Subunit Interactions within an Expressed Regulatory Domain of Chicken Skeletal Myosin

LOCATION OF THE NH2 TERMINUS OF THE REGULATORY LIGHT CHAIN BY FLUORESCENCE RESONANCE ENERGY TRANSFER*

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The abbreviations used are: S1, myosin subfragment-1; RD, regulatory domain; ELC, essential light chain; RLC, regulatory light chain; HC, heavy chain peptide; LC, light chain; 1,5-IAEDANS, N-iodoacetyl-N-(5-sulfo-1-naphthyl)ethylenediamine; DDPM, N-(4-dimethylamino-3,5-dinitrophenyl)maleimide; DTT, dithiothreitol; DANS, dansyl moiety of 1,5-IAEDANS; DDP, dimethylamino-dinitrophenyl moiety of DDPM; FRET, fluorescence resonance energy transfer; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

The regulatory domain (RD), or neck region of the myosin head, consists of two classes of light chains that stabilize an α-helical segment of the heavy chain. RD from chicken skeletal muscle myosin was prepared in Escherichia coli by coexpression of a 9-kDa heavy chain fragment with the essential light chain. Recombinant regulatory light chain (RLC), wild type or mutant, was added separately to reconstitute the complex. The affinity of RD for divalent cations was determined by measuring the change in fluorescence of a pair of heavy chain tryptophans upon addition of calcium or magnesium. The complex bound divalent cations with high affinity, similar to the association constants determined for native myosin. The intrinsic fluorescence of the tryptophans could be used as a donor to measure the fluorescence resonance energy transfer distance to a single labeled cysteine engineered at position 2 on RLC. Dansylated Cys² could also serve as a donor by preparing RLC with a second cysteine at position 79 which was labeled with an acceptor probe. These fluorescence resonance energy transfer distances (24–30 Å), together with a previous measurement between Cys² and Cys¹⁵⁵ (Wolff-Long, V. L., Tao, T., and Lowey, S. (1995) J. Biol. Chem. 270, 31111–31118) suggest a location for the NH² terminus of RLC that appears to preclude a direct interaction between the phosphorylatable serine and specific residues in the COOH-terminal domain.

Our initial goal was to see if a correctly folded RD complex, consisting of two light chains and a heavy chain peptide, could be prepared in an expression system. Not only would this approach eliminate microheterogeneity introduced by proteolysis, but the opportunity would arise to study light chain-heavy chain interactions by site-directed mutagenesis. To date it has not been possible to express sufficient quantities of a striated muscle myosin for biophysical studies. We show here that homogeneous RD, retaining the high affinity nonspecific calcium/magnesium site found in all muscle myosins, can be prepared in good yield in a bacterial expression system. Moreover, by labeling a single engineered cysteine (Cys²) with 1,5-IAEDANS at the NH² terminus of RLC, it was possible to determine the fluorescence resonance energy transfer distance between Cys² and a cluster of two tryptophans located at the sharp bend of the heavy chain α-helix, which served as a donor to IAEDANS. Dansylated Cys² could also be used as a donor by engineering a second cysteine at position 79, which was subsequently labeled with an acceptor molecule. These two FRET distances, together with a previously determined distance between Cys² and Cys¹⁵⁵ (7), were used to define an approximate position for the NH² terminus of RLC. The first 19 residues of RLC are not visible in the crystal structure of chicken S1, probably due to their flexibility (1). The structure of scallop RD is without 11 NH²-terminal RLC residues, since these are removed by proteolysis during the preparation of RD (2). The location of the missing sequence by fluorescence resonance energy transfer, despite the low resolution of this technique, provides a structural framework for considering how the actomyosin interaction can be enhanced by phosphorylation of a single serine on a light chain far removed from the active site.

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construct has an NH₂-terminal tag of 4 residues (MARI) from the vector followed by Pro² as the first residue of LC1 instead of Ala. Sequencing of the cDNA revealed a six-base deletion (encoding Pro-Pro) in the 5'-coding region, which results in six pairs in Pro-Ala instead of the seven pairs found in tandem in the native protein. Comparison of LC1 sequences from different species indicates a highly conserved region in the NH₂-terminal regions (11), and the deletion was considered unimportant for the experiments presented here.

A construct with both LC1 and wild-type RLC in the same pWY vector was also designed. The pWY/LC1 clone was digested with PstI/BamHI and the 700-base pair PstI/BglII fragment from pT7-7/LC2 encoding RLC (12) was ligated into this clone. Thus, the cDNA for each light chain was positioned in tandem with the promoter and stop codon. This construct was used to express both light chains together with the pT7-7 vector containing the 9-kDa heavy chain fragment. Protein expression was in BL21(DE3), and bacterial cultures were grown at 37 °C for 16–18 h in the presence of ampicillin and/or kanamycin as described in Wolf-Lang et al. (13).

Preparation of RLC—The expression and purification of wild-type and mutant chicken skeletal RLC in the vector pT7-7 has been described (14). Wild-type RLC contains two endogenous Cys residues at positions 126 and 155. In some RLC mutants, e.g. Cys¹²⁶/Cys¹⁵⁵ and Cys², the endogenous tryptophan at position 138 was changed to phenylalanine in order to eliminate the contribution of Trp fluorescence. In other mutants the endogenous Cys residues were selectively removed by mutagenesis to alanine, as in Cys¹²⁶Cys², and new Cys residues were introduced at positions 2 and 79 as in Cys¹⁵⁵Cys⁷⁹. Table I gives a description of all the RLCs, and the location of specific residues is shown in the ribbon diagram of the regulatory domain in Fig. 1.

Purification of the Regulatory Domain—When the 9-kDa heavy chain fragment and ELC are coexpressed, they are found in inclusion bodies, whereas simultaneous expression of heavy chain fragment together with RLC and ELC results in a soluble complex in the cytoplasm. An advantage of the first method is that the soluble bacterial proteins can be readily separated from the inclusion bodies by centrifugation, followed by washing of the pellet with a mild detergent (14). The relatively pure protein in the inclusion bodies can then be used to prepare complexes with a variety of unlabeled or labeled mutant RLCs. Although simultaneous expression of the three chains is in some ways a simpler procedure, a major drawback is the presence of a much higher concentration of contaminating bacterial proteins that are difficult to remove. For this reason, and since we usually wished to incorporate a labeled RLC into the regulatory complex, the first preparative procedure was adopted.

The cell pellet from a 1-liter culture of Escherichia coli BL21(DE3) coexpressing 9-kDa HC and LC1 was lysed, and the inclusion bodies isolated as described (14). The washed pellet was resuspended in 8 M guanidine, 10 mM DTT in PBS (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 3 mM NaN₃), to which RLC (20–25 mg unchromatographed, or purified and labeled) was added with stirring at room temperature for 1 h. Insoluble cell debris was removed by centrifugation (100,000 × g for 30 min), and the proteins were renatured by dialysis against PBS, 1 mM DTT, 1 mM MgCl₂ at 4 °C. Denatured insoluble proteins were removed by centrifugation, and the supernatant was dialyzed against 10 mM sodium phosphate, pH 7.5, 1 mM MgCl₂, 1 mM DTT, 3 mM NaN₃ for 4 h, clarified, and applied to a hydroxyapatite column (1.5 × 10 cm) equilibrated with the same buffer. The proteins were eluted with a linear gradient (10–200 mM sodium phosphate in a total volume of 200 ml), and the fractions containing the regulatory complex were pooled and further purified on a DEAE-Sephacel (Pharmacia) column (1 × 10 cm) equilibrated in 10 mM imidazole, pH 7.0, 20 mM NaCl, 2 mM MgCl₂, 1 mM DTT, 1 mM NaN₃. After eluting the proteins with a linear gradient to 0.5 M NaCl (total volume 100 ml), the fractions were analyzed by SDS-PAGE and pooled. Trace amounts of remaining impurities were removed by gel filtration chromatography on a Superose 12 column (1 × 30 cm, FPLC, Pharmacia). The yield was 10–15 mg of complex.

Labeling of RLC—Purified RLC (lyophilized with sucrose) was dissolved in 8 M guanidine, PBS, 10–20 mM DTT, and stirred at room temperature for 1 h in order to completely reduce the cysteines before dialysis against PBS, 50 μM DTT, with at least one change of buffer. The single Cys mutant, Cys² (Table I), was reacted with 2.5–5-fold molar excess of 1,5-IAEDANS (Molecular Probes) over the Cys residues with stirring at room temperature for 4 h in the dark before stopping the reaction by adding DTT to a final concentration of 10 mM. The double Cys mutant, Cys¹²⁶/Cys⁻⁷⁹, at a concentration of 5–7 mg/ml, was reacted with a 3-fold molar excess of 1,5-IAEDANS for 2 min on ice. The reaction was stopped with 50 mM DTT, and the sample dialyzed against 50 mM sodium phosphate, pH 7.0, 0.2 mM EDTA, 1 mM DTT, and 3 mM NaN₃. The protein was chromatographed...
graphed on a DEAE-Sephadex (FPLC, Pharmacia) column (1.5 × 10 cm) equilibrated in the above buffer and eluted with a linear gradient to 300 mM sodium phosphate (total volume 150 ml). Fractions containing unreacted, singly labeled (C2DAN/C79), and doubly labeled RLC were identified by urea-PAGE. C2DAN/C79 (in PBS, 50 mM DTT) was subsequently incubated with a 4-fold molar excess of DDPM (Aldrich) at room temperature for 4 h to obtain C2DAN/C79DDP. Iodoacetamide was used to block any unreacted cysteines in control experiments. The light chains were dialyzed exhaustively against PBS to remove excess reagent before being used to prepare the RD complex.

Spectrophotometric analysis of the labeled light chains was used to determine the extent of labeling. Protein concentration was determined by the method of Bradford (15) using wild-type RLC as a standard (absorbance of 0.5 at 280 nm, 1 mg/ml). A molar extinction coefficient of 6200 M⁻¹ cm⁻¹ at 340 nm was used for IAEDANS-labeled proteins. The ratio of DANS to light chain was ~0.9. The molar extinction coefficient for DDPM-labeled RLC was taken as 2930 M⁻¹ cm⁻¹ at 442 nm (7). The ratio of DDPM to RLC was approximately 1.

**Titrations with Divalent Cations—** RLC and the regulatory domain (with either wild-type or mutant RLCs) were dialyzed against 0.1 mM Hepes, pH 7.2, 0.1 mM NaCl, 1 mM NaN₃ for 10–14 h and clarified at 100,000 × g for 30 min. EGTA was added to a final concentration of 1 mM before titration with calcium ions. Similarly, 1 mM EDTA was used in titrations with magnesium ions. Proteins were diluted to an absorbance of less than 0.05 to reduce the effect of light scattering (and the Raman scattering was removed by subtraction of a buffer blank). Small aliquots (2–20 μl) of the stock CaCl₂ or MgCl₂ solution were delivered by a microsyringe (Hamilton Co.) into 2 ml of the protein solution with constant stirring. A SLM 48000 spectrofluorometer was used to measure tryptophan fluorescence (emission maximum at 340 nm) upon excitation at 285 nm. The emission spectra were recorded from 300 to 400 nm at 1-nm intervals with 2-s integration at each wavelength. The sample compartment temperature was maintained at 20 °C. Percent decrease in tryptophan fluorescence (after volume correction) was plotted against -log free metal ions (pat) to obtain the concentration (equivalent to 1/Kₘ), the association constant at which the half-maximal change in fluorescence occurs. The program used to calculate free metal ion concentrations was based on the methodology of Storer and Cornish-Bowden (16).

**Energy Transfer Measurements—** Steady-state fluorescence energy transfer measurements were performed on an ISS PC1 photon counting spectrophotometer equipped with a rhodamine B (4 mg/ml in ethanol with a RG 630 filter) quantum counter. Spectra were recorded in ratio mode to correct for lamp intensity fluctuations. The spectral bandwidth for excitation and emission were 5 nm. Emission spectra were recorded at 1-nm intervals with 2-s integration at each wavelength, and were later corrected with software supplied by ISS. A 305 nm cut-off filter was used in the emission path for tryptophan spectra. When IAEDANS was used as a donor, 320 and 470 nm cut-off filters were placed in the excitation and emission paths, respectively.

The cluster of two tryptophans in the heavy chain peptide of RD could be used as an intrinsic energy donor. The acceptor was a single cysteine on RLC labeled with 1,5-IAEDANS. For preparation of RD, RLC mutants were used in which tryptophan residue 138 was replaced with cysteine on RLC labeled with 1,5-IAEDANS. For preparation of RD, the co-elution of the light and heavy chains from the DEAE-Sephacel (FPLC, Pharmacia) column was monitored by SDS-PAGE analysis (23). The extinction coefficients of the recombinant RDs were determined by measuring the absorbance at 280 nm and determining the concentration by the method of Bradford (15). Sedimentation velocity runs were made in a Beckman Model E analytical ultracentrifuge. Myosin light chains were phosphorylated as described previously (24).

**RESULTS**

**Purification and Characterization of Recombinant RD—** The 9-kDa heavy chain fragment (HC) could not be obtained in any significant amount when expressed without the light chains, as determined by SDS-PAGE analysis of the crude extracts from overnight cultures (data not shown). Therefore, it was necessary to coexpress HC with both light chains, ELC (LC1 isoform) and RLC (LC2) (Fig. 2A, lane 1) or with ELC alone (lane 2). In the latter method, HC and ELC were segregated into inclusion bodies, which could be readily separated from the bacterial proteins (lane 3). After resuspension in 8 M guanidine, RLC was added to solubilize the complex upon subsequent dialysis into a benign solvent (lane 4). RD was purified by ion-exchange chromatography on hydroxypatite (lane 5) followed by DEAE-Sephael (lane 6) to remove any last trace of impurities. The recombinant RLC added to the HC/ELC complex was one of several mutants described in Table I. The purified RD remained stable at all concentrations (up to 20 mg/ml) when stored at 4 °C.

Various methods were used to establish the composition of RD. The co-elution of the light and heavy chains from the ion-exchange columns during purification suggested that they form a complex. Upon gel filtration chromatography, RD eluted between the chymotryptic S₁ (~100 kDa) and the free light chains (~20 kDa) consistent with a molecular mass of ~50

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kDa. The sedimentation velocity pattern for purified RD showed a single, homogeneous peak with a sedimentation coefficient of 3.3 S (Fig. 2B). The extinction coefficients of RDs made with wild-type and Cys126/Cys155 were 0.5 and 0.4, respectively, which agrees well with the theoretical values based on the number of tryptophans and tyrosines in a 1:1:1 complex (25). Although the ratio of the two LCs and HC as determined by densitometry of the Coomassie Blue-stained SDS gels was 1:1:2, it is probable that the large number of charged residues in the heavy chain peptide caused a disproportionate absorption of dye.

**Intrinsic Fluorescence of Heavy Chain Tryptophans**—There are three tryptophans in wild-type RD, two in the heavy chain peptide (Trp629-Pro830-Trp831) and one in wild-type RLC (Trp138) (Fig. 1). A mutant RLC was prepared in which Trp 138 was replaced by Phe so that Trp fluorescence from the heavy chain could be used as a single reporter group or donor site in FRET experiments. Tryptophan emission spectra for RD containing wild-type or mutant RLC were compared with free wild-type RLC (Fig. 3). RD(mut) containing two tryptophans from the heavy chain showed far less fluorescence than wild-type RLC which has only a single tryptophan. The diminished intensity of fluorescence suggests intramolecular quenching by the heavy chain tryptophans due to their close proximity in the structure (WPW). In contrast, the single Trp138 in wild-type RLC appears to increase its fluorescence in the more hydrophobic environment of the heavy chain complex, as judged by the enhanced fluorescence of RD(wt) compared with the sum of the fluorescence intensity of wild-type RLC and RD(mut). In a denaturing solvent such as guanidine hydrochloride, the fluorescence intensity is more proportional to the tryptophan content of the protein: i.e. the fluorescence of RD(wt) is equal to the sum of the fluorescence of RD(mut) and wild-type RLC (Fig. 3).

The emission maxima in guanidine hydrochloride showed a red shift (353 nm) compared with the emission maxima at 343 and 340 nm for WT and RDs, respectively, in a benign solvent. This can be explained by the increased exposure of the Trps to solvent in an unfolded, denatured state.

**Binding of Divalent Metal Ions to RLC and RD**—Titration with divalent cations were carried out to determine if expressed RD would bind calcium and magnesium with affinities similar to those found for native myosin. First, the intrinsic fluorescence of Trp138 in RLC was measured as a function of calcium (or magnesium) to determine the binding of these ions to free RLC (data shown only for Ca2+ binding) (Fig. 4, A and B). The total decrease in fluorescence intensity was ~18% for Ca2+ and ~12% for Mg2+ ions. The association constants of 105 M^{-1} for Ca2+ and 10^3 M^{-1} for Mg2+ obtained here agree well with those reported by Alexis and Gratzer (26) for rabbit skeletal RLC. Despite the considerable distance between the cation binding site in the NH2-terminal domain and the single Trp in the COOH terminus of RLC, the conformational change associated with metal binding must be transmitted to the Trp, as evidenced by a change in signal.

Similarly, the decrease in fluorescence for RD(wt) or RD(mut) as a function of calcium (or magnesium) was used to determine the affinity of RLC for metal ions in the heavy chain bound state. Association constants of 10^{-7}-10^8 M^{-1} for Ca2+, and 10^6 for Mg2+ were obtained (Fig. 4, C and D). The smaller fluorescence change with RD(wt) compared with RD(mut) can be attributed to the high level of background fluorescence contributed by Trp138 in wild-type RLC. The large fluorescence change in RD(mut) (~32%), despite the low absolute intensity of the signal, suggests that the heavy chain tryptophans are perturbed to a greater degree by metal binding than the single Trp in RLC. This may be related to the hydrophobic interactions of residues in the NH2-terminal domain of RLC with the

![Fig. 2. Purification of RD. A, HC peptide (9 kDa) was coexpressed with ELC (LC1) and RLC (LC2) (lane 1) or with ELC alone (lane 2). The latter method segregated HC and ELC into inclusion bodies, which were separated from the bacterial proteins (lane 3), and resuspended in 8 M guanidine. Addition of RLC solubilized the complex (lane 4), which was purified on hydroxylapatite (lane 5) followed by DEAE-Sephacel (lane 6). The added RLC, labeled or unlabeled, was one of several mutants described in Table I. B, sedimentation velocity pattern for purified RD showed a single, homogeneous peak.](image-url)
tryptophans in the COOH terminus of the heavy chain (2). The binding constants obtained here are in good agreement with earlier values \((3 \times 10^7 \text{ M}^{-1} \text{ for Ca}^{2+})\) determined by more direct binding methods (27).

Fluorescence Energy Transfer between Heavy Chain Tryptophans and IAEDANS-labeled RLC—The intrinsic fluorescence of the two heavy chain tryptophans can also be used as a donor in energy transfer experiments. By introducing a single IAEDANS acceptor probe into the Cys\(^2\) residue of RLC, we could measure the energy transfer distance between the cluster of Trp at the bend of the heavy chain helix and the NH\(_2\) terminus of RLC. The tryptophan emission spectra of RD (excitation at 295 nm) prepared either with or without the IAEDANS-labeled RLC are shown in Fig. 5A. The tryptophan emission is reduced by about 9% when Cys\(^2\) is labeled with the IAEDANS acceptor (RD(C2DAN)). Although donor quenching is small, the large enhancement in IAEDANS fluorescence at 500 nm compared with the free labeled light chain (C2DAN) is strong qualitative evidence for energy transfer from the heavy chain tryptophans to the dansyl probe on RLC.

We also examined the excitation spectra for several constructs that contained either no tryptophan or had tryptophans located at different distances from the acceptor probe, IAEDANS (Fig. 5B). The appearance of a tryptophan component between 280 and 300 nm in the excitation spectrum of IAEDANS (emission measured at 500 nm) is strong evidence that energy transfer has occurred. C2DAN has no tryptophan and therefore shows little fluorescence when excited in the region of 290 nm. C126DAN shows strong fluorescence when the single tryptophan at position 138 is excited due to the close proximity of the labeled Cys\(^{126}\). Similarly, the tryptophan cluster (WPW) on the heavy chain shows energy transfer, although much weaker, to the dansylated Cys\(^2\) in RD(C2DAN).

The efficiency of energy transfer \((E)\) between the heavy chain Trp and the IAEDANS probe on RLC, as determined from emission spectra, is given in Table II. In order to convert \(E\) into a distance, the quantum yield \((Q_d)\) and the overlap integral \((J)\) had to be determined for RD(mut) (see “Materials and Methods”). The low value of 0.02 obtained for \(Q_d\) is not surprising since the tryptophan emission is unusually low for this complex (see Fig. 3). The overlap integral for the Trp/IAEDANS pair was calculated to be \(6.1 \times 10^{-15} \text{ M}^{-1} \text{ cm}^3\). The orientation factor, \(k^2\), used in the calculations was the isotropically averaged value of 2/3. Measurements of the limiting anisotropy of the Trp donor and IAEDANS acceptor in RD gave values of 0.14

**FIG. 4. Binding of calcium ions to RLC and RD.** A and B, the decrease in tryptophan fluorescence intensity (emission maximum at 340 nm) of RLC as a function of calcium ions was used to measure the binding of Ca\(^{2+}\) to free RLC. Emission spectra at the beginning (EGTA, solid line) and at the end of the titration (calcium, dotted line) are shown. Similarly, the data for RD(wt) and RD(mut) are shown in C and D. The greater percentage fluorescence change with RD(mut) indicates that the heavy chain tryptophans are the major source of the fluorescence change upon metal binding. Excitation was at 295 nm.

**FIG. 5. Fluorescence energy transfer between heavy chain tryptophans and IAEDANS-labeled RLC.** A, corrected tryptophan emission spectra (excitation at 295 nm) of RD in the absence (RD(Cys\(^2\), solid line) and presence (RD(Cys-2DAN), dotted line) of acceptor. Although donor quenching is small (~10%), the large enhancement in acceptor fluorescence at 500 nm compared with the free labeled light chain (Cys-2DAN, broken line) is strong qualitative evidence for energy transfer from the heavy chain tryptophans to the dansylated Cys\(^2\). B, excitation spectra (emission monitored at 500 nm) of dansylated RD (dotted line) shows a tryptophan component between 270 and 300 nm which is evidence for energy transfer. C2DAN (broken line) has no tryptophan and therefore shows little or no fluorescence when excited in this region. C126DAN (solid line) shows strong fluorescence when the single Trp at position 138 is excited due to its close proximity to the dansyl probe. The molar protein concentration was the same for all species.
to 0.16, indicating considerable randomization of the probe orientation (21). A value of 2/3 for the orientation factor has generally been used in distance calculations, with no major errors in distance resulting from this assumption. Based on the above parameters, \( R_0 \), the distance at which \( E \) is 50\%, was 16 Å which yields a distance of 23–24 Å between -WPW- and Cys\(^2\) in RD. This distance did not change significantly upon phosphorylation of RLC, or in the presence or absence of divalent cations.

As a control, the distance between Trp\(^{138}\) and Cys\(^{126}\) was determined in the RLC mutant, Cys\(^{126}\) (see Table I) using IAEDANS as the acceptor. Between 80 and 85\% quenching was observed in the presence or absence of metal ions. A \( Q_d \) of 0.14 was obtained for the single Trp in RLC, which is close to the value reported for free tryptophan (17). The value for the overlap integral was assumed to be the same as for the above, particularly, since a similar \( J \) value was reported for a Trp cluster and single IAEDANS-labeled Cys mutant in a membrane protein (20). The range of anisotropy values measured for Trp\(^{138}/\text{IAEDANS}\) in RLC was also similar to RD, and a value of 2/3 was taken for the orientation factor. These parameters led to a value of 22 Å for \( R_0 \), from which a distance of 16–17 Å was calculated (Table II). This FRET distance agrees well with the distance of 18 Å for the corresponding residues in the crystal structure of scallop RD (2), and with an earlier distance determination by fluorescence spectroscopy (28).

Fluorescence Energy Transfer between Donor/Acceptor Probes on RLC Bound to the Heavy Chain—To fix the position of Cys\(^2\) in RD, a minimum of three distances needs to be determined. In an earlier study, the FRET distance between Cys\(^2\) and Cys\(^{155}\) in the heavy chain-bound RLC was measured (7). For a third distance, Cys\(^2\) and Cys\(^{79}\) in RLC were labeled with IAEDANS and DDPM as donor and acceptor probes, respectively, and reconstituted with heavy chain peptide in RD. The greater reactivity of Cys\(^2\) compared with Cys\(^{79}\) again enabled us to prepare the RLC with donor and acceptor probes in a 1:1 stoichiometry (7). The emission spectra of RD containing mutant RLC labeled with donor, IAEDANS, at position 2 (C\(^2\)DAN/C\(^{79}\)) and RD containing RLC with donor and acceptor (C\(^2\)DAN/C\(^{79}\)DDP) is shown in Fig. 6. The fluorescence of IAEDANS is strongly quenched by the non-fluorescent acceptor, DDPM, see Table II. In the presence of guanidine hydrochloride no energy transfer occurs, and the fluorescence intensity of the light chains is the same in the presence or absence of acceptor (dotted and broken lines superimpose).

**DISCUSSION**

The structure and function of myosin have been studied historically by a reductionist approach: first, myosin was cleaved by proteolytic enzymes into heavy meromyosin and light meromyosin, which allowed a separation of the enzymatic properties of this macromolecule and the active globular head region, subfragment-1 (S1), was cleaved from the long ß-helical rod (29). Although proteolytic procedures could be used to reduce S1 even further (5, 30), it became far simpler to prepare the “motor domain” by molecular biolog-

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**TABLE II**

| Sample          | Preparation | Donor/location | Acceptor/location | \( E \) | \( R_0 \) | \( R \) |
|-----------------|-------------|----------------|-------------------|------|--------|------|
| RD(C2)          | 1           | Trp/WPW        | IAEDANS/Cys\(^2\) | 8.3  | 16\(^a\) | 23.9 |
|                 | 2           |                |                   | 8.8  | 23.6   |      |
|                 | 3           |                |                   | 9.1  | 23.5   |      |
| RD(C2/C79)      | 1\(^b\)     | IAEDANS/Cys\(^2\) | DDPM/Cys\(^{79}\) | 45.0 | 29\(^c\) | 30.0 |
|                 |             |                |                   | 42.0 | 30.6   |      |
|                 |             |                |                   | 45.0 | 30.0   |      |
|                 |             |                |                   | 50.0 | 29.0   |      |
|                 |             |                |                   | 52.0\(^d\) | 28.7   |      |
| RLC             | 1           | Trp\(^{138}\)  | IAEDANS/Cys\(^{126}\) | 80.0 | 22     | 17.5 |
|                 | 2           |                |                   | 86.0 | 16.2   |      |
|                 | 3           |                |                   | 86.0 | 16.2   |      |

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- \( R_0 \) is lower in RD than in RLC, because of the low quantum yield, \( Q_d \) = 0.02, measured for the two tryptophans in the heavy chain as compared to \( Q_d \) = 0.14 for the single tryptophan in RLC. An orientation factor, \( k \), of 2/3 was assumed. The overlap integral, \( J \), for the Trp/IAEDANS pair in RD and RLC was taken as \( 6.1 \times 10^{-15} \text{ mol}^{-1} \text{cm}^3 \).
- Measurements at varying concentrations from 1.5 to 4 \( \mu \)M.
- \( R_0 \) is taken from Wolff-Long et al. (7).
- A single lifetime measurement of this pair was made in the laboratory of Dr. Terence Tao at Boston Biomedical Research Institute.

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**Fig. 6. Fluorescence energy transfer between donor and acceptor probes on RLC bound to the heavy chain.** Emission spectrum (excitation at 340 nm) of RD containing mutant RLC labeled with donor (RD[C\(^2\)DAN/C\(^{79}\), solid line] shows strong quenching when the non-fluorescent acceptor DDPM is attached to position 79 (RD[C\(^2\)DAN/C\(^{79}\)DDP, broken line]). In guanidine hydrochloride no energy transfer occurs, and the fluorescence intensity of the light chains is the same in the presence or absence of acceptor (dotted and broken lines superimpose).
ical methods (31). Here we describe the first preparation of the “regulatory domain” or neck region of chicken skeletal myosin S1 by recombinant methods.

The regulatory domain appropriately derived its name from the structure of the invertebrate, scallop myosin, a molecule that is regulated by a Ca\(^{2+}\)-specific site in the neck region of the myosin head (32). The RD consists of an essential (ELC) and a regulatory (RLC) light chain, which together stabilize the S1 heavy chain in an \(\alpha\)-helical conformation as it joins the \(\alpha\)-helical coiled-coil rod (1). The crystal structure of scallop RD (2) first showed that this multisubunit interaction creates a Ca\(^{2+}\)-binding site on the NH\(_2\) terminus of the ELC, a result which could not be predicted from the sequence, since it fails to correspond to a typical EF-hand (2). The only other myosins regulated by Ca\(^{2+}\) are the “unconventional” myosins which contain primarily calmodulin in the neck region of the head (33).

Unlike the invertebrate and unconventional myosins, the vertebrate myosin IIs are regulated to varying degrees by a phosphorylated serine in the NH\(_2\) terminus of the RLC (34). Smooth and nonmuscle myosins are completely inactive in the dephosphorylated state, whereas the activity of skeletal myosin is only modulated by the state of phosphorylation (4). This raises the intriguing question of which properties of myosin dictate whether a single phosphorylation site in the regulatory domain can accelerate product release in the motor domain? In a recent study, it was suggested that the RLC must possess certain arginine residues in its COOH-terminal domain which can bond to the phosphoserine in the NH\(_2\) terminus (35). Only thick filament-regulated myosins have conserved arginine residues in the E to H-helices (nomenclature refers to calmodulin) of RLC that can serve as possible candidates for such an interaction. This might explain why skeletal RLC, which lacks these conserved Arg residues, cannot replace smooth RLC in the regulation of smooth muscle myosin (36).

Location of the NH\(_2\) Terminus of RLC—The two FRET distances determined here, together with an earlier determination of the distance between Cys\(^{2}\) and Cys\(^{155}\) in RLC exchanged into myosin (7), define a locus for Cys\(^{2}\), which marks the beginning of the sequence for RLC (Fig. 7). The 19 residues in RLC, that are not visible in the crystal structure of chicken S1, appear to lie at an angle to the first A-helix of the EF-hand that forms the Mg\(^{2+}\)-binding site. The region of closest approach between the phosphoserine and the remainder of the RLC molecule would be in the vicinity of the D/E linker segment that joins the NH\(_2\) and COOH-terminal lobes. Although, theoretically, Cys\(^{5}\) could lie 180° away from this location, this orientation is preferred insofar as it maximizes side chain interactions between the NH\(_2\)-terminal peptide and the linker region. Interestingly, the NH\(_2\) terminus of troponin C is located in approximately the same position relative to the A-helix and the central linker helix (37). An Arg residue in the NH\(_2\) terminus of TnC has been shown to form a H-bond with a Glu residue in the D-helix of the NH\(_{2}\)-terminal domain (37). Assuming that skeletal and smooth RLC share a similar structure, the localization studies described here preclude a direct interaction between the phosphoserine and specific arginine residues in the COOH-terminal domain. However, before discussing alternative mechanisms for the activation of myosin’s activity, one should question: 1) how faithfully the recombinant regulatory domain mimics the neck region of the native myosin molecule, and 2) how reliable are resonance energy transfer measurements in determining distances?

The structural determination of the regulatory domain of scallop myosin was carried out on a complex produced by a series of proteolytic digestions of the native protein (2, 5).
Despite the absence of the motor domain, the structure of scallop RD was remarkably similar in its overall features to that of chicken S1 (1), implying that RD is a stable folding unit, and that the structure of RD in all myosin IIs is highly conserved. The remarkable stability of RD is evident from its ability to fold spontaneously upon replacement of a denaturing solvent with a benign solvent (5). The recombinant chicken RD used in this study was also prepared by reconstitution out of a denaturant, although the same complex could be prepared by simultaneous expression of the two LCs and the heavy chain peptide, which avoids the use of denaturants. The only functional property that can readily be measured is the binding affinity of RD for divalent cations. The good agreement between the binding constants obtained here and those reported for myosin, provides strong evidence that RD retains its native properties and is a good model system. An important advantage in using RD for structural studies compared with S1 is that the endogenous tryptophans on the heavy chain can be used as a donor group without introducing potentially disruptive extrinsic probes. Thr^{79} was chosen for mutagenesis to Cys, because its position on the D-helix is solvent-exposed, and a probe at this site would be expected to have a minimal effect on RLC/heavy chain interactions.

The accuracy of distance measurements by resonance energy transfer is thought to be limited by assumptions about the orientation factor, k^2 (see “Discussion” in Wolff-Long et al. (7) and references therein). Even with this uncertainty, usually less than 20%, FRET distance measurements are in a range that is sufficiently narrow to determine whether an interaction is likely to occur between the NH_2 and COOH termini of RLC. An unexpected experimental problem encountered here was the low quantum yield of the two neighboring tryptophans in the heavy chain peptide. This led to a low value for R_0 (16 Å), and therefore very little quenching of the Trp fluorescence by the acceptor probe (<10%). To perform these measurements, it was necessary to prepare RD with a high degree of purity since any contamination by fluorescent impurities would seriously compromise the data. The reproducibility of the results, and the demonstration of acceptor excitation, enhanced our confidence in the validity of these distance determinations.

**Regulation by Phosphorylation**—Protein phosphorylation stabilizes different conformational states of regulated molecules in order to enhance or repress biological activity. A classic example of such a mechanism is glycogen phosphorylase, whose crystal structure has been solved in both the active and inactive state. This brings us back to the question of why mutating six residues in the COOH-terminal domain of skeletal RLC to those present in smooth RLC (of which four were Arg) had the effect of restoring regulation to a smooth myosin heavy chain, which was locked permanently in the off state with wild-type skeletal RLC (36). Instead of proposing a direct coordination between the phosphoserine and Arg residues (35), we suggest that the mutated skeletal RLC interacts more strongly with smooth heavy chain than wild-type skeletal RLC, with the consequence that phosphorylation can now activate this chimeric myosin. This alternative explanation pictures myosin regulation to be based on a complex series of molecular interactions between the RLC and the heavy chain that can be perturbed by phosphorylation, and transmitted to the active site through the regulatory domain.

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