       | 11.58           | 0.215| 27.6     | 34.7 |
| C2DAN/C155 + MHC (1)         | 15.48       | 9.40            | 0.393| 27.7     | 29.7 |
| C2DAN/C155 + MHC (2)         | 17.13       | 10.16           | 0.407| 28.1     | 30.0 |
| C2DAN/C155 + MHC + 10 mM ATP (1) | 16.41   | 10.54           | 0.358| 27.9     | 30.8 |
| C2DAN/C155 + MHC + 5 mM ATP (2) | 17.18   | 10.88           | 0.367| 28.1     | 31.4 |
| C2DAN/C155 + MHC + 10 mM ATP (2) | 17.15   | 11.32           | 0.340| 28.2     | 30.8 |
| C2DAN/C155 + MHC + actin (2) | 17.45       | 11.00           | 0.369| 28.2     | 30.8 |
| C2DAN/C155 + MHC + actin (2) | 17.64       | 10.37           | 0.412| 28.3     | 30.0 |
| C2DAN/C155, free             | 16.20       | 10.07           | 0.378| 27.9     | 30.3 |
| C2DAN/C155 + MHC             | 18.81       | 8.37            | 0.555| 28.6     | 27.5 |
| [C73/C155]DAN, free          | 16.92       | 11.21           | 0.337| 28.1     | 31.4 |
| [C73/C155]DAN + MHC          | 18.69       | 10.62           | 0.432| 28.5     | 29.9 |
| [C73/C155]DAN + MHC + 5 mM ATP | 18.55     | 10.30           | 0.445| 28.5     | 29.6 |
| [C73/C155]DAN + MHC + 10 mM ATP | 18.43   | 9.51            | 0.484| 28.5     | 28.9 |
| [C73/C155]DAN + MHC + actin    | 18.22       | 9.31            | 0.489| 28.4     | 28.6 |
| [C73/C155]DAN + MHC + actin    | 18.42       | 10.05           | 0.454| 28.5     | 29.4 |

* Number within the parentheses refers to measurements carried out on two independent preparations.

* Repetitive measurements on the same preparation.
decayed C2DAN/C155 contains primarily a nondecaying component, consistent with the fact that the majority of the RLCs are bound to MHC with a rotational relaxation time that is too long to be measured. Similar anisotropy decay behavior was observed for all the DAN-labeled RLCs used in this work, including those that are also labeled with the DDP acceptor. The zero time anisotropy, A_0, of free and MHC-bound C2DAN/C155 are similar, with a value of 0.22 (Table III). The steady-state polarization ratio of C2DAN/C155 increased from a relatively low value of 1.13 to 1.44 upon binding to MHC (Table III), again consistent with the majority of the labeled RLC being bound to MHC. The polarization ratio and zero time anisotropy values for free and MHC-bound C155DAN and [C73/C155]DAN were also measured and shown in Table III. As for C2DAN/C155, the polarization ratio increased but A_0 was invariant with binding to MHC.

DISCUSSION

Choice of Labeling Sites—The aim of this study was to determine if a structural change can occur in the vicinity of the head/rod junction of myosin. The relative ease with which the RLC is exchanged into myosin provides a simple means to introduce spectroscopic probes into the neck region of S1, irrespective of whether the exchange is into isolated molecules, or single fibers. We reasoned that any underlying structural change in the neck region might be reflected by a change in the distance between the probes of the bound light chain. Until recently, the only pair of Cys residues available for spectroscopic labeling in vertebrate myosin light chains was Cys^{126} and Cys^{155}, both in the COOH-terminal domain of RLC. Crosslinking studies have shown that these residues are in sufficiently close proximity to form a disulfide bond in the free light chain (Huber et al., 1989; Katoh and Lowey, 1989). The Cys at position 126 can be selectively labeled due to its greater reactivity of Cys^{2} relative to Cys^{155} allowed us to selectively label Cys^{2} with either the donor or the acceptor. That selective labeling was achieved was clearly demonstrated by the labeling stoichiometries and by the proteolytic digestion patterns of the labeled RLC. Owing to the similarity in their reactivities, selective labeling of Cys^{73} or Cys^{155} in C73/C155 was not achievable, and the digestion patterns show that the probes were randomly distributed between the two Cys residues. This did not hamper the distance determination for the following reasons: 1) The fluorescence decay of [C73/C155]DAN is monoexponential, evidently owing to the similarity in environment between the Cys residues. This allowed us to take the lifetime of [C73/C155]DAN as 0.14–0.22, Table III) that are considerably smaller than the maximum value of 0.4, indicating considerable randomization of the probe’s transition moment orientation. From the A_0 values in Table III, the axial depolarization factors and the limits for R could be calculated. Using Stryer’s formalism (Stryer, 1978), which does not take into account the rotation of the acceptor, the uncertainty limits for R are 23–31%. Using Dale and Elings’s formalism as described in Torgerson and Morales (1984), and using the DAN depolarization factors as an approximation of the acceptor depolarization factors, the uncertainty limits are 15–26%. Second, similar distances were obtained when the locations of the donor and acceptor probes were switched on C2/C155. It is highly unlikely that the distance would remain unchanged if the transition moments of the probes were fixed. Finally, the same conclusion could be reached for C73/C155; here energy transfer is measured in both directions in a single experiment owing to the random distribution of the donor/acceptor sites. There was no indication of additional decay components which would arise if heterogeneity existed in transfer efficiency. All of these observations validate the use of an isotropic model for k^2.

Functional Properties—If these mutant light chains are to be useful for the incorporation of probes into myosin molecules in residues at positions 2 or 73 in the NH_{2}-terminal domain, while the endogenous Cys at position 155 near the COOH-terminus was retained as the second member of each pair.

Specificity of Labeling—In the case of C2/C155, the higher reactivity of Cys^{2} relative to Cys^{155} allowed us to selectively label Cys^{2} with either the donor or the acceptor. That selective labeling was achieved was clearly demonstrated by the labeling stoichiometries and by the proteolytic digestion patterns of the labeled RLC. Owing to the similarity in their reactivities, selective labeling of Cys^{73} or Cys^{155} in C73/C155 was not achievable, and the digestion patterns show that the probes were randomly distributed between the two Cys residues. This did not hamper the distance determination for the following reasons: 1) The fluorescence decay of [C73/C155]DAN is monoexponential, evidently owing to the similarity in environment between the Cys residues. This allowed us to take the lifetime of [C73/C155]DAN as 0.14–0.22, Table III) that are considerably smaller than the maximum value of 0.4, indicating considerable randomization of the probe’s transition moment orientation. From the A_0 values in Table III, the axial depolarization factors and the limits for R could be calculated. Using Stryer’s formalism (Stryer, 1978), which does not take into account the rotation of the acceptor, the uncertainty limits for R are 23–31%. Using Dale and Elings’s formalism as described in Torgerson and Morales (1984), and using the DAN depolarization factors as an approximation of the acceptor depolarization factors, the uncertainty limits are 15–26%. Second, similar distances were obtained when the locations of the donor and acceptor probes were switched on C2/C155. It is highly unlikely that the distance would remain unchanged if the transition moments of the probes were fixed. Finally, the same conclusion could be reached for C73/C155; here energy transfer is measured in both directions in a single experiment owing to the random distribution of the donor/acceptor sites. There was no indication of additional decay components which would arise if heterogeneity existed in transfer efficiency. All of these observations validate the use of an isotropic model for k^2.

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![Figure 5. Anisotropy decay curves of C2DAN/C155 in the presence and absence of MHC. The anisotropy decay curves of C2DAN/C155, free (lower curve) and MHC-bound (upper curve), were measured under the same experimental conditions as described in the legend to Fig. 4. The anisotropy decay of the RLC is much slower in the presence than in the absence of MHC, verifying that the RLC protein is bound to the MHC. Qualitatively similar results were found for all of the labeled light chains used in this study (data not shown).](http://www.jbc.org/)

| Samples | R_0  | A_0  |
|---------|------|------|
| C2DAN/C155, free | 1.13 | 0.218 |
| C2DAN/C155 + MHC | 1.44 | 0.224 |
| C155DAN, free | 1.18 | 0.142 |
| C155DAN + MHC | 1.35 | 0.149 |
| [C73/C155]DAN, free | 1.25 | 0.195 |
| [C73/C155]DAN + MHC | 1.56 | 0.211 |

TABLE III
Polarization parameters

R_0 is the steady-state polarization ratio, A_0 is the zero time anisotropy obtained from the anisotropy decay curves.
solution and in fibers, it is important to establish whether the mutations and the probes affect the functional properties of myosin. Chemical modification of the reactive SH1 thiol of the heavy chain has been shown to inhibit the actin-activated ATPase activity of myosin and impair the sliding velocity of actin in the in vitro motility assay (Root and Reisler, 1992). It was shown previously that RLC reacted with fluorescein on its endogenous Cys residues did not inhibit the Ca \textsuperscript{2+} - or K\textsuperscript+ / EDTA-activated ATPase of myosin (Katoh and Lowey, 1989). However, the ATPase activity of skeletal muscle myosin is not particularly sensitive to the presence or absence of RLC, and a more stringent test is to determine the effect of light chain modification on actin sliding velocity in the motility assay (Lowey et al., 1993). As our results show, neither mutagenesis nor labeling of the Cys residues affected the motility compared with wild type RLC. By these criteria, light chains are well suited for the purpose of introducing reporter groups into myosin.

Comparison with Calmodulin—For free RLC our results indicate that the distance measured by RET between residues 2 in the NH\textsubscript{2}-terminal segment and residue 155 in the H-helix is about 35 Å. For comparison the \( \alpha \)-carbon-to-\( \alpha \)-carbon distance between residues 3 in the NH\textsubscript{2}-terminal segment and residue 146 in the H-helix of free calmodulin is 37 Å (Persechini and Kretsinger, 1988). Although these residues are not at equivalent locations, and one must consider the substantial (–20%) amount of error in RET-determined distances, the similarity in these two distances suggests that the disposition of the the NH\textsubscript{2} and COOH-terminal residues in these two proteins are similar and that like free calmodulin, free RLC is likely to adopt a relatively extended conformation. This is also consistent with conclusions drawn from earlier physical studies (Stafford and Szent-Györgyi, 1978; Alexis and Gratzer, 1978).

It has been found that when calmodulin binds a peptide analog derived from one of its substrates, myosin light chain kinase, its two domains are brought into close proximity to each other (Ikura et al., 1992; Meador et al., 1992). The distance between residues 3 and 146, e.g. decreases from 37 to 25 Å for the free versus bound states. We found, however, that the RET-measured distance between residues 2 and 155 decreases by only –5 Å from 35 Å for free versus 30 Å for MHC-bound RLC (Table II), indicating that unlike calmodulin, RLC remains relatively extended (although not necessary with the same conformation) when it binds to MHC. The recently determined crystal structures directly verified that the conformation of MHC-bound RLC is extended (Rayment et al., 1993; Xie et al., 1994).

It is noteworthy that when these distances were remeasured using a different preparation of labeled recombinant light chain, we obtained values of 34 and 30 Å for free versus MHC-bound RLC. This indicates that owing to such favorable conditions as the ability of specific labeling and mono-exponential donor decay in the absence of acceptor, the reproducibility of our measurements is within –1 Å.

Comparison with Crystal Structures—The distance between residues 73 and 155 decreased negligibly from 31 to 30 Å for free versus MHC-bound RLC. This latter value can be compared with the distances between the corresponding residues in the crystal structures of myosin fragments. They are 33.6 Å in chicken skeletal S1 (Rayent et al., 1993) and 33.4 Å in the regulatory domain of scallop myosin (Xie et al., 1994), taking Pro\textsuperscript{64} and Thr\textsuperscript{145} as equivalent residues in scallop myosin RLC. The good agreement between the value from the solution measurements and the crystal structures enhances our confidence in the reliability of RET for distance determinations, as was indicated by a comparison of RET distances in actin with the crystal structure (Miki et al., 1992).

The distance between Cys\textsuperscript{2} and Cys\textsuperscript{155} cannot be compared with the crystal structure, because the first 19 residues of RLC are not visible in the electron density map for chicken S1 (Rayent et al., 1993), and there is no comparable sequence in scallop RLC. The distance of 30 Å determined by the RET measurements is therefore of particular interest insofar as it permits one to speculate about the approximate location of the amino terminus of RLC. From the crystal structures it could be deduced that the distance between Phe\textsuperscript{20} at the beginning of the A-helix and Cys\textsuperscript{155} near the center of the H-helix is only 20 Å. That the RET-determined distance between Cys\textsuperscript{2} and Cys\textsuperscript{155} is 10 Å longer indicates that the NH\textsubscript{2}-terminal segment of RLC extends away from the COOH-terminal domain back toward some region of the NH\textsubscript{2}-terminal domain, possibly in the vicinity of the linker region that connects the NH\textsubscript{2}- and COOH-terminal lobes of RLC.

Invariance with Ligands—We found no detectable change in the distance between Cys\textsuperscript{2} and Cys\textsuperscript{155} for MHC-bound C2/C155 when saturating nucleotides were present. This was qualitatively obvious from the decay curves, which were identical before and after the addition of ATP. Quantitatively, duplicated measurements on two separate preparations yielded values that differed from each other and from that in the absence of ATP by no more than 1 Å, the estimated precision of these measurements. Likewise, we found no change in the distance between Cys\textsuperscript{73} and Cys\textsuperscript{155} of RLC when the myosin was bound to actin. Entirely similar results were obtained for the distance between Cys\textsuperscript{73} and Cys\textsuperscript{155} in C73/C155: no change was detected within an estimated uncertainty of –1 Å. These results indicate that if there are significant conformational changes in this region of myosin during the contraction cycle, these changes are not reflected in the gross conformation of RLC. The absence of structural changes in RLC with ATP and actin validates the use of RLC to introduce probes into muscle fibers for monitoring the orientation of the head during contraction.

Conclusions and Perspectives—The results presented here show that it is possible to incorporate spectroscopic probes into myosin by introducing Cys residues into the RLC by site-directed mutagenesis. These modifications do not appear to affect the functional properties of myosin, an essential prerequisite for structural studies involving spectroscopic probes. Now that the crystal structures of S1 and the regulatory domain are available (Rayent et al., 1993; Xie et al., 1994), it will be possible to ask more directed questions by constructing specific sites within the RLC, as well as between the ELC and the RLC. For example, we now know that Cys\textsuperscript{126} is located at the interface of the RLC and the ELC; by engineering a Cys into an appropriate position on the ELC, it may be possible to detect a relative movement between the light chains upon ATP hydrolysis. It was previously shown that a probe bound to the endogenous COOH-terminal Cys of the ELC changed its fluorescence signal in parallel to the rate of myosin ATP hydrolysis (Marsh and Lowey, 1980; Marsh et al., 1982). This result is highly suggestive of a relative movement of the light chains within the neck region of myosin. Although the RET technique can only give low resolution structural information, it may be able to detect small changes in intra and interlight chain distances occurring during the contractile cycle and thereby yield valuable biological insights into the mechanism of regulation.

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