Proximity Relationships between Sites on Myosin and Actin. Resonance Energy Transfer Determination of the Following Distances, Using a Hybrid Myosin: Those between Cys-55 on the *Mercenaria* Regulatory Light Chain, SH-1 on the *Aequipecten* Myosin Heavy Chain, and Cys-374 of Actin†

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ABSTRACT: Resonance energy transfer measurements have been made on hybrid myosins in order to map distances between sites on the regulatory light chain, heavy chain, and actin as well as to assess potential conformational changes of functional importance. Using scallop (*Aequipecten*) myosin hybrid molecules possessing clam (*Mercenaria*) regulatory light chains, we have been able to map the distance between Cys-55 on the regulatory light chain and the fast-reacting thiol on the myosin heavy chain (SH-1). This distance is shown to be ~6.4 nm, and it is not altered by the presence or absence of Ca^{2+}, MgATP, or actin. Experiments performed at low ionc strength on heavy meromyosin (HMM) derived from these hybrid myosins gave results similar to those performed on the soluble parent myosin preparations. The distances between Cys-374 on actin and each of the above sites were also measured. *Mercenaria* regulatory light-chain Cys-55, within the hybrid myosin molecule, was found to be >8.0 nm away from actin Cys-374. Scallop heavy-chain SH-1 is shown to be ~4.5 nm away from actin Cys-374, in broad agreement with earlier measurements made by others in nonregulatory myosins. The significance of our results is discussed with respect to putative conformational changes within the region of the heavy chain connecting SH-1 to the N-terminal region of the light chain.

The determination of the spatial distribution of sites upon the surface of the myosin head not only is an important objective in its own right but also is a prerequisite to an understanding of the spatio-dynamic relationship between sites on actin and those on myosin during the cross-bridge cycle. Conformational events associated with ATP hydrolysis and actin interaction may manifest themselves as ligand-dependent alterations in distances connecting appropriate pairs of sites. Relatively few sites are available for specific labeling on the myosin head; a number of these, including the active site, the fast-reacting thiols, and Cys-177 of the essential light chain, have been exploited by many in experiments on vertebrate skeletal muscle myosin [for reviews, see Cooke (1986) and dos Remedios et al. (1987)].

In regulatory myosins, conformational aspects of regulation may also be monitored in this way. Invertebrate myosins offer the added advantage that direct calcium binding to the myosin head triggers the onset of actin-activated MgATPase activity; thus, regulatory changes are linked to events at the active site and the actin binding site by some complex allosteric mechanism [for a review, see Szent-Gyorgyi and Chantler (1986)]. Furthermore, the facile and reversible removal of regulatory light chains from scallop myosin (Kendrick-Jones et al., 1976; Chantler & Szent-Gyorgyi, 1980) and the incorporation of labeled, foreign regulatory light chains into scallop myosin, resulting in functional hybrids (Kendrick-Jones et al., 1976; Sellers et al., 1980), make is possible to use sites on the regulatory light chains as topographic markers (Chantler & Tao, 1986; Chantler & Kensler, 1989).

In this study, we use resonance energy transfer (RET)[1] to explore the proximity relationship between Cys-55 on the *Mercenaria* regulatory light chain and SH-1 on the scallop hybrid myosin (into which the *Mercenaria* light chain was incorporated). We measured this novel distance to be ~6.4 nm. We have examined the effects of calcium, MgATP, and actin on the efficiency of energy transfer between these two sites in an attempt to observe perturbations in this distance in response to the addition of physiological ligands. Further, we have used probe-labeled actin in order to investigate the proximity of actin Cys-374 to the two myosin head sites we have used in our experiments. We compared our results to those of others who have used vertebrate skeletal muscle myosin for SH-1 labeling. The results presented in this paper are consistent with current views of myosin head topography. This work has been presented in preliminary form (Park et al., 1990).

Materials and Methods

Protein Preparations. Myofibrils, extracted from the scallop (*Aequipecten irradians*) striated adductor muscle, myosin from the same source, and regulatory light chains from the clam

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[1] Abbreviations: DABMI, *N*-[4-[[4-(dimethylamino)phenyl]azo]-phenyl]maleimide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; RET, resonance energy transfer; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HMM, heavy meromyosin; 1,5-IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; S-1, myosin subfragment 1.

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(*Mercenaria mercenaria*) red adductor muscle were all prepared by methods now standard in our laboratory (Chantler & Szent-Gyorgyi, 1978; Chantler & Bower, 1988). Rabbit skeletal muscle actin was prepared by established procedures (Kendrick-Jones et al., 1970).

Desensitization of myofibrils or myosin was performed by using 10 mM EDTA for 5 min at an elevated temperature (25 °C for myosin; 35 °C for myofibrils) as described earlier (Chantler & Szent-Gyorgyi, 1980; Chantler & Tao, 1986). *Aequipecten* HMM was prepared by using a protocol based on standard procedures (Chantler et al., 1981; Craig et al., 1980). Myosin (10 mg/mL in 0.6 M NaCl, 10 mM phosphate, 5.0 mM $MgCl_2$, and 1.0 mM $CaCl_2$, pH 7.0) was mixed with trypsin (1:400 weight ratio of trypsin/myosin) for 1 min at 23 °C, the reaction being terminated by the addition of soybean trypsin inhibitor (2:1 weight ratio of inhibitor to trypsin). Dialysis against a low ionic strength buffer brought about precipitation of myosin rods and any remaining undigested myosin, leaving HMM in the supernatant after centrifugation.

Pure hybrid myosin molecules, possessing one modified *Mercenaria* regulatory light chain on each myosin head, were constructed from scallop myosin or myofibrils by using earlier published techniques (Sellers et al., 1980; Chantler & Tao, 1986; Chantler & Bower, 1988). SH-1 modification of myosin was usually performed on intact myosin at low ionic strength prior to desensitization procedures (in the case of hybrid myosins), but the same end result could also be achieved upon modification of intact myosin at high ionic strength (see Results).

Protein Modifications. Mercenaria regulatory light chains were reacted with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine (1,5-IAEDANS) or *N*-[4-[[4-(dimethylamino)phenyl]azo]phenyl]maleimide (DABMI) (both from Molecular Probes Inc., Eugene, OR) essentially as described previously (Chantler & Tao, 1986). Briefly, light chains possessing free sulfhydryl groups but in the absence of a reducing reagent were reacted with 1,5-IAEDANS (5 molar ratio of label to protein) or DABMI (2 molar ratio of label to protein) in 8 M guanidine hydrochloride, 50 mM Tris, 40 mM NaCl, and 3 mM NaN_3, pH 7.5, at room temperature for 2 h, under nitrogen with gentle stirring. The reaction was terminated with the addition of a 100 molar excess of dithiothreitol (DTT) over the reagent. The mixture was then passed over a Sephadex G-25 column and the breakthrough peak pooled and lyophilized prior to resuspension in, and dialysis against, 10 mM phosphate, 20 mM NaCl, and 6 mM NaN_3, pH 7.0. The stoichiometry of labeling was determined by using an extinction coefficient of 6.0×10^3 M^{-1} cm^{-1} at 337 nm for 1,5-IAEDANS (Hudson & Weber, 1973) and of 2.48×10^4 M^{-1} cm^{-1} at 460 nm for DABMI (Tao et al., 1983). We obtained labeling in the range 0.6–1.0 mol of probe/mol of light chain.

The labeling of Cys-374 of actin with DABMI was performed essentially as described earlier (Tao et al., 1983). In essence, labeling was performed overnight in the G-actin state in 2 mM HEPES, 0.25 mM ATP, and 0.1 mM $CaCl_2$, pH 7.5, using a 2-fold molar ratio of DABMI to actin. Separation from unreacted reagent and additional purification were achieved by repeated cycles of polymerization and depolymerization. Labeling stoichiometry, assessed by using the extinction coefficients referred to above, was 0.92 mol of probe/mol of actin monomer.

The reactive SH-1 thiol of scallop myosin was labeled with a 2-fold molar ratio of either 1,5-IAEDANS or DABMI per myosin molecule using techniques similar to those described by Titus (1988), but using nitrogen as opposed to an argon atmosphere. The reaction took place at 4 °C with gentle stirring for either 2 h or overnight in 40 mM NaCl, 5 mM phosphate, 1.0 mM $MgCl_2$, and 3.0 mM NaN_3, pH 7.0. Separation from unreacted reagent was achieved by repeated washing cycles in the above buffer, the thick filaments being spun down after each wash. Similar specific labeling was also achieved by modification of myosin solubilized in a high ionic strength buffer containing 0.6 M NaCl but otherwise similar to the above. Labeling stoichiometry was assessed by using the extinction coefficients referred to above and always found to be in the range 0.9–1.1 mol of label/mol of myosin heads. Upon proteolysis, label was confined to the myosin head as expected. Detailed analysis of the products of the labeling reaction under these conditions confirms specific labeling at the SH-1 site (Dr. Margaret Titus, personal communication).

Fluorescence Measurements. Most fluorescence results were obtained by lifetime measurements carried out on a modified ORTEC 9200 nanosecond fluorometer using techniques described earlier (Tao & Cho, 1979; Chantler & Tao, 1986). Fluorescence lifetimes were extracted from the decay curves by methods-of-moments analysis. The transfer efficiency (E) was calculated according to eq 1 where τ_D and τ_{DA} are the

$$E = 1 - (\tau_{DA}/\tau_D) \quad (1)$$

donor fluorescence lifetimes in the absence and presence of acceptor, respectively. Steady-state fluorescence measurements were carried out on a Perkin-Elmer MPF-66 instrument, the sample being maintained at a constant temperature in the range 15–17 °C. Corrected emission spectra were obtained in the ratio mode upon excitation at the long-wavelength excitation maximum (~340 nm for 1,5-IAEDANS). The absorbance was kept below 0.05 at all wavelengths studied, and a 350-nm cutoff filter was sometimes used to reduce scattered light. The efficiency of energy transfer by donor quenching was calculated from the decrease in fluorescence intensity at the emission maximum brought about by the presence of acceptor:

$$E = 1 - (I_{DA}/I_D) \quad (2)$$

where I_{DA} and I_D are donor emission intensities in the presence and absence of acceptor, respectively. Emission maxima were sometimes determined relative to normalized spectra obtained in the presence of 8 M guanidine hydrochloride (Chantler & Tao, 1986).

The interprobe distance (R) was calculated from transfer efficiencies by using equations originally developed by Forster (1948, 1959) and reviewed by Fairclough and Cantor (1978) and Stryer (1978):

$$R = R_0(E^{-1} - 1)^{1/6} \quad (3)$$

$$R_0^6 = (8.78 \times 10^{-5})\kappa^2 n^{-4} q J \text{ (in Å}^6) \quad (4)$$

where R_0 is the critical transfer distance, or distance at which $E = 0.5$, κ^2 is the orientation factor, taken as $^2/_3$ here, an assumption whose validity will be discussed, and q is the quantum yield. q was calculated from τ_D by using earlier accurately determined values obtained with 1,5-IAEDANS-labeled tropomyosin (Tao et al., 1983); i.e., $q = 0.53(\tau_D/13.3)$. J is the overlap integral obtained from the normalized donor emission and acceptor absorption spectra by numerical integration [i.e., $J = \int F(\lambda)\epsilon(\lambda)\lambda^4 \, d\lambda / \int F(\lambda) \, d\lambda$] where F is the corrected fluorescence spectrum of the donor and ϵ is the molar extinction coefficient of the acceptor).

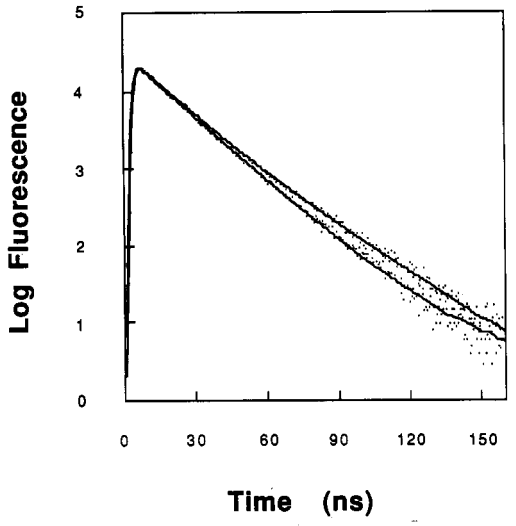

FIGURE 1: Fluorescence decay curves of *Mercenaria* regulatory light chain labeled at Cys-55 with 1,5-IAEDANS by itself (RLC^{DAN}, lower curve) and reconstituted with desensitized scallop myosin (RLC^{DAN}–myosin, upper curve). Dots are experimental points. Solid lines are calculated curves using decay parameters derived from method-of-moments analysis. The fluorescence decay of RLC^{DAN} alone is essentially monoexponential (τ_1 = 14.89 ns, A_1 = 0.983; τ_2 = 32.35 ns, A_2 = 0.017), while that of RLC^{DAN}–myosin is biexponential (τ_1 = 14.08 ns, A_2 = 0.836; τ_2 = 24.50 ns, A_2 = 0.164), where τ's are the lifetimes and A's are the fractional amplitudes of the fluorescence decay, respectively.

Anisotropy decay (Tao, 1978) and acrylamide quenching (Tao & Cho, 1979) measurements were carried out as described previously, the measurements being made at 25 °C.

Other Procedures. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed by using standard procedures (Matsudaira & Burgess, 1978). Protein concentrations were determined either by the Folin–Lowry procedure (Lowry et al., 1951) or by the Bradford method (Bradford, 1976), using bovine serum albumin as standards. Binding studies, performed under the same conditions as the fluorescence measurements, were undertaken alongside steady-state experiments involving actin. Material was spun down at >100000*g* for 30 min, the supernatant was separated from the pellet, and the relative proportions of actin and myosin in both were analyzed and quantitated by fluorescence emission and SDS/acrylamide gel electrophoresis.

Results

Fluorescence of 1,5-IAEDANS Attached to Cys-55 of the Mercenaria Regulatory Light Chain, either on the Light Chain Alone or Bound to the Hybrid Myosin. The fluorescence decay curve of 1,5-IAEDANS attached to the sole sulfhydryl (Cys-55) of the *Mercenaria* regulatory light chain was measured in the absence and presence of desensitized scallop myosin (scallop myosin from which all indigenous regulatory light chain had been removed). Figure 1 shows that whereas the decay of the donor-labeled light chain alone is essentially monoexponential (τ = 14.9 ns), the presence of the heavy chain induced the appearance of a long-lived component (τ = 24.5 ns) in addition to a component with the shorter decay constant (τ = 14.1 ns). Acrylamide quenching studies (Figure 2) indicate that this long-lived component is considerably less quenchable by acrylamide in the medium than either the short-lived component of the decay of hybrid myosin or that of the donor-labeled light chain by itself.

Anisotropy decay measurements (Figure 3) show that the free labeled light chain can tumble fairly rapidly [with a rotational correlation time (ϕ) corresponding to ~6 ns]. This

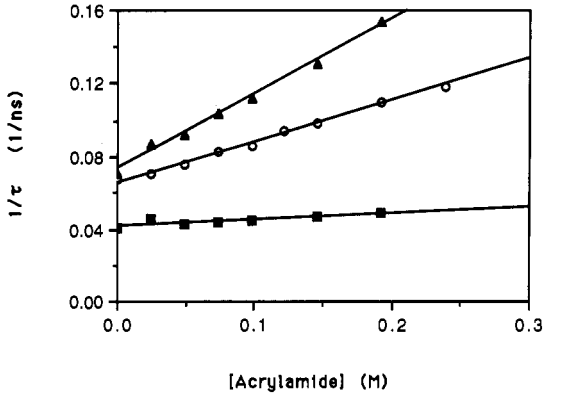

FIGURE 2: Acrylamide quenching of the fluorescence of donor-labeled (1,5-IAEDANS) *Mercenaria* regulatory light chain (RLC^{DAN}) either alone or following reconstitution with desensitized scallop myosin (RLC^{DAN}–myosin). The rate of fluorescence decay ($k = 1/\tau$) is plotted against the molar concentration of acrylamide, and the resulting curves are fitted according to the Stern–Volmer equation: $1/\tau = 1/\tau_0 + k_q[Q]$, where τ and τ_0 are the fluorescence lifetimes in the presence and absence of quencher, respectively, [Q] is the acrylamide concentration, and k_q is the Stern–Volmer quenching rate constant. For RLC^{DAN} alone (open circles), $k_q = (2.25 \pm 0.05) \times 10^8$ M^{-1} s^{-1} and τ_0 = 15.17 ns. For the short-lived (major) component of RLC^{DAN}–myosin (closed triangles), $k_q = (4.11 \pm 0.16) \times 10^8$ M^{-1} s^{-1}, τ_0 = 13.65 ns, and average fractional amplitude = 0.800 (range of 0.745–0.836). For the long-lived (minor) component of RLC^{DAN}–myosin (closed squares), $k_q = (0.338 \pm 0.089) \times 10^8$ M^{-1} s^{-1}, τ^0 = 23.88 ns, and average amplitude = 0.200.

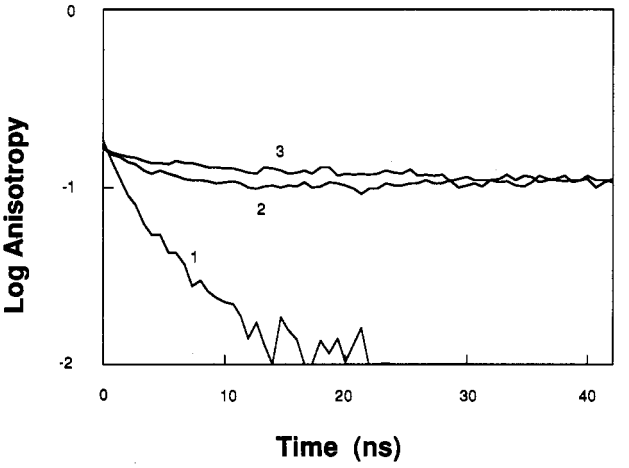

FIGURE 3: Anisotropy decay curves of 1,5-IAEDANS-labeled *Mercenaria* regulatory light chain either alone (RLC^{DAN}, curve 1) or after (RLC^{DAN}–myosin, curve 2) reconstitution with desensitized scallop myosin and of scallop myosin labeled by donor (1,5-IAEDANS) at SH-1 ($SH\text{-}1^{DAN}$–myosin, curve 3). ϕ_c = 6 ns for RLC^{DAn} alone, and ϕ_c > 240 ns for both RLC^{DAN}–myosin and $SH\text{-}1^{DAN}$–myosin, where ϕ_c is the rotational correlation time.

tumbling motion is slowed down considerably when the light chain is reconstituted within the myosin molecule (ϕ > 240 ns). Earlier measurement of the limiting anisotropy using steady-state Perrin plots (Chantler & Tao, 1986) demonstrated that 1,5-IAEDANS bound at this site exhibits rapid depolarization on a subnanosecond time scale. Extrapolation of the decay curves shown here to zero time give values consistent with this conclusion, allowing, as they do, an estimate of the limiting anisotropy, albeit less precise.

Fluorescence of 1,5-IAEDANS Attached to SH-1 of Scallop Myosin. The fluorescence decay of 1,5-IAEDANS attached to SH-1 of scallop myosin is essentially monoexponential with a lifetime of 19–21 ns (Figure 4; Tables I and II) (as seen in Table I, two-exponential analysis yielded a second component of negligible amplitude with only small changes

Table I: Fluorescence Decay Parameters of the DAN Donor in the Absence and Presence of the DAB Acceptor[a]

sample	A_1	τ_1 (ns)	A_2	τ_2 (ns)	χ^2/N
SH-1^{DAN}–myosin, Ca^{2+}	1.0	20.75			3.53
	0.933	19.39	0.067	32.39	1.63
SH-1^{DAN}–RLC^{DAB}–myosin, Ca^{2+}	1.0	19.21			3.92
	0.926	18.02	0.074	28.70	2.36
SH-1^{DAN}–myosin, EGTA	1.0	20.98			3.69
	0.997	20.64	0.003	62.32	2.52
SH-1^{DAN}–RLC^{DAB}–myosin, EGTA	1.0	19.25			3.72
	0.991	18.78	0.009	43.13	3.39
SH-1^{DAN}–myosin, ATP	1.0	21.26			3.94
	0.999	21.12	0.001	68.44	3.52
SH-1^{DAN}–RLC^{DAB}–myosin, ATP	1.0	19.31			4.04
	0.997	19.00	0.003	55.91	3.36
SH-1^{DAN}–myosin–F-actin	1.0	20.26			3.32
	0.913	18.75	0.087	30.25	1.58
SH-1^{DAN}–RLC^{DAB}–myosin–F-actin	1.0	18.93			5.04
	0.757	16.26	0.243	24.46	2.91
SH-1^{DAN}–myosin–F-actin	1.0	21.45			3.89
	0.922	20.01	0.078	32.20	3.15
SH-1^{DAN}–myosin–F-actin^{DAB}	1.0	18.74			10.52
	0.535	11.57	0.465	22.92	1.79
RLC^{DAN}–myosin	1.0	16.86			9.07
	0.859	14.38	0.141	25.40	2.38
RLC^{DAN}–SH-1^{DAB}–myosin	1.0	15.04			8.38
	0.914	13.34	0.086	24.72	4.60
RLC^{DAN}–myosin	1.0	16.39			11.02
	0.730	12.27	0.270	22.41	1.63
RLC^{DAN}–myosin–F-actin^{DAB}	1.0	15.65			7.43
	0.832	13.12	0.168	22.89	3.00

[a] Parameters were determined from the decay curves by one- and two-exponential method-of-moments analyses. A_1 and A_2 are fractional amplitudes. τ_1 and τ_2 are lifetimes. χ^2/N is the mean squared residual where $\chi^2 = \sum_{i=1}^{N}(c_i - e_i)^2/e_i$, c_i and e_i are the ith calculated and experimental points, respectively, and N is the number of points.

Table II: Parameters of RET between Probes Attached at Cys-55 of *Mercenaria* Regulatory Light Chain, SH-1 of Scallop Myosin Heavy Chain, and Cys-374 of F-Actin[a]

donor location	acceptor location	condition	τ_D (ns)	τ_{DA} (ns)	E	R_0 (nm)	R (nm)
SH-1	RLC	Ca^{2+}	19.39	18.02	0.071	4.3	6.6
SH-1	RLC	EGTA	20.64	18.78	0.090	4.3	6.3
SH-1	RLC	ATP	21.12	19.00	0.100	4.3	6.2
SH-1	RLC	actin	18.75	16.26	0.133	4.3	5.9
RLC	SH-1	Ca^{2+}	14.38	13.34	0.072	4.0	6.1
SH-1	actin	Ca^{2+}	20.01	11.57	0.422	4.3	4.5
RLC	actin	Ca^{2+}	12.27	13.12	−0.07	4.0	>8.0

[a] τ_D and τ_{DA} are donor fluorescence lifetimes in the absence and presence of acceptor, respectively, using major decay component lifetimes derived from two-exponential method-of-moments analysis (Table I). E is the transfer efficiency, defined as $E = 1 - (\tau_{DA}/\tau_D)$. R_0 is the critical transfer distance assuming that the orientation factor $\kappa^2 = {}^2/_3$. R is the apparent distance, calculated from $R = R_0(E^{-1} - 1)^{1/6}$. The donor is the DAN moiety of 1,5-IAEDANS; the acceptor is the DAB moiety of DABMI.

in the major component lifetime). This is identical with that of vertebrate skeletal muscle myosin labeled at SH-1 with 1,5-IAEDANS (Mendelson et al., 1973; Tao & Lamkin, 1981). This lifetime is invariant with whether labeling was carried out at high or low ionic strengths, or with subsequent desensitization (i.e., removal of light chain) and resensitization (i.e., readdition of light chain) (data not shown).

The anisotropy decay curve for scallop myosin labeled at SH-1 with 1,5-IAEDANS is seen in Figure 3. The decay exhibits a slow tumbling rate ($\phi > 240$ ns) similar to that of the label attached to myosin-bound light chain.

RET between Cys-55 of the Mercenaria Regulatory Light Chain and SH-1 on the Hybrid Myosin Heavy Chain. Resonance energy transfer between Cys-55 of the *Mercenaria* regulatory light-chain bound to myosin and SH-1 on the myosin heavy chain was measured in both directions (i.e., either with the donor, 1,5-IAEDANS, attached to Cys-55 and the acceptor, DABMI, bound to SH-1 or with the donor attached to SH-1 and the acceptor bound to Cys-55). Examples of decay curves are shown in Figure 4, and lifetimes are tabulated in Table I. Quenching of donor fluorescence is readily apparent by inspection of the decay curves. When method-of-moments analysis was applied to the decay curves and the results were inserted into the Forster equations (see Materials and Methods), the most probable distance of sep-

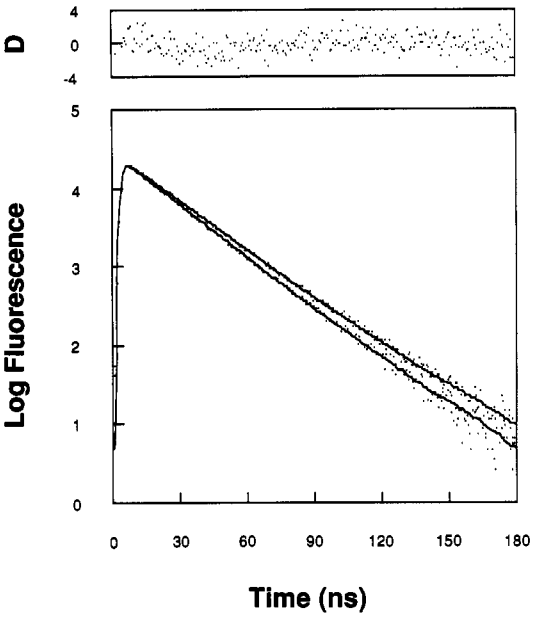

FIGURE 4: RET determination of the distance between SH-1 on the scallop myosin heavy chain and Cys-55 of *Mercenaria* regulatory light chain. Top and bottom curves are for SH-1^{DAN}–myosin and SH-1^{DAN}–RLC^{DAB}–myosin, respectively, both in the presence of calcium. Dots are experimental points; solid lines are calculated fits using parameters derived from two-exponential method-of-moments analysis (Table I). The top panel is the weighted deviation function for the top curve, defined as $D = (c_i - e_i)/e_i^{1/2}$, where c_i and e_i are the ith calculated and experimental points, respectively.

aration between these two sites was calculated to be 5.9–6.6 nm (Table II). At high ionic strength, the efficiency of energy transfer was invariant with respect to the presence or absence of 0.1 mM $CaCl_2$, 1.0 mM MgATP, or a 10-fold molar ratio of actin to myosin.

Similar results were obtained at low ionic strength using HMM derived from the labeled myosins. The intensities ($\lambda_{em,max}$ = 472 nm; λ_{ex} = 342 nm) of the emission spectra of both donor–acceptor-labeled and donor-labeled HMM were not altered by the presence or absence of the above ligands (data not shown).

RET between Cys-55 of the Mercenaria Regulatory Light Chain on the Hybrid Myosin and Cys-374 on F-Actin. The efficiency of energy transfer between Cys-55 of the *Mercenaria* regulatory light chain bound to myosin and Cys-374 sites on F-actin was negligibly small (Table II), indicating that the distance separating these sites is, in all probability, >8.0 nm. Similar results were obtained in steady-state experiments, both at high ionic strength using myosin and at low ionic strength using HMM. This efficiency was in no way altered by the presence or absence of calcium.

RET between SH-1 on the Heavy Chain of Scallop Myosin and Cys-374 on F-Actin. The fluorescence decay of scallop myosin labeled at SH-1 with 1,5-IAEDANS in the presence of F-actin labeled at Cys-374 with DABMI is composed of two components: One component is short-lived with τ = 11.6 ns; the other is long-lived with τ = 22.9 ns, the latter being comparable to the unquenched lifetime of τ = 20.0 ns (Table I). Taking the short-lived lifetime as τ_{DA} and the unquenched lifetime as τ_D, the Forster equations yielded a distance of ~4.5 nm (Table II). This distance was unaltered by the presence or absence of calcium.

Discussion

These experiments were initiated with a view to increasing our understanding of the complex allosteric mechanism which must control polypeptide or domain motion within the head of a regulatory myosin. Calcium binding to specific sites on an invertebrate myosin head primes the molecule for interaction with actin (Chantler et al., 1981). This information is relayed to the active site and the actin binding site by a process involving relative movement between the regulatory and the essential light chain, such events being initiated even in the complete absence of actin (Hardwicke et al., 1983; Wells & Bagshaw, 1985). The ability to form functional myosin hybrids possessing probe-labeled light chains using scallop myosin as a carrier (Chantler & Szent-Gyorgyi, 1978; Sellers et al., 1980; Chantler & Tao, 1986) makes such hybrids ideal vehicles for energy-transfer studies. *Mercenaria* regulatory light chains possess a single sulfhydryl group (Cys-55) which may be labeled specifically and has been localized to within 3.5 nm of the head–rod junction (Chantler & Kensler, 1989). Hence, this residue is well situated, when probe-labeled and placed within a hybrid myosin, to monitor potential ligand-induced changes in the spatial relationships between itself and SH-1, on the myosin heavy chain, or Cys-374 on actin, by RET.

The monoexponential fluorescence decay exhibited by the donor-labeled *Mercenaria* regulatory light chain alone (Figure 1) is consistent with a single population of labeled sites. These Cys-55 sites are relatively exposed to the aqueous medium as reflected by the relatively short lifetime and the large quenching constant derived from the acrylamide quenching studies (Figure 2). Combination of the probe-labeled light chain with myosin heavy chain induces the appearance of a second long-lived component (Figure 1) which is considerably less quenchable by acrylamide than the short-lived component of either the bound or the free light chain (Figure 2). It would appear that when the light chain is bound to the heavy chain a proportion of the probe (~20%) at any one time interacts directly with the heavy chain, which serves to shield the probe from the aqueous medium. This could indicate that the region of the bound light chain in the vicinity of Cys-55 is somewhat flexible and can flip from one environment to another with respect to the heavy chain.

Anisotropy decay measurements (Figure 3) show that the free 1,5-IAEDANS-labeled light chain undergoes a rapid tumbling motion as expected for a molecule of M_r 18000. The rate of this tumbling motion is reduced considerably when the light chain becomes incorporated into the myosin molecule when it is comparable to that of myosin labeled at SH-1 with 1,5-IAEDANS. These results indicate that the majority of the added probe-labeled light chain is bound to the desensitized myosin, consistent with data obtained by urea/acrylamide gel electrophoresis (Chantler & Szent-Gyorgyi, 1980; Sellers et al., 1980). It may be noted that the decay appears to be biexponential at early time points. This could indicate the presence of some unbound light chain. Alternatively, it would indicate a minor population of molecules possessing a more mobile region in the vicinity of Cys-55, consistent with the acrylamide quenching studies (see above). For the majority of the population of molecules, the Cys-55 region of the light-chain polypeptide chain backbone appears to be immobilized relative to the heavy chain.

The specificity of labeling of scallop myosin SH-1 by 1,5-IAEDANS has been determined (Titus et al., 1986; Dr. Margaret Titus, personal communication). Our labeling protocols were based on their techniques. Localization of label within head polypeptides upon proteolysis (data not shown), to produce either S-1 or HMM, is consistent with the anticipated specificity. Furthermore, fluorescence lifetimes for IAEDANS-labeled SH-1 scallop myosin were similar to those obtained by others on vertebrate myosins (Mendelson et al., 1973; Botts et al., 1979; Tao & Lamkin, 1981; Dalbey et al., 1983), suggestive of identical label specificity. However, the need to incorporate a labeled light chain into desensitized SH-1-labeled scallop myosin caused us to investigate whether or not the immediate environment of the labeled SH-1 site was affected by desensitization and resensitization or whether the labeled site presented different decay characteristics depending on whether labeling was carried out at low or high ionic strength. No significant difference in probe lifetime could be demonstrated irrespective of labeling intact myosin at either low or high ionic strength, or upon subsequent desensitization and resensitization of the labeled species (data not shown). Desensitization of 1,5-IAEDANS SH-1-labeled scallop myosin appeared to be equally facile to desensitization of the unmodified species.

The main novel distance determined in this study by RET is between Cys-55 of the *Mercenaria* regulatory light chain bound to scallop myosin and SH-1 located on the scallop myosin heavy chain. With the donor attached to Cys-55 of the *Mercenaria* regulatory light chain and the acceptor attached to SH-1, an apparent interprobe distance of 6.4 nm was obtained. When the locations of the donor and acceptor were switched, a distance of 6.1 nm was obtained, in good agreement with the former distance (Table I). Both distances were calculated under the assumption that the orientation factor, κ^2, takes on the isotropically averaged value of $^2/_3$, the most probable value for κ^2 (Hillel & Wu, 1976). The validity of this assumption was discussed in detail in our previous work

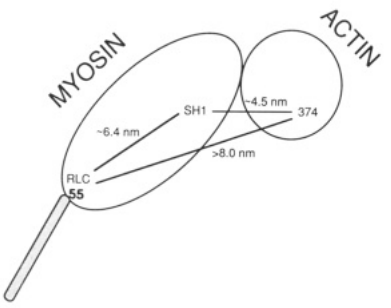

FIGURE 5: Schematic diagram depicting the proximity relationship between SH-1 of the heavy chain, Cys-55 of the regulatory light chain, and Cys-374 of actin.

(Chantler & Tao, 1986), in which the limiting anisotropy for 1,5-IAEDANS-labeled *Mercenaria* regulatory light chain was measured by steady-state fluorescence depolarization to be 0.21 (time-dependent anisotropy decay measurements carried out in this work yielded a similar value). This value is significantly smaller than the theoretical maximum of 0.4, indicating considerable randomization of the probe's transition moment orientation, and partially justifies our use of $\kappa^2 = {}^2/_3$. A quantitative treatment using Stryer's formalism (Stryer, 1978) yielded $0.70 < \alpha < 1.31$, with an 80% probability that $0.75 < \alpha < 1.13$, where $\alpha = R/R_{2/3}$, $R_{2/3}$ is the apparent distance calculated by using $\kappa^2 = {}^2/_3$, and R is the actual distance. Applied to the present case in which $R_{2/3} = 6.3$ nm, we obtain ranges in R of $4.8 < R < 9.0$ nm (100% probability) and $5.6 < R < 8.4$ nm (80% probability). The above calculations were carried out under the assumption that the orientation of the acceptor's transition moment remains unchanged. In reality, some randomization of the acceptor's transition moment can be expected, so the ranges in R can be expected to be even smaller.

It is noteworthy that the same apparent distance was obtained when we switched the locations of the donor and acceptor. If the donor and acceptor transition moments are completely fixed, it would be highly fortuitous if one were to obtain the same κ^2 and apparent distance upon switching locations. The fact that experimentally we obtained nearly the same apparent distance is therefore in support of our isotropic model for κ^2.

The data shown in Table II indicate that, for myosin, the efficiency of energy transfer does not change irrespective of the presence or absence of 0.1 mM calcium, ±1.0 mM MgATP, ±actin (a 10 molar ratio of actin to myosin). Because high ionic strength may abolish calcium-sensitive changes within the myosin molecule, as judged by intrinsic fluorescence measurements (Wells et al., 1985), we have also performed experiments at low ionic strength on HMM derived from hybrid myosins. Such preparations gave similar results to those of the parent myosin. One must be cautious, however, in interpreting these results as evidence for a lack of large-scale conformational change within the region of the heavy chain connecting SH-1 to the N-terminal portion of the regulatory light chain, during normal regulation. There is evidence to suggest that calcium sensitivity is compromised upon labeling at SH-1 (Titus & Szent-Gyorgyi, 1986). Our results suggest that if any part of the putative conformational relay system remains after SH-1 modification, then its mechanism does not involve a change in proximity between sites located at SH-1 and the N-terminal region of the regulatory light chain.

The efficiency of energy transfer between Cys-55 of the regulatory light chain and Cys-374 residues on F-actin was negligibly small (Table II), suggesting a distance between probes of >8.0 nm. This efficiency did not appear to be affected by the presence or absence of calcium (0.1 mM) or MgATP (1.0 mM) at either high (myosin) or low (HMM) ionic strength. However, at such low efficiencies of energy transfer, it is difficult to rule out any movement which may lead to a greater separation between probes. At any event, it is clear that under all circumstances Cys-55 on the regulatory light chain is remote from Cys-374 on actin.

The efficiency of energy transfer between SH-1 of scallop myosin and Cys-374 on F-actin gave rise to an estimated distance of around 4.5 nm, which was not altered in any way by the presence or absence of calcium (0.1 mM) or MgATP (1.0 mM) at either high (myosin) or low (HMM) ionic strength. This is the first time that this distance has been measured in a regulatory myosin although it has been measured by RET using rabbit skeletal muscle myosin by at least three different groups (Takashi, 1979; Trayer & Trayer, 1983; Miki & Wahl, 1984). Our distance is comparable to those measured on skeletal myosin, the latter ranging from 5.0 through 6.0 nm.

The results presented in this paper have been incorporated into a simple, self-consistent schematic diagram (Figure 5), which shows that Cys-55 of the regulatory light chain is relatively close to the neck region of the myosin head and SH-1 is located in the middle portion of the S-1 head, ~6.4 nm from Cys-55. Actin binds near the tip of S-1; Cys-374 of actin is ~4.5 nm from SH-1 and considerably further (>8.0 nm) from Cys-55. This scheme can be readily incorporated into more complex schemes devised from RET and other studies (Botts et al., 1984; dos Remedios et al., 1987; Tokunaga et al., 1987). Cys-55 on the *Mercenaria* regulatory light chain has been localized by biotin–avidin labeling and rotary-shadowing electron microscopy to be 1.5–3.5 nm from the head–rod junction of scallop striated adductor myosin (Chantler & Kensler, 1989), while SH-1 in vertebrate skeletal muscle myosin has been localized by using the same technique to be 13 nm from the head–rod junction (Sutoh et al., 1984; Tokunaga et al., 1987). This implies that the distance between Cys-55 and SH-1 cannot be less than 9.5 nm, considerably larger than the 6.4 nm that we obtained here. It should be noted, however, that both RET and rotary-shadowing electron microscopy yield measurements which are subject to fairly large errors [up to 20% for both sets of measurement in this case (Stryer, 1978; Sutoh et al., 1984)], perhaps too large for meaningful quantitative comparisons. Qualitatively, it is clear that both techniques support the scheme we propose in Figure 5.

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Discontinuous Release of Heat at Successive Steps of Oxygenation in Human and Bovine Hemoglobin at pH 9.0†

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ABSTRACT: We have measured the temperature dependence of the oxygen-binding isotherms of human and bovine hemoglobin at pH 9.0 in 0.1 M borate buffer. In both hemoglobins the ionization of the Bohr protons is finished at this pH; therefore, their heat does not interfere with the measurements. Two sets of curves have been obtained, which have been analyzed by either singular or global procedures for estimating the enthalpy changes of subsequent steps of oxygenation. The data indicate that in human hemoglobin the reaction with oxygen is enthalpy driven for steps 1, 2, and 4 while it is entropy driven for step 3. In bovine hemoglobin this phenomenon is even more evident: steps 2 and 4 are enthalpy driven while steps 1 and 3 are entropy driven. The discontinuous distribution of heat at subsequent steps of oxygenation suggests that the T to R transition in hemoglobin is not a monotonic process and involves conformations with novel characteristics.

Cooperativity of oxygen binding in hemoglobin implies the transition from a low to a high-affinity conditions, which indicates a progressive modulation of the free energy of the reaction. The intrinsic reactivity of the heme iron for oxygen is constant; therefore, the changes are due to allosteric modulations of the thermodynamic parameters of the system. Thus, measuring the temperature dependence of the oxygen affinity may provide information on the heat of the conformational changes that modulate the system. The distribution of these heats at subsequent steps of oxygen binding may reveal critical conformational events of the system during oxygenation.

The data available in the literature are uncertain whether the enthalpy of oxygenation is linear with successive oxygenation steps. Data presented by Imai (1979) indicate that at alkaline pH the enthalpy is sharply less exothermic for the third step of oxygenation. Calorimetric measurements of Parodi-Monreale et al. (1987) indicate linearity for steps 1,

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