Fluorescence Energy Transfer between Subfragment-1 and Actin Points in the Rigor Complex of Actosubfragment-1†

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ABSTRACT: The fast-reacting thiol (SH_1) of myosin subfragment-1 (S-1) was covalently and specifically labeled with (iodoacetamido)fluorescein (IAF), while Cys-373 of actin was also covalently and preferentially labeled with *N*-(iodoacetyl)-*N'*-(1-sulfo-5-naphthyl)ethylenediamine (1,5-IAEDANS). The method of fluorescence energy transfer was used to examine the spatial proximity between the two sites, i.e., SH_1 and Cys-373, in the rigor complex of acto-S-1. Approximately 30% fluorescence energy transfer was observed from the 1,5-IAEDANS on actin as a donor to the IAF on S-1 as an acceptor in their rigor complex; under certain assumptions this corresponds to a distance of ca. 6.0 nm.

It is currently postulated that the thrust in muscle contraction originates in a change in relation between the S-1[1] moiety and actin (in the organized system this is expressed as a change in the attitudinal angle of S-1 referred to the axis of the actin filament). To examine for such a change, it is essential that we begin collecting pairs of proximal points, one on S-1 and one on actin; if it occurs, the postulated change might then be defined by noting that some pairs of points separate, while others come together. This report is a beginning on this difficult task. While our results are semiquantitative, we do show that a chemically defined point on myosin S-1 is proximal—within fluorescence energy transferring distance—to a chemically defined point on actin.

Of course, there have been previous observations made that indirectly suggest myosin–actin proximities. For example, recently we[2] showed that in the acto-S-1 rigor complex the reactivities of SH_1 on S-1 and of Cys-373 on actin are reciprocally affected over what they are in the uncomplexed molecules; i.e., the former reactivity is depressed, and the latter is enhanced (Duke et al., 1976). However, it could always be thought that such an effect is transmitted over a great distance by some structural distortion within the molecule. In this work we have placed specifically a resonance energy acceptor (fluorescein) on SH_1 and a donor (dansyl) on Cys-373, and then we have shown that ca. 30% of the energy absorbed on the dansyl is transferred to fluorescein—an event that would be very unlikely if the two points were more than about 10 nm from one another (Förster, 1948, 1959).

Byproducts of this investigation were that (1) this labeling of either actin or S-1 affects the Mg^{2+}–ATPase activity of their complex, (2) this labeling of SH_1 actually improves the Ca^{2+}–ATPase activity of S-1 if the enzyme is subjected to increasing K^+ and Cl^-, and (3) certain strategies intended to reveal the role of the orientational factor in energy transfer—neutral ion structure disruption and interchange of fluorophores—have no effect on the energy transfer efficiency.

Experimental Procedures

Materials

Myosin was prepared from rabbit back muscle according to Tonomura et al. (1966). S-1 was prepared from myosin digested by insoluble papain in the absence of Mg^{2+} according to Lowey et al. (1969). Actin was prepared from acetone powder of rabbit skeletal muscle by using the methods of Spudich & Watt (1971). L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone (TPCK)–trypsin was prepared according to the methods of Carpenter (1967). IAF [5-(iodoacetamido)fluorescein] and 1,5-IAEDANS [*N*-(iodoacetyl)-*N'*-(1-sulfo-5-naphthyl)ethylenediamine] were synthesized by R. P. Haugland while he was in our laboratory. [^{3}H]-1,5-IAEDANS was also synthesized by R. P. Haugland according to the procedures of Huang et al. (1975). Papain, trypsin, ATP, TPCK, and N^{α}-*p*-tosyl-l-lysine chloromethyl ketone hydrochloride were purchased from Sigma Chemical Co. All other chemicals were analytical grade.

Methods

Labeling of S-1 and Actin. S-1 (40 μM) was labeled with a 10-fold molar excess of IAF in a solution of 0.15 M KCl and 50 mM Tes buffer at pH 7.0 and 0 °C for 30 min in the dark. The labeling was terminated upon addition of a 100-fold molar excess of β-mercaptoethanol over dye. Subsequently, the S-1 labeled with IAF was separated from free dye by a Sephadex G-25 column equilibrated with a solution of 0.15 M KCl and 50 mM Tes at pH 7.0 and 4 °C. Typically, S-1 labeled as described above possesses about a fourfold higher Ca^{2+}–ATPase activity than the unlabeled control, while EDTA–ATPase decreases to about 30% of control (Figure 1), showing clearly that the fast-reacting thiol (SH_1) of S-1 is specifically labeled with IAF as already well-known by us and other workers using other alkylating reagents (Sekine & Kielley, 1964; Seidel et al., 1970; Takashi et al., 1976). The amount of IAF bound to S-1 was mostly 0.7–0.8 mol of fluorophores per mol of S-1 as described below.

Acto–S-1 (40 μM actin and 20–40 μM S-1) was labeled in a solution of 0.1 M KCl, 5 mM $MgCl_2$, 2 mM EGTA, 50 mM Tes, and 0.4 mM 1,5-IAEDANS at pH 7.0 and 22–23 °C and stirred gently in the dark for 50 min. An addition of a 100-fold

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[1] Abbreviations used: [^{3}H]-1,5-IAEDANS, *N*-(iodo[^{3}H]acetyl)-*N'*-(1-sulfo-5-naphthyl)ethylenediamine; IAF, 5-(iodoacetamido)fluorescein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid; Tes, 2-[*N*-[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Cys-373, cysteinyl residue in position 373 of the amino acid sequence of actin; S-1, subfragment-1 of myosin; HMM, heavy meromyosin; NEM, *N*-ethylmaleimide; SH_1, fast-reacting thiol on the heavy chain of myosin; 1,5-IAEDANS–actin, F-actin labeled with 1,5-IAEDANS; IAF–S-1, S-1 labeled with IAF.

[2] With respect to the location of 1,5-IAEDANS on actin, we originally thought that it was Cys-10. However, further studies (amino acid analysis) have convinced us that it is Cys-373.

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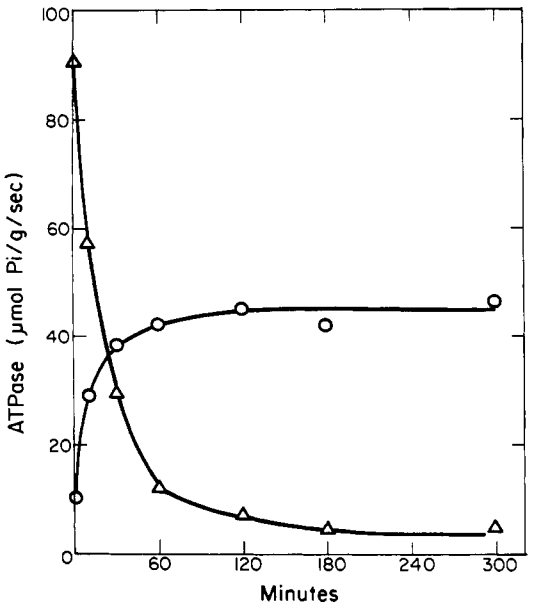

FIGURE 1: Effect of labeling on the ATPase of S-1. S-1 (40 μM) was incubated with a 10-fold molar excess of IAF in a solution containing 0.15 M KCl and 50 mM Tes buffer at pH 7.0 and 0 °C in the dark. ATPase measurement was performed in 0.6 M KCl, 50 mM Tris-HCl at pH 8.0, either (○) 10 mM $CaCl_2$ or (△) 5 mM EDTA, 1 mM ATP, and 0.09 μM IAF–S-1 at 25 °C.

molar excess of β-mercaptoethanol over dye to stop the reaction was followed by dissociation of actin from S-1 by 10 mM ATP, 64 mM $MgCl_2$, and 60 mM Tes in final concentration, at pH 7.5 and 4 °C. It takes only several minutes to form a white actin precipitate under the above conditions. F-Actin was then centrifuged at 160000*g* for 60 min. The actin pellet was gently homogenized in a glass homogenizer and redissolved in 0.15 M KCl and 10 mM Tes at pH 7.0, and then the ATP–$MgCl_2$ solution described above was added to remove a slight amount of contaminant S-1 and was followed by centrifugation as described before. The resulting pellet was gently homogenized in a buffer of 5 mM Tris-HCl, 0.2 mM $CaCl_2$, and 0.2 mM ATP at pH 8.0 and dialyzed against the above buffer in order to depolymerize F-actin into G-actin at 4 °C for 48 h. The resulting solution was centrifuged at 160000*g* for 2 h to remove denatured actin, and then the clear supernatant was polymerized by addition of solid KCl to bring the KCl concentration to 0.1 M at 0 °C overnight. The alternative way to prepare labeled F-actin was the following. The final F-actin pellet was directly placed in a glass homogenizer and homogenized gently in 0.15 M KCl and 10 mM Tes at pH 7.0 and 0 °C, then the actin was dialyzed against the above buffer at 4 °C overnight, and subsequently the resulting F-actin solution was diluted to about 2–3 mg/mL. This was followed by centrifugation at 15000*g* for 60 min. The supernatant was routinely used for labeled F-actin.

In 0.15 M KCl, 20 mM Tris-HCl, and 2 mM $MgCl_2$ at pH 8.5, fluorescence anisotropy decay of F-actin labeled with 1,5-IAEDANS prepared by the above method showed a correlation time of 500–800 ns at 4 °C, which is in good agreement with the correlation time obtained by using *N*-3-pyrenemaleimide or dansyl-L-cysteine attached to F-actin presumably at the same site reported by other workers (Wahl et al., 1975; Kawasaki et al., 1976). Furthermore, this actin showed essentially a single band with a molecular weight of 42 000 in $NaDodSO_4$-containing, 7.5% acrylamide gel electrophoresis. Labeled actin was tryptically digested. From the peptide map there was isolated a dipeptide, Cys-Phe, in which the Cys had been alkylated by 1,5-IAEDANS; no other peptides were significantly labeled (Duke et al., 1976). This shows that the labeling site on actin is Cys-373, near the C terminal of the protein.

Protein Concentration. Myosin ($A_{1cm}^{1\%}$ = 5.70) and S-1 ($A_{1cm}^{1\%}$ = 7.70) (Young et al., 1965) concentrations were estimated from optical density measurements, while G-actin concentration was measured by absorbance at 290 nm ($A_{1cm}^{1\%}$ = 6.30), according to Houk & Ue (1974). In each case an appropriate correction for light scattering was made. In the case of protein labeled with dye, both actin and S-1 concentrations were determined by the Folin phenol method (Lowry et al., 1951) using bovine serum albumin as the standard. This method was also used to determine the concentration of unlabeled proteins.

Measurement of the Amount of Bound Dye. The concentration of IAF bound to S-1 was determined by absorbance measurements in the presence of 5 M urea in 20 mM Tris-HCl at pH 8.5 and 22 °C by using an extinction coefficient $\epsilon_{496} = 7.7 \times 10^4\ M^{-1}\ cm^{-1}$. The concentration of 1,5-IAEDANS bound to actin was estimated in one of two ways. In the primary method we used the radioactivity of [^{3}H]-1,5-IAEDANS and the known concentration of actin. On this radioactively labeled actin (in 0.12 M KCl, 20 mM Tris-HCl, 5 mM sodium phosphate, and 2 mM $MgCl_2$ at pH 8.5 and 4 °C), we also measured the fluorescence (excitation 340 nm; emission 470 nm) not only at the same concentration of labeled actin but also at many others. From these latter data we constructed a calibration curve relating amount of label to concentration of labeled actin. Our secondary method consisted of using this calibration curve to relate the fluorescence of an unknown actin sample to the concentration of labeled actin.

In the various experiments the concentratin of dye bound to protein ranged from 0.75 to 0.9 mol of 1,5-IAEDANS per actin monomer and from 0.7 to 0.8 mol of IAF per mol of S-1.

Molecular weights of S-1 and G-actin are assumed to be 115 000 (Lowey et al., 1969) and 42 000 (Elzinga et al., 1973).

Tryptic Digestion of Labeled Actin. The labeled actin (25 mg, 0.595 μmol) was dissolved in 5 mL of 6 M urea, 50 mM Tris-HCl, pH 8.5, and 22.4 mM β-mercaptoethanol. After the actin stood at 0 °C overnight, about a 100-fold molar excess of iodoacetamide over the thiol groups of actin was added, and the mixture was left at 25 °C for 60 min. The excess iodoacetamide was quenched by addition of approximately a 1.2-fold molar excess of β-mercaptoethanol over iodoacetamide. The solution was exhaustively dialyzed (at least 3 days) against 0.1 M NH_4HCO_3 at pH 8.3 and 4 °C. TPCK–trypsin in 1 mM HCl (5 mg/mL) was added in the proportion of 0.75 mg of trypsin per 50 mg of actin in order to digest the labeled actin, and the digestion was allowed to continue for 24 h with trypsin being added at 6-h intervals to give a total amount of trypsin equal to 6% of the actin. The digest was then lyophilized.

Peptide Maps and Amino Acid Analysis. Approximately 2 mg of the lyophilized tryptic digest in 0.05 mL of either water or 0.1 M acetic acid was applied to Whatman 3 MM paper (50 × 75 cm) and subjected to descending (first-dimension) chromatography in 1-butanol–acetic acid–water (4:1:5 v/v/v) at room temperature and subsequently to transverse (second-dimension) electrophoresis in formate–acetate buffer (pH 1.9) at 2000 V and 20 °C for 50 min. The strongly fluorescent spot was cut out from the paper and eluted with 0.5–1.0 mL of 0.1 N NH_4OH overnight at room temperture, and the eluant was lyophilized. The peptide was hydrolyzed with 6 N HCl under vacuum for 24 h at 110 °C. Amino acid

analysis was conventionally performed according to the methods of Spackman et al. (1958).

Steady-State Fluorescence Measurements. Steady-state fluorescence measurements were made at 4 °C with a Hitachi Perkin-Elmer MPF-4 fluorescence spectrophotometer equipped with corrected spectrum capability, a temperature-controlled cell holder, and a built-in magnetic stirrer.

Neglecting a slight difference in depolarization by attached and free labels (Shinitzky, 1972) (and therefore slightly overestimating R), we used a comparative method (Parker, 1968) to measure the quantum yield of 1,5-IAEDANS–actin in 0.15 M KCl, 20 mM Tris-HCl, and 2 mM $MgCl_2$ at pH 8.5 and 4 °C. Equation 1 gives the ratio of quantum yields, Q, as a

$$Q_1/Q_2 = (F_1/F_2)(A_2/A_1) \quad (1)$$

function of the areas under the corrected emission spectra, F, and the absorbances at the exciting wavelength, A, for two different fluorescence compounds. Quinine sulfate in 0.1 N H_2SO_4 was used as a standard and was assumed to have an absolute quantum yield[3] of 0.70 (Scott et al., 1970). The areas of the corrected emission spectra were determined by cutting out from the recorder paper and weighing the areas. In the energy transfer experiments, usually five sets of measurements were performed under the identical conditions, i.e., 1,5-IAEDANS–actin alone, 1,5-IAEDANS–actin plus S-1, 1,5-IAEDANS–actin plus IAF–S-1, actin plus IAF–S-1, and IAF–S-1 alone. The protein concentrations used here ranged from 0.5 to 3 μM; these corresponded to absorbances of less than 0.02 at the exciting wavelength. Energy transfer is indicated by a decrease in the fluorescence of donor and an increase in the fluorescence of the acceptor in the acto–S-1 rigor complex containing the two kinds of fluorophore.

Fluorescence Lifetime and Polarization Anisotropy Measurements. These were performed in a "dual-beam" time-resolving fluorescence anisotropy decay apparatus described elsewhere (Mendelson et al., 1975). In this instrument exciting wavelengths are selected by Corning 7-60 and 0-52 glass filters which give a mean excitation wavelength of 375 nm, and fluorescence wavelengths are selected by a Corning 0-51 glass filter and by two specially made Corion Corp. (Serial No. 644980) filters which pass light of wavelength greater than 450 nm. The readings of the two photomultipliers (proportional to $I_\parallel$ and $I_\perp$) are combined to give either anisotropy $[(I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)]$ or total $(I_\parallel + 2I_\perp)$. Adequate accumulation of data usually takes 20–30 min. Experiments are performed at 4 °C, using concentrations like those in steady-illumination experiments.

ATPase Measurements. ATPase activity was measured as the amount of P_i liberated as a function of time (Morales & Hotta, 1960; Lin & Morales, 1977).

Energy Transfer Efficiency. The protein samples used for this purpose were all freshly prepared. The fluorescence measurements were carried out within 48 h in most cases. For the determination of the transfer efficiency between a donor-labeled actin and an acceptor-labeled S-1 in the acto–S-1 rigor complex, three sets of measurements were performed by using the samples[4] as shown:

actin labeled with 1,5-IAEDANS (I)

acto–S-1 from 1,5-IAEDANS–actin and IAF–S-1 (II)

acto–S-1 from 1,5-IAEDANS–actin and S-1 (III)

These three samples contain the same concentration of 1,5-IAEDANS–actin, respectively. For determination of the transfer efficiency, typically an excitation wavelength of 340 nm and an emission wavelength of 465 nm were selected, the extent of donor fluorescence quenching was measured, and then the transfer efficiency was computed as follows. We let the total concentration of actin and its fraction labeled be A_0 and f_A, respectively, and the total concentration of S-1 and its fraction labeled be S_0 and f_S, respectively. We let X be the concentration of the acto–S-1 complex. Knowing the binding constant, K_a [we assume K_a to be 2×10^6 M^{-1} for complex formation, according to the data by Highsmith et al. (1976)], we calculate the concentration of the complex, X, and of free actin as $A_0 - X$. We introduce several more symbols, viz., F_A for the molar fluorescence of free, labeled actin, F_X for the molar fluorescence of labeled actin bound to unlabeled S-1, and F_X' for the molar fluorescence of labeled actin bound to labeled S-1.

In experiment I, we observe the total fluorescence at 465 nm (F^I) as

$$F^I = f_A A_0 F_A \quad (2)$$

Knowing A_0 and f_A experimentally, we get from eq 2

$$F_A = \frac{F^I}{f_A A_0} \quad (3)$$

In experiment II, we observe the total fluorescence of donor at 465 nm (F^{II}) as

$$F^{II} = f_A f_S X F_X' + f_A(1 - f_S)X F_X + f_A(A_0 - X)F_A \quad (4)$$

Here $f_A f_S X$ and $f_A(1 - f_S)X$ are the concentrations of complex in which both partners are labeled and that in which actin is labeled and S-1 is not labeled; $f_A(A_0 - X)$ is the concentration of actin which is free and labeled. In experiment III, we similarly observe the total fluorescence of donor at 465 nm (F^{III}) as

$$F^{III} = f_A X F_X + f_A(A_0 - X)F_A \quad (5)$$

Here $f_A X$ is the concentration of complex in which actin is labeled and S-1 is not labeled; $f_A(A_0 - X)$ is the concentration of actin which is free and labeled. From eq 5, we obtain

$$F_X = \frac{F^{III} - f_A(A_0 - X)F_A}{f_A X} \quad (6)$$

since everything else on the right-hand side of eq 6 is known. Now, knowing F_X from eq 6, we substitute it back into eq 4 and obtain the only remaining unknown

$$F_X' = \frac{F^{II} - f_A(1 - f_S)X F_X - f_A(A_0 - X)F_A}{f_A f_S X} \quad (7)$$

Finally, we compute the transfer efficiency (E) as

$$E\ (\%) = 100(F_X - F_X')/F_X \quad (8)$$

Results and Discussion

(1) Characterization of S-1 and Actin Labeled with Dye. KCl Dependence of Ca^{2+}–ATPase of S-1 Labeled with IAF. The Ca^{2+}–ATPase activity of S-1 labeled with IAF as described under Methods was measured in three different KCl

[3] In the calculation of R described below, 0.55 (Melhuish, 1964) is sometimes used instead of 0.70; if we do so, R changes slightly, from 6.0 to 5.8 nm.

[4] We attempted to estimate the transfer efficiency by measuring a decrease of donor lifetime. However, with our particular filtering system, the direct contribution of the acceptor fluoresence to the observed made quantitative interpretation too difficult. We also tried to monitor the transfer by measuring an enhancement in the emission from the acceptor, but wavelengths always overlapped and the correction process required measurements on four or five samples in addition to those required for measuring transfer efficiency by steady-state donor quenching, namely, IAF–S-1 alone and unlabeled F-actin plus IAF–S-1.

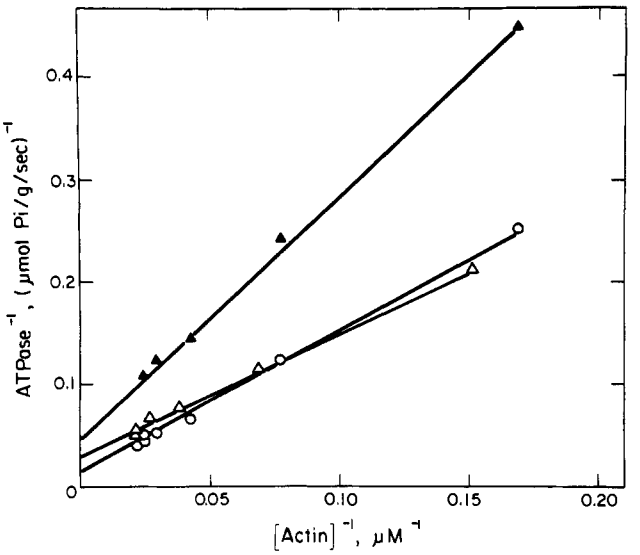

FIGURE 2: Double-reciprocal plot of actin-activated Mg^{2+}–ATPase of S-1 as a function of actin concentration. ATPase measurement was performed in 39.3 mM KCl, 1.75 mM ATP, 2.18 mM $MgCl_2$, 43.7 mM Tris-HCl at pH 8.0, and either 0.53 μM IAF–S-1 or 0.53 μM S-1 at 25 °C. Symbols are (○) for actin plus S-1, (△) for 1,5-IAEDANS–actin plus IAF–S-1, and (▲) for actin plus IAF–S-1. For each plot the Mg^{2+}–ATPase activity of S-1 in the absence of actin has been subtracted from the measured Mg^{2+}–ATPase activity of acto-S-1. The ATPase activity of unlabeled S-1 in the absence of actin, 1.09 μmol g^{-1} s^{-1}; the ATPase activity of IAF–S-1 in the absence of actin, 1.14 μmol g^{-1} s^{-1}.

concentrations, viz., 30, 50, and 0.6 M KCl, including also 50 mM Tris-HCl, 1 mM ATP, and 10 mM $CaCl_2$ at pH 8.0 and 25 °C. The Ca^{2+}–ATPase activity of labeled S-1 increases with increasing KCl concentration, while the Ca^{2+}–ATPase activity of unlabeled S-1 decreases markedly with increasing KCl concentration. For example, Ca^{2+}–ATPase activity of unlabeled S-1 at 0.6 M KCl was about 4.4-fold lower than that of S-1 labeled with IAF. In contrast, at 30 mM KCl the Ca^{2+}–ATPase activity of unlabeled S-1 was about 2.3-fold higher than that of S-1 labeled with IAF.

Warren et al. (1966) reported that myosin in which SH_1 had been reacted with PCMB (*p*-(chloromercuri)benzoate) suffered a much lesser drop in ATPase activity with increasing [KCl] than did native myosin. Although the reactivity of IAF toward SH_1 is more specific than that of PCMB, it is likely that the phenomenon observed here is basically the same as that observed by Warren et al. (1966).

Mg^{2+}-Activated Acto-S-1 ATPase Reconstituted from S-1 Labeled with IAF and Actin Labeled with 1,5-IAEDANS. Actin-activated Mg^{2+}–ATPase of S-1 reconstituted from actin and S-1 was measured in 39.3 mM KCl, 43.7 mM Tris-HCl (pH 8.0), 1.75 mM ATP, and 2.18 mM $MgCl_2$, at 25 °C (Figure 2). The Mg^{2+}–ATPase activity of acto–S-1 made from IAF–S-1 and unlabeled F-actin was about 30% of the activity of acto–S-1 made from both unlabeled proteins. Calculation of the percent inhibition of the *labeled* enzyme depends upon the degree of labeling. This quantity can be precisely estimated by using radioactive labels, but radioactive IAF is not yet available; degree of labeling is ca. 0.8 + 0.05. If we use this average degree of labeling, then in the particular experiment of Figure 2 the percent inhibition of the labeled enzyme is 88%. This is in good agreement with the report (Silverman et al., 1972) that the Mg^{2+}–HMM ATPase of NEM-labeled HMM showed very little actin activation compared to the maximum activation shown with native acto-HMM but is in seeming contradiction to the report that actin-activated Mg^{2+}–ATPase of myosin, instead of S-1 (or

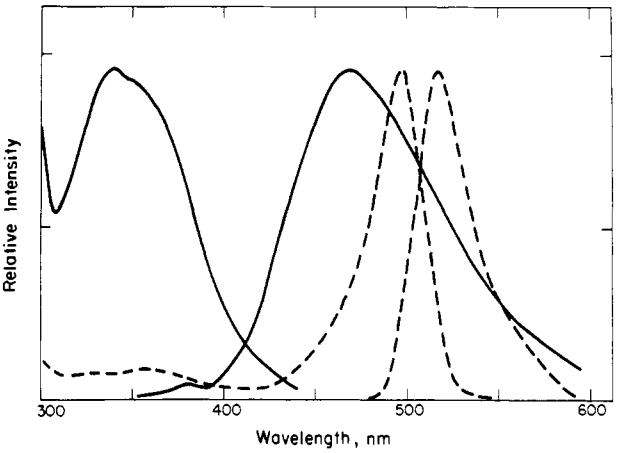

FIGURE 3: Fluorescence excitation and emission spectra of 1,5-IAEDANS-labeled actin and IAF-labeled S-1. All measurements were performed in 0.14 M KCl, 10 mM sodium phosphate buffer at pH 7.0, and either 0.43 μM IAF–S-1 [(---) 0.79 mol of IAF per mol of S-1] or 1.04 μM 1,5-IAEDANS–actin [(—) 0.79 mol of 1,5-IAEDANS per mol of actin] at 4 °C. In order to facilitate comparison, we have normalized all spectra to the same maximum intensity. Actually, the IAF intensities are much greater than those of 1,5-IAEDANS.

HMM), made from unlabeled F-actin and myosin labeled with 1,5-IAEDANS, had the same V_{max} as control actomyosin at low salt concentration (Mendelson et al., 1975; Lin & Morales, 1977). The effect of labeling on the rigor complex is probably quite different. For example, there is an experiment in which Highsmith et al. (1976) studied the binding affinity of 1,5-IAEDANS–S-1 to unlabeled F-actin in the rigor complex by its fluorescence anisotropy decay and found that the affinity constant of "native S-1" was unaffected by labeling. Also, that labeled actin retains an affinity for unlabeled S-1 was shown directly in a filtration experiment. We complexed the two proteins in a solution containing 0.1 M KCl, 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 10 μM actin, and 5 μM S-1 at 23 °C and subjected the system to Millipore filtration (a Millipore filter of 0.45-μm pore size was used). After equilibration, Mg^{2+}–ATP was added, and under the same filtration conditions a large amount of S-1 was released, as detected by total protein measurement [by the Folin phenol method (Lowry et al., 1951)] in the filtrate. Further evidence that labeled actin complexes with S-1 is Figure 2, which shows that 1,5-IAEDANS–actin appears to enhance the actin-activated Mg^{2+}–ATPase activity of S-1 (V_{max}) more than the unlabeled actin does. More importantly, in the experiment of Figure 2, the V_{max} of acto-S-1 reconstituted from 1,5-IAEDANS–actin and IAF–S-1 is ca. 47%. This apparent inhibition varies with preparation; sometimes it has been as small as 20%. It is evident that V_{max} of IAF–S-1 is consistently lower than that of unlabeled S-1.

In summary, it can be stated that the labeling of either S-1 or actin does not abolish the ability of S-1 to bind to actin in the absence of ATP nor does it abolish the cyclic interaction of S-1 with actin and ATP.

Fluorescence Properties of IAF Attached to S-1 and of 1,5-IAEDANS Attached to Actin. Figure 3 shows spectra of IAF-labeled S-1. The absorption spectrum has a maximal absorption at 498 nm, and the emission spectrum has a maximum at 520 nm. As expected from fluorescein derivatives in general (Mercola et al., 1972), a striking pH dependence of a fluorescence intensity in IAF attached to S-1 was observed. The fluorescence intensity nearly doubles as the pH is increased between 6.0 and 8.2, and the fluorescence intensity stays constant while the pH is between 8.2 and 9.0. For this

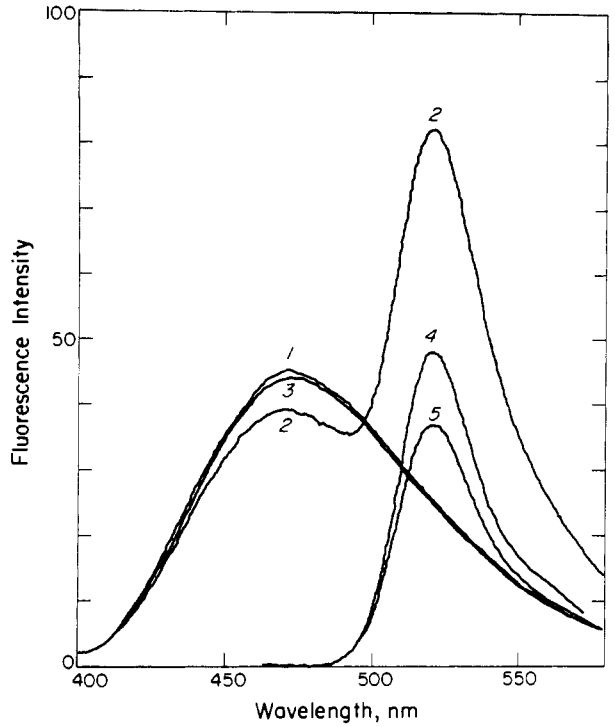

FIGURE 4: Emission spectra of IAF–S-1 and 1,5-IAEDANS–actin. Fluorescence measurement was performed in 0.15 M KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl at pH 8.5, 1.5 μM S-1, and 3.0 μM actin at 4 °C. Curve 1 is the spectrum for 1,5-IAEDANS–actin plus S-1, curve 2 is the spectrum for 1,5-IAEDANS–actin plus IAF–S-1, curve 3 is the spectrum for 1,5-IAEDANS–actin alone, curve 4 is the spectrum for actin plus IAF–S-1, and curve 5 is the spectrum for IAF–S-1 alone.

reason, we measured the fluorescence in IAF at pH 8.5, unless stated otherwise. No effect of calcium or magnesium ions on the fluorescence of labeled S-1 was observed up to 0.625 mM Ca^{2+} or up to 3.2 mM Mg^{2+} (at which conditions our experiments were performed). Figure 3 also shows an absorption spectrum of 1,5-IAEDANS-labeled F-actin in 0.14 M KCl and 10 mM sodium phosphate buffer at pH 7.0 and 4 °C with a maximum at 338 nm and an emission spectrum with a maximum at 470 nm. Neither an effect of the pH in the range 4–10 nor an effect of divalent cations such as Ca^{2+} and Mg^{2+} on the fluorescence of labeled actin was observed (not shown). This was to be expected from the studies of Hudson & Weber (1973) on the properties of free 1,5-IAEDANS solution. As can be seen in Figure 3, there is a significant overlap between the emission spectrum of 1,5-IAEDANS attached to actin and the absorption spectrum of IAF attached to S-1, suggesting that it is very advantageous to use 1,5-IAEDANS fluorophore as a donor and IAF as an acceptor to examine for fluorescence energy transfer between two fluorophores (Huang et al., 1975).

(2) Fluorescence Energy Transfer. If we consider first the emission of the actin-attached 1,5-IAEDANS fluorophore at 470 nm (excited at 340 nm), Figure 4 shows that labeled actin alone emits according to curve 3. When this labeled actin is mixed with *unlabeled* S-1[5] and some complex forms, the emission is enhanced by 3–4% (curve 1). On the other hand, if the labeled actin is mixed with IAF-*labeled* S-1, the emission is quenched by 7–9% (curve 2). Now we consider the emission of the S-1-attached IAF fluorophore at 520 nm (excited at 340 nm). Curve 5 shows this emission for labeled S-1 alone. When labeled S-1 is mixed with *unlabeled* actin (curve 4), the emission is enhanced by about 30%; however, when it is mixed with *labeled* actin (curve 2), the emission is much stronger. Comparing curves 3 and 1 at 470 nm and curves 5 and 4 at 520 nm, we see that the environments at Cys-373 and SH_1, respectively, are changed by acto-S-1 formation. By subtracting out these nontransfer effects, we see further that as a result of acto-S-1 formation donor emission is quenched (curve 1 to curve 2) and acceptor emission is enhanced. These latter effects show qualitatively that energy transfer is occurring when both protein components are labeled. To make a quantitative statement, one must take into account incomplete labeling and the fact that at binding equilibrium the various components are at different concentrations; that is the object of the data analysis. In order to use transfer efficiency (E) data obtained at different concentrations of the labeled proteins, and to assure ourselves that the analysis described under Methods is correct, we calculated *E per mole of doubly labeled acto-S-1 complex* in the solvent 0.15 M KCl, 20 mM Tris-HCl, pH 8.5, and 2 mM $MgCl_2$ at 4 °C. Throughout a range of total [S-1] to total [actin] ratio from 0.5 to 2.0, this specific value of E remained approximately constant, at 25–30%. This shows that, in an acto-S-1 complex, SH_1 on S-1 and Cys-373 on actin are within transfer distance of one another. In trying to make this assertion more metric and quantitative, we encounter the difficulty of evaluating the so-called orientational factor. Since both constituents of the complex, and the complex itself, have long rotational correlatonal times, it is unreasonable to assume that either of our fluorophores is totally in a randomizing motion. Faced with this problem, we attempted to change the orientational factor, anticipating a change of the transfer efficiency. This was attempted in two ways. Warren et al. (1966) reported that neutral salts such as NaCl, NaBr, $NaNO_3$, and $NaClO_4$ inhibited the enzymatic activity of a wide variety of enzymes including myosin and concluded that these effects are due to a conformational change of the surface structure of the myosin molecule by the binding of such ions. We might then expect a change of the orientational factor of fluorophores attached to S-1. In turn, we might expect that the energy transfer efficiency between two sites might be altered considerably. So we tested the effect of $NaClO_4$ as a structure-disrupting salt on the energy transfer efficiency in 20 mM Tris-HCl, 2 mM $MgCl_2$, 1.5 μM IAF–S-1, and 1.5 μM 1,5-IAEDANS–actin at pH 8.5 and 21 °C. We tried three different $NaClO_4$ concentrations, i.e., 0.15 M KCl, 0.05 M $NaClO_4$ plus 0.10 M KCl, and 0.1 M $NaClO_4$ plus 0.05 M KCl. However, in the three sets of experiments, the energy transfer efficiency remained unchanged, at 33–39%. As a second attempt to change the orientational factor, we interchanged the 1,5-IAEDANS donor on actin with IAF acceptor on S-1. If this interchange were in favor of the relative orientation of two fluorophores, the energy transfer efficiency should be enhanced, but if this were not in favor of the relative orientation, the transfer efficiency should be diminished. The quenching of the fluorescence at 470 nm was monitored upon mixing S-1 labeled with 1,5-IAEDANS and actin labeled with IAF in either 0.15 M KCl or 0.15 M NaCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 1.5 μM S-1 (2.6 mol of 1,5-IAEDANS per mol of S-1), and 1.5 μM actin (1.5 mol of IAF per mol of actin) at pH 8.5 and 4 °C. This interchange of fluorophores also left the transfer efficiency unchanged. If both fluorophores were in randomizing motion, then we would not expect the interchange to matter, and K^2 should be 2/3. We have already said that neither fluorophore could be *totally* in such motion, for then the rotational correlation time (ϕ)

[5] We have found as well that S-1 labeled with iodoacetamide (IAA) at the SH_1 group similarly enhances the fluorescence of the 1,5-IAEDANS–actin.

Table I: Energy Transfer Parameters

donor–acceptor part	Cys-373 to SH_1
λ_f (nm)[a]	468
λ_a (nm)[a]	498
Q_D[b]	0.63
$J \times 10^{13}$ (cm^3 M^{-1})	1.73
R_0 (nm)[c]	5.2
E (%)	30
R (nm)[d]	6.0

[a] λ_f is the fluorescence emission maximum of donor, and λ_a is the absorption maximum of acceptor. [b] Q_D is the quantum yield of the donor in the absence of acceptor. [c] R_0, the distance at which the transfer efficiency is 50%, is computed according to Förster's equation (Förster, 1965): $R_0^6 = (8.785 \times 10^{-25}) \times K^2 Q_D n^{-4} J$ cm^6. In this equation, n is the refractive index of the medium, Q_D is the quantum yield of the donor in the absence of acceptor, K^2 is the orientation factor for dipole–dipole transfer, and J is the spectral overlap integral of the donor fluorescence and the acceptor absorption. In the calculations, K^2 was assumed to be 2/3. [d] R, the distance between the donor–acceptor pair, is given by $R = R_0(E^{-1} - 1)^{1/6}$. In this equation, E is the transfer efficiency and R_0 is the "critical transfer distance" at which E is 50%.

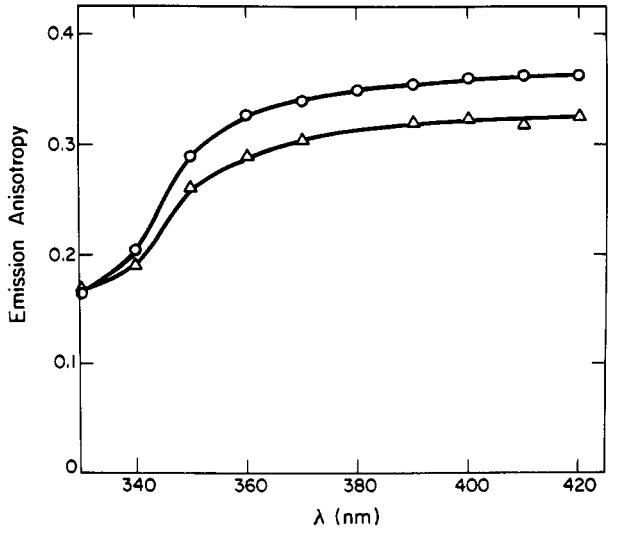

FIGURE 5: Emission anisotropy spectra of 1,5-IAEDANS and 1,5-IAEDANS attached to S-1. Emission (△) was observed through 2 M NaNO and Corning 3-71 filters in a solution containing 10 μM 1,5-IAEDANS–S-1, 0.0214 M KCl, 7.14 mM Tris-HCl (pH 7.6), and various concentrations of sucrose (0, 10, 20, 30, and 40% w/v) at 5 °C. The values for 1,5-IAEDANS alone (○) were taken from the data reported by Hudson & Weber (1973). The emission anisotropy r was obtained from a plot of $1/P$ against T/η upon extrapolation to $T/\eta = 0$ at each wavelength, where T is the absolute temperature and η is the solvent viscosity.

should be much smaller (e.g., for actin, $\phi \ll$ 500–800 ns), but it is possible that each fluorophore is in a rapid equilibrium between a bound (fixed) state and a mobile (fluorophore-randomizing) state. The condition $K^2 = 2/3$ would then apply at least to the latter state; the calculations of Table I show in that case that the distance between Cys-373 and SH_1 is ca. 6.0 nm. To decide whether it is reasonable to ascribe motion to S-1-bound label, we measured the emission anisotropy of 1,5-IAEDANS attached to S-1 whose rotational motion had been minimized by a high concentration of sucrose. These measurements were made in the same high-quality spectrofluorometer that Hudson & Weber (1973) used to measure the emission anisotropy of a true "glass" of free 1,5-IAEDANS. Figure 5 shows that at all wavelengths the anisotropy of the label attached to the protein is less than that of the label in the glass, thus indicating that indeed there is some motion of the label relative to S-1.

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