Fluorescence Energy Transfer in Myosin Subfragment-1†

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ABSTRACT: Fluorescent probes have been selectively introduced into skeletal muscle myosin subfragment-1 and the fluorescence emission characteristics of the labeled products studied. The fluorophores employed were the thiol-specific reagents *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid and 5-(iodoacetamido)fluorescein, the spectral properties of which render them a particularly effective donor–acceptor pair in Förster energy-transfer studies. Alkali 1 light chain, labeled at a single cysteine with either of these probes, was incorporated into chymotryptic subfragment-1 by the exchange procedure of Wagner & Weeds [Wagner, P. D., & Weeds, A. G. (1977) *J. Mol. Biol. 109*, 455–473]. The resultant, fluorescently labeled subfragment-1 was isolated by ion-exchange chromatography. Determination of the extent of incorporation by extinction and fluorescence indicated that greater than 80% of the subfragment-1 population possessed a fluorescently labeled alkali 1 light chain. The introduction of labeled alkali 1 did not perturb the K^+-, Ca^{2+}-, or actin-activated adenosine triphosphatases of subfragment-1. The addition of adenosine triphosphate (ATP), liganded by various cations, to this singly labeled subfragment-1 induced a 6–10% decrease in the fluorescence intensity of the extrinsic chromophore. An intensity decrease of ~4% was obtained when the hydrolysis of ATP was complete, and also upon direct addition of adenosine diphosphate. The ATP analogue adenylyl imidodiphosphate induced a decrease of ~7% in intensity. The addition of F-actin to the subfragment-1 in the presence of MgATP elicited no further fluorescence intensity change. A second, appropriate fluorophore was introduced into the singly labeled subfragment-1 at the SH_1 thiol on the heavy chain. Förster energy transfer was observed between this labeled site and the fluorophore previously introduced on the alkali 1 light chain. The measured efficiency of energy transfer indicated that the two fluorophores were ~40 Å apart. The same value was obtained upon reversal of the donor and acceptor attachment sites, suggesting that the uncertainty in the calculated distance introduced by the choice of orientation factor is probably less than 20%. Steady-state observations did not reveal any obvious change in this distance upon the addition of MgATP and then F-actin to the doubly labeled subfragment-1.

Myosin from fast-twitch skeletal muscles possesses two classes of low molecular weight light chains: 2 mol of alkali light chains and 2 mol of DTNB[1] light chains per mol of myosin (Weeds & Lowey, 1971; Lowey & Risby, 1971). The function of these different light chains remains obscure.

The DTNB light chains are not essential for ATPase activity (Weeds, 1969; Weeds & Lowey, 1971); indeed, active subfragments of myosin can be prepared that contain little or no DTNB light chain (Margossian et al., 1975; Weeds & Taylor, 1975). The DTNB light chain will functionally replace a light chain of some invertebrate myosins that is known to modulate the ATPase in response to calcium ions (Kendrick-Jones et al., 1976). It has also been implicated in a calcium-sensitive modulation of the actomyosin interaction in striated muscle myosin (Margossian et al., 1975).

The second class of light chains, the alkali light chains, can be removed from the parent myosin by exposure to alkaline pH (Kominz et al., 1959). Their removal results in a complete loss of ATPase activity (Stracher, 1969; Dreizen & Gershman, 1970). Rabbit fast-twitch myosin possesses two types of chemically homologous alkali light chains: the alkali 1 light chain of molecular weight 20 700 and the alkali 2 light chain of molecular weight 16 500 (Frank & Weeds, 1974). These occur in a ratio of 1.3 mol of alkali 1 and 0.7 mol of alkali 2 per mol of myosin, thus suggesting the existence of myosin isoenzymes (Sarkar, 1972; Weeds et al., 1975).

The band pattern of alkali light chains on gel electrophoresis is characteristic of muscle type (Lowey & Risby, 1971; Sarkar et al., 1971). If a fast muscle is surgically innervated with a motor nerve normally associated with a slow muscle, the changed frequency of nerve impulses results in a light-chain pattern and an actin-activated ATPase activity more characteristic of a slow muscle. The reciprocal transformation of a slow muscle can also be effected upon cross-reinnervation with a fast motor nerve (Weeds et al., 1974; Srêter et al., 1974). Similar transformations in light-chain pattern and ATPase are also induced by long-term artificial electrical stimulation of fast muscles (Srêter et al., 1973; Salmons & Srêter, 1976). The alkali light-chain pattern changes during development of the animal (Chi et al., 1975; Pelloni-Mueller et al., 1976), and it appears to differ as between normal and dystrophic mice (John, 1976).

These studies have shown, therefore, that myosins from different muscle types exhibit differences in alkali light-chain pattern which are accompanied by changes in ATPase characteristics. Recent work has clarified this relationship; Wagner & Weeds (1977) have developed a procedure for dissociating and recombining myosin subunits which can be used to produce subfragment-1 hybrids containing a heavy chain and an alkali light chain from different myosin sources. The dissociating conditions employed did not result in any loss in ATPase activity of the treated subfragment-1. A detailed study of the ATPase characteristics of various hybrids indicated that the

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[1] Abbreviations used: A_{337nm}^{1cm}, absorbance at 337 nm in a 1-cm path length cell; ADP, adenosine diphosphate; AMPPNP, adenylyl imidodiphosphate; ATP, adenosine triphosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; 1,5-IAEDANS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid; IAF, 5-(iodoacetamido)fluorescein; $NaDodSO_4$, sodium dodecyl sulfate; S1(A1) and S1(A2), myosin subfragment-1 species containing only the alkali 1 and only the alkali 2 light chain, respectively; Tris, tris(hydroxymethyl)-aminomethane.

0006-2960/80/0419-0774$01.00/0

actin-activated ATPase is linked to the type of alkali light chain and that the magnesium ion dependent ATPase is influenced by the heavy-chain component. The success of the exchange procedure of Wagner & Weeds (1977) pointed to the possibility of reintroducing fluorescently labeled alkali light chains into subfragment-1. The spectroscopic characteristics of this fluorescently labeled subfragment-1 could then be studied in the presence of physiological ligands, and the introduction of a second, appropriate fluorophore would enable the distance between the two labels to be determined by energy-transfer efficiency measurements. The alkali light chain used in the exchange procedure was conveniently labeled at its single thiol group. The second label was introduced into subfragment-1 at the SH_1 thiol on the heavy chain. In this way, it was possible to study the fluorescence characteristics of both singly and doubly labeled subfragment-1 species.

The object of the study was to gain some insight into the relative location of the alkali 1 light chain on the myosin head and to search for any conformational changes manifested in the presence of physiological ligands.

Materials and Methods

Preparation of Proteins. Fast-twitch myosin was prepared from the back and leg muscles of New Zealand white rabbits by following the method of Holtzer & Lowey (1959). Subfragment-1 was prepared by digestion of myosin using α-chymotrypsin, and the two isoenzymes were isolated by the procedure of Weeds & Taylor (1975). The column-purified isoenzymes were dialyzed against 10 mM potassium phosphate and 1 mM DTT, pH 6.5, at 4 °C, diluted with the same buffer to 1 mg/mL, and 4 mg of sucrose was added for each milligram of subfragment-1 (Yount & Koshland, 1963). When the sucrose had dissolved, the solution was frozen in a dry ice–propanol mixture and lyophilized. The lyophilized subfragment-1 was stored as a powder at −20 °C. A comparison of subfragment-1 before and after lyophilization showed that its ATPase characteristics were unaltered by the lyophilization procedure (see Table II).

Rabbit light chains were prepared by the method of Perrie & Perry (1970) as modified by Holt & Lowey (1975). Fractionation of the light chains was achieved either by the ion-exchange chromatography procedure of Holt & Lowey (1975) or by the ethanol fractionation method of Perrie et al. (1973) followed by ion-exchange chromatography. The only modification of the procedure of Perrie et al. (1973) was that upon addition of ethanol to 18% (v/v) the solution was left on ice overnight. This was necessary to ensure complete precipitation of the DTNB light chains. The isolated light chains were lyophilized as described above for subfragment-1.

F-actin was prepared as described in Margossian & Lowey (1973). Some preparations were the gifts of Dr. S. S. Margossian.

Protein concentrations were determined by extinction using the following coefficients: subfragment-1, $E_{280}^{1\%} = 7.5$ cm^{-1} (Wagner & Weeds, 1977); alkali 1 light chains, $E_{280}^{1\%} = 2.3$ cm^{-1}; F-actin, $E_{280}^{1\%} = 11.0$ cm^{-1} (West et al., 1967). Proteins were clarified by centrifugation prior to absorption measurements, and the absorbance at 340 nm was subtracted from the absorbance at 280 nm to compensate for light-scattering artefacts.

The coefficient employed for the alkali 1 light chain was calculated from absorbance measurements performed on a Beckman Acta III spectrophotometer and a Kjeldahl determination of the protein concentration (performed by Dr. S. S. Margossian). The nitrogen content of the protein was assumed to be 16%. The same extinction coefficient was obtained when the protein concentration was determined by amino acid analysis on a Durrum D500 analyzer.

The molecular weights employed for the various proteins were the following: subfragment-1, 110 000; actin, 41 700; alkali 1, 20 700.

Fluorescent Labels. *N*-[[(Iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid (1,5-IAEDANS) (M_r 434) and 5-(iodoacetamido)fluorescein (IAF) (M_r 515) were purchased from Molecular Probes, Plano, TX. In one experiment, tritiated 1,5-IAEDANS, a gift of Dr. R. H. Fairclough, was used. The extinctions of 1,5-IAEDANS at 337 and 280 nm were taken as 6.8×10^3 and 1.06×10^3 M^{-1} cm^{-1}, respectively (Hudson & Weber, 1973). The concentration of IAF was calculated by using an extinction of 4.87×10^4 M^{-1} cm^{-1} at 495 nm. This value was obtained by comparing the measured absorbance at 495 nm of alkali 1 labeled with IAF and the protein concentration determined by amino acid analysis and agrees well with the value of 4.26×10^4 M^{-1} cm^{-1} at 495 nm determined for a fluorescein isothiocyanate conalbumin conjugate by Tengerdy & Chang (1966). A value of 1.4×10^4 M^{-1} cm^{-1}, determined from the ratio of the absorbances at 495 and 280 nm of a solution of IAF, was used for the extinction at 280 nm.

Both 1,5-IAEDANS and IAF are thiol-specific reagents, the reaction involving, as with iodacetamide, the release of hydrogen iodide. The high quantum yield of 1,5-IAEDANS, the fact that its emission spectrum overlaps the absorption spectrum of IAF, and the high extinction of the IAF combine to render these two fluorophores particularly suited to Förster energy-transfer studies (Fairclough & Cantor, 1978).

Fluorescent Labeling of the Alkali 1 Light Chain. Lyophilized alkali 1, with the sucrose used in the lyophilization still present, was dissolved in 7 M guanidine hydrochloride, 2 mM DTT, 2 mM EDTA, and 0.5 M Tris, pH 8.0, at room temperature at a concentration of ~10 mg/mL. The protein was incubated in this solvent, under nitrogen, at room temperature for 2 h. Solid 1,5-IAEDANS or IAF was then added to a final concentration of 0.016 M and, if necessary, 1 M Tris, pH 11, was added to restore the pH to 8.0. The reaction was allowed to proceed in the dark at room temperature and under nitrogen for 2 h. The reaction was terminated by the addition of β-mercaptoethanol to a final concentration of 0.1 M. The mixture was then dialyzed extensively against 10 mM potassium phosphate, pH 6.5, at 4 °C and finally against 50 mM imidazole, pH 7.0, at 4 °C to remove unreacted fluorophore. Imidazole (Sigma) was recrystallized from ethanol to remove impurities and was conserved by only using it in the final dialyses. The labeled light chain was subsequently passed down a Whatman DE-52 ion-exchange column (1.5 × 30 cm, gradient 100 × 100 mL, 0.05–0.30 M NaCl in 50 mM imidazole, pH 7.0, at 4 °C) to remove residual unreacted label.

The extent of the labeling reaction was determined by extinction, amino acid analysis, and peptide mapping. In one experiment, radioactive counting was also used.

Extinction. Labeling was quantified by employing the extinction coefficients cited above. The problems associated with extinction are elaborated upon under Discussion (see also Figure 3).

Amino Acid Analysis. Preparation of the labeled alkali 1 for amino acid analysis was achieved in the following manner. The light chain (~1 mg) was dialyzed against water and dried down under vacuum. A 5.7 M HCl and 1% β-mercaptoethanol solution (0.5 mL) was added to the residue, and the sample was divided equally among four vials which were evacuated and sealed. The samples were placed in a heating block and

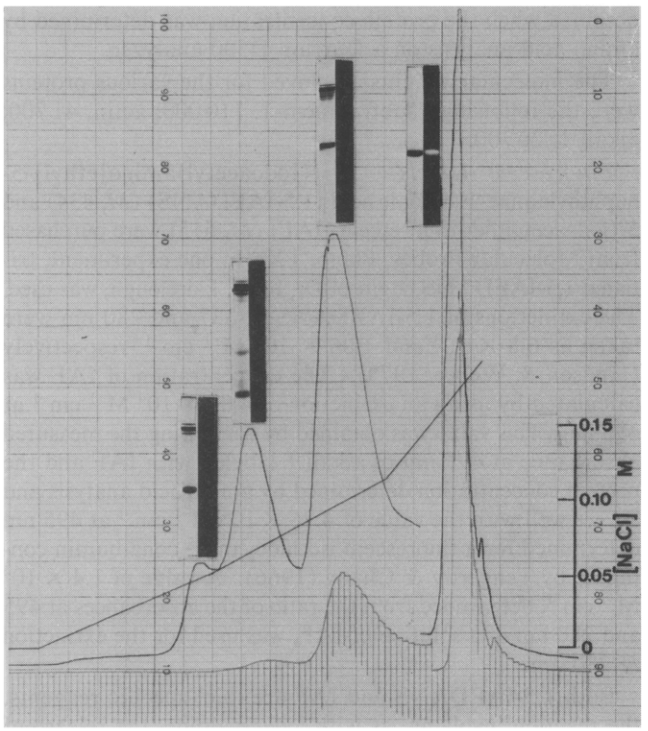

FIGURE 1: Elution profile of S1(A1) labeled with IAF on the alkali 1 light chain. The four sets of gels refer to the pooled fractions of the four peaks. In the case of the peak containing fluorescently labeled subfragment-1, the five peak fractions were pooled. The photograph on the left of each pair shows the gel stained with Coomassie Brilliant Blue. The photograph on the right shows the same gel before staining, illuminated by UV light, thus revealing the presence of any fluorescent bands. The elution was monitored at 280 (single solid line) and 510 nm (solid line with vertical displacements produced by the recorder "event marker"). The absorbance ranges were 0.32 at both wavelengths. Column flow rate 40 mL/h. Fraction volume 1.5 mL.

heated at 110 °C for 24–110 h. One sample was removed every 24 h for analysis. In this manner, it was possible to monitor any progressive degradation of the residues and to compensate for this loss in the calculation of the number of alkylated cysteine residues. The analyses were performed on a Durrum D500 amino acid analyzer.

Peptide Mapping. Labeled alkali 1 was prepared for peptide mapping analysis by dialysis against 0.5% ammonium bicarbonate, pH 8.2, followed by hydrolysis at 37 °C for 4 h in the presence of trypsin at a light chain/trypsin weight ratio of 100:1. Hydrolysis was terminated by freezing in a dry ice–propanol mixture, and the sample was lyophilized. The lyophilized powder was then dissolved in ~50 μL of pyridine–acetic acid buffer (volume ratio of pyridine/acetic acid/H_2O = 1:0.03:9), pH 6.5, and ~2 mg of protein was applied to Whatman 4MM chromatography paper. Electrophoresis was performed at 2 kV for 1.5 h. The strip of paper containing the peptides was then cut out, sewn onto a second sheet of chromatography paper, and subjected to electrophoresis in a direction perpendicular to the first electrophoretic migration. The solvent employed was pyridine–acetic acid buffer (volume ratio of pyridine/acetic acid/H_2O = 1:10:189), pH 3.4, and the electrophoresis was run at 2 kV for 1.5 h. The electrophoreses were performed in a high-voltage electrophoresis apparatus from Savant Instruments, Inc. Fluorescent peptides were localized by illumination with an ultraviolet lamp and other peptides by staining with ninhydrin–cadmium acetate stain (0.25% w/v ninhydrin in acetone and 1 g of cadmium acetate plus 50 mL of glacial acetic acid plus 100 mL of H_2O, in the ratio ninhydrin/cadmium acetate = 17:3).

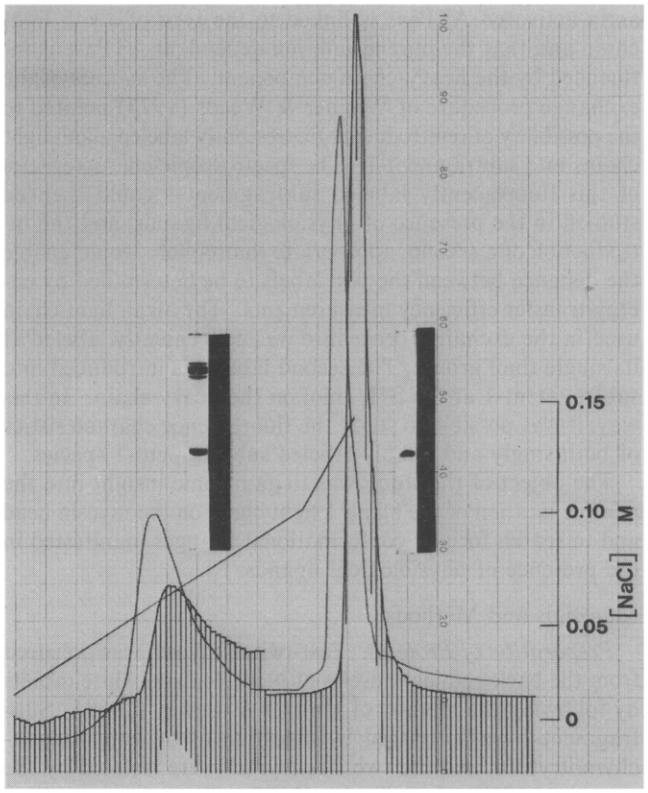

FIGURE 2: Elution profile of S1(A1) labeled with 1,5-IAEDANS on the alkali 1 light chain. The two pairs of gels refer to the pooled fractions of the two major peaks and indicate the polypeptide band pattern and the location of the fluorophore. The elution was monitored at 280 (single solid line) and 340 nm (solid line with vertical displacements). The absorbance ranges were 0.08 at 340 nm and 0.64 at 280 nm prior to elution of the excess alkali light chains (last peak), at which point the ranges were expanded to 1.28 absorbance units. The back fractions of the first peak were pooled as fluorescently labeled subfragment-1. The front half of the first peak contained unlabeled S1(A1) and S1(A2). Column flow rate 40 mL/h. Fraction volume 1.5 mL.

Preparation of Labeled Subfragment-1. The incorporation of alkali 1 light chains into subfragment-1 was performed as described by Wagner & Weeds (1977). All manipulations were performed at 4 °C. Chymotryptic S1(A2) (~2 mg/mL = 20 μM) was mixed with free, fluorescently labeled alkali 1 light chain (~4 mg/mL = 200 μM) in 0.1 M imidazole, 2 mM DTT, and 2 mM EDTA, pH 7.0. Solid ammonium chloride was added to a final concentration of 4.7 M (assuming a 20% volume increase). After being stirred for 15–20 min at 4 °C, during which time the ammonium chloride had dissolved, the mixture was dialyzed against 50 mM imidazole and 1 mM DTT, pH 7.0.

The exchange mixture was applied to a Whatman DE-52 ion-exchange column (1.5 × 30 cm) equilibrated in the same buffer. A gradient of 0–0.15 M NaCl (100 × 100 mL) in 50 mM imidazole and 1 mM DTT, pH 7.0, was applied, followed by a step of 1.0 M NaCl in the same buffer. The elution was monitored at 280 nm (for protein) and either 310 (for 1,5-IAEDANS) or 510 nm (for IAF) by using two Altex Model 153 analytical UV detectors and a Linear Instruments Research Model 300 series recorder. The elution profiles obtained were dependent upon which of the two fluorophores was attached to the alkali 1 light chain. An elution profile for the exchange involving alkali 1 labeled with IAF is shown in Figure 1, and a profile for the exchange involving 1,5-IAEDANS-labeled alkali 1 is shown in Figure 2.

Polyacrylamide–NaDodSO$_4$ Gel Electrophoresis. The purity of the proteins, both the starting materials and the

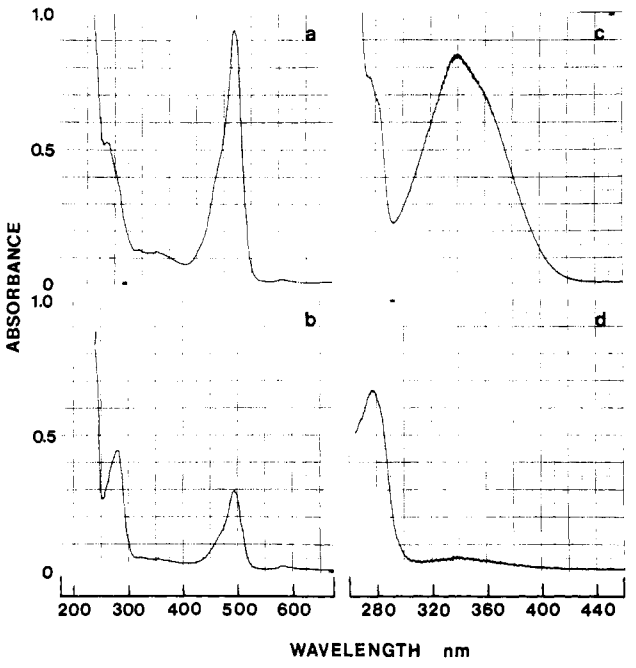

FIGURE 3: Absorption spectra of labeled alkali 1 and subfragment-1. (a) Alkali 1 labeled with IAF; (b) subfragment-1 containing alkali 1 labeled with IAF; (c) alkali 1 labeled with 1,5-IAEDANS; (d) subfragment-1 containing alkali 1 labeled with 1,5-IAEDANS.

products, was studied by 10% polyacrylamide gel electrophoresis in the presence of 0.1% $NaDodSO_4$ (Weber & Osborn, 1969). Fluorescent bands were observed by illumination with an ultraviolet lamp and were photographed by using Polaroid 57 film and Kodak Wratten filter no. 56. The protein bands were subsequently stained with 0.025% Coomassie Brilliant Blue R250 in 25% methanol and 10% acetic acid. The gels were destained by diffusion or electrophoresis in 10% acetic acid and 10% methanol.

Quantification of the Extent of Incorporation of Labeled Alkali 1 into Subfragment-1. An accurate determination of the extent of incorporation by extinction in the case where subfragment-1 contains alkali 1 labeled with 1,5-IAEDANS is difficult because of the low molar extinction and poorly resolved maximum of this fluorophore. The greater molar extinction of IAF and its well resolved maximum permit a more reliable determination of the incorporation when this probe is used (Figure 3).

In both cases, the contribution of the fluorophore to the absorbance at 280 nm was corrected for by using the extinction coefficients previously cited. It was, therefore, possible to determine the absorbance at 280 nm due to the protein alone, and hence the incorporation could be quantified. It should be noted that in the case of IAF the maximum fraction of the absorbance at 280 nm that derives from the label is 15% and for 1,5-IAEDANS the contribution is 1.3%.

The extent of incorporation was also quantified by relating the fluorescence intensity of the probe on the alkali 1 bound to subfragment-1 to that obtained for the free light chain (where quantification is more straightforward). This method involves the assumption that the chemical environment of the fluorophore is the same for the free and bound states of alkali 1. The need for this assumption was removed in later experiments as the fluorescence measurements were recorded under denaturing conditions (5 M guanidine hydrochloride).

A calibration curve of fluorescence intensity vs. the absorbance of the fluorophore at its maximum was obtained for the free, labeled alkali 1 upon excitation at 337 nm for 1,5-IAEDANS and 495 nm for IAF. The fluorescence intensity of a known concentration of subfragment-1 possessing labeled alkali 1 was then observed under the same instrumental settings, and the measured fluorescence intensity was used to derive the concentration of fluorophore from the calibration curve.

Close agreement was consistently found between the extinction and fluorescence determinations of the extent of fluorophore incorporation into subfragment-1. In one experiment, tritiated 1,5-IAEDANS was employed, and radioactive counting confirmed the reliability of these two methods. Availability of the radioactive fluorophore limited this method to one exchange experiment.

ATPase Measurements. ATPase assays were performed at pH 8.0 by using a Radiometer SBR2 titrigraph thermostated at 25.0 ± 0.1 °C. The assay volume was 10 mL, and the quantities of subfragment-1 used in the various assays varied between 0.025 and 0.100 mg. Ca^{2+}-ATPase was measured in 0.6 M KCl, 10 mM $CaCl_2$, 1 mM Tris, and 1 mM ATP; K^+-ATPase was measured in 0.6 KCl, 1 mM EDTA, 1 mM Tris, and 1 mM ATP; and actin-activated ATPase was measured in 6 mM KCl, 1.5 mM $MgCl_2$, 1 mM Tris, and 1 mM ATP.

F-actin (~15 mg/mL) was added to final concentrations of 0.05–1.50 mg/mL. Changes in the ionic strength of the assay medium were not taken into account (the maximum increase in the KCl concentration was to 16 mM). Dilution effects were corrected for in the construction of double-reciprocal plots.

In the case of the assays performed on subfragment-1 labeled at the SH_1 thiol, the solvent conditions for the K^+- and Ca^{2+}-ATPase assays were as described above. The assays, however, were performed at 37 °C, the temperature at which Sekine & Kielley (1964) conducted the first studies of the effect of SH_1 thiol modification on myosin ATPase.

Introduction of a Second Fluorophore into Subfragment-1. Labeling of the SH_1 thiol, by IAF or 1,5-IAEDANS, was achieved by the addition of a small aliquot of a concentrated solution (~5 mM) of the fluorophore to a solution of subfragment-1 (1–3 mg/mL) in 50 mM Tris, pH 7.9, at 4 °C. The molar ratio of fluorophore to subfragment-1 was in the range 0.75–0.80:1. The reaction was allowed to proceed in the dark, on ice, for 20–24 h and was terminated by the addition of β-mercaptoethanol to 0.01 M.

The elevation of the Ca^{2+}-ATPase of myosin upon incubation of the protein with β-mercaptoethanol, first noted by Hartshorne & Morales (1965), was not observed at the low concentrations of β-mercaptoethanol and short incubation periods employed in this investigation. In later experiments β-mercaptoethanol was replaced by DTT. The labeled subfragment-1 was then dialyzed extensively against 50 mM Tris, pH 7.9, at 4 °C.

The degree of alkylation of the SH_1 thiol was estimated by extinction measurements (as described above) and ATPase measurements. Alkylation of the SH_1 thiol results in a characteristic loss of K^+-ATPase and an augmentation of the Ca^{2+}-ATPase (Sekine & Kielley, 1964; Takashi et al., 1976). In theory, the percentage loss of K^+-ATPase corresponds directly to the percentage alkylation of the SH_1 thiol. By monitoring the loss of the K^+-ATPase, it is possible to estimate the degree of alkylation obtained in the reaction.

Fluorescence Measurements. Fluorescence measurements were performed with a Perkin-Elmer MPF 44 instrument equipped with a thermostated cell housing. All measurements were made at 25.0 ± 0.1 °C in 50 mM Tris, pH 7.5, unless

otherwise stated. Subfragment-1 concentrations were in the range 0.1–0.5 μM (~0.01–0.05 mg/mL), thus ensuring that the maximum extinction of either fluorophore at the excitation wavelength did not exceed 0.01. Fluorescence cells of 1-cm path length were used, and the sample volume was 3 mL.

Buffer was introduced into the cell by using a volumetric pipet, and a small aliquot (10–30 μL) of a stock subfragment-1 solution (1–5 mg/mL) was then added. The addition of cations and nucleotides was achieved by using an automatic micropipet and small volumes (10–30 μL) of concentrated stock solutions. Dilution effects were considered negligible. In all comparative measurements involving different subfragment-1 species, the protein concentration and the optical parameters were maintained constant. Fluorescent intensity measurements were expressed in terms of divisions of recorder chart paper. Complete excitation and emission spectra were not corrected for fluctuations in the xenon lamp intensity, variability in photomultiplier response, and other instrumental variables.

Fluorescence of Singly Labeled Subfragment-1. In the study of the effects of nucleotides upon the extrinsic fluorescence of subfragment-1 labeled on the alkali 1 light chain, small volumes (generally 15 μL) of stock solutions of $MgCl_2$, $CaCl_2$, or $MnCl_2$ were added to 3 mL of 50 mM Tris to bring the final cation concentration to 5 mM. An aliquot of labeled subfragment-1 was added, and the extrinsic fluorescence intensity was measured under a given set of optical conditions. An aliquot of a stock nucleotide solution was added, and the extrinsic fluorescence intensity was remeasured.

In the case of the hydrolysis of ATP, where a time course was followed, the sample was continuously excited at the absorption maximum of the fluorophore present on the alkali 1 and the emission was monitored at a wavelength near the emission maximum of the fluorophore. The time required for complete hydrolysis of the added ATP was generally less than 5 min. Addition of a second aliquot of ATP, upon hydrolysis of the first, confirmed that the activity of the subfragment-1 was unaffected by continuous irradiation over this and longer time periods (Figure 4).

Fluorescence of Doubly Labeled Subfragment-1. The efficiency of energy transfer between donor (1,5-IAEDANS) and acceptor (IAF) was determined by donor quenching in the manner described below. The fluorescence characteristics of four subfragment-1 species were studied: (1) subfragment-1 labeled with both donor and acceptor; (2) subfragment-1 labeled with donor only; (3) subfragment-1 labeled with acceptor only; (4) unlabeled subfragment-1. Optical conditions were chosen such that the measured fluorescence intensities of species (3) and (4) were zero: viz., excitation and emission slits 5–10 nm, 310-nm cutoff filter, and excitation/emission wavelength pairs 330/440, 340/450, and 350 nm/460 nm, respectively. For each excitation/emission wavelength pair, the intensity of emission was measured on identical concentrations of subfragment-1 species (1) and (2).

Determination of the Quantum Efficiency, ϕ, of 1,5-IAEDANS. The quantum efficiency, ϕ, of a fluorescent donor is defined as ϕ = (the number of quanta emitted)/(the number of quanta absorbed). ϕ is a function of the chemical environment of the fluorophore, and this parameter was, therefore, determined for 1,5-IAEDANS both when present on the alkali 1 light chain and when bound to the heavy chain at the SH_1 thiol.

The procedure used was essentially that given by Parker (1968). It has been assumed in the application of this method that no anisotropy of fluorescence emission exists and, therefore, that the errors described by Shinitzky (1972) will not be introduced into the calculations. The absorbance at 337 nm of a stock solution of subfragment-1 labeled with 1,5-IAEDANS was recorded in a Beckman Acta III spectrophotometer. A known dilution of this solution was made and a complete, uncorrected emission spectrum was recorded upon excitation at 337 nm. The absorbance at 337 nm of a solution of quinine bisulfate (~4×10^{-6} M) in 0.05 M H_2SO_4 was then measured by using the 0.1 slide-wire of the spectrophotometer. This solution was transferred to a fluorescence cell, and a complete, uncorrected emission spectrum was recorded under the same optical conditions as for the subfragment-1. The areas under the emission spectra were measured by using an electronic integrator (electronic graphics calculator, Numonics Corp.), and the quantum efficiency of 1,5-IAEDANS, ϕ, was calculated from

(area under emission spectrum of 1,5-IAEDANS)/(area under emission spectrum of quinine bisulfate) = ϕ (A_{337nm}^{1cm} of subfragment-1)/[0.70(A_{337nm}^{1cm} of quinine bisulfate)]

where 0.70 is the quantum efficiency of quinine bisulfate in 0.05 M H_2SO_4 (Scott et al., 1970).

Determination of the Overlap Integral, J. The overlap integral, J, describes the relationship between the fluorescence emission of the donor and the extinction of the acceptor [see Fairclough & Cantor (1978)]. The expression used to calculate J in these studies was

$$J = \frac{\int_0^\infty F_D(\lambda) E_A(\lambda) \lambda^4 \, d\lambda}{\int_0^\infty F_D(\lambda) \, d\lambda}$$

where F_D is the fluorescence intensity of the donor, expressed in arbitrary intensity units, E_A is the molar extinction of the acceptor in M^{-1} cm^{-1}, and λ is the wavelength in nanometers.

The raw data from which J was calculated were an uncorrected emission spectrum of the donor, 1,5-IAEDANS, and the absorption spectrum of the acceptor, IAF. An overlap integral was calculated for two subfragment-1 species: (1) subfragment-1 labeled with donor at the SH_1 thiol and by acceptor on the alkali 1; (2) subfragment-1 labeled with acceptor at the SH_1 thiol and by donor on the alkali 1. The calculation was performed on a Texas Instruments SR-60 calculator using a program written by Dr. W. F. Stafford, III. The values of J calculated were in good agreement with those obtained by Huang et al. (1975) using a corrected emission spectrum of 1,5-IAEDANS and a fluorescein derivative as the acceptor. For this reason it was not considered essential to employ corrected spectra in the present calculations.

Results

Fluorescent Labeling of the Alkali 1 Light Chain. Determination of the stoichiometry of labeling of the alkali 1 light chain with either 1,5-IAEDANS or IAF, by extinction and by amino acid analysis, indicated that ~1 mol of fluorophore was incorporated into 1 mol of light chain. A summary of amino acid analysis data is given in Table I.

Technical difficulties were encountered in the peptide mapping because of streaking of the labeled peptide due to the hydrophobic nature of the fluorophore. It was, however, possible to demonstrate the presence of a single major fluorescent spot on the peptide map of each of the labeled light chains.

Incorporation of Fluorescently Labeled Alkali 1 into

Table I: Extent of Thiol Labeling by Amino Acid Analysis[a]

expt no.	fluorophore attached to alkali 1	mol of carboxymethylated Cys per mol of alkali 1
1	1,5-IAEDANS	0.95
2	IAF	0.84
3	1,5-IAEDANS	0.84
4	IAF	0.88
5	IAF	ND

[a] For each experiment the total number of moles of Glu, Ala, Gly, Asp, Lys, and Phe divided by the number of moles of carboxymethylated cysteine was plotted against hydrolysis time. Each plot consisted of three or four points corresponding to the amount of carboxymethylated cysteine at different times during the acid hydrolysis of the labeled alkali 1 light chain (see Materials and Methods). Extrapolation to zero time and comparison of this ratio with the ratio derived from the amino acid composition ((Frank & Weeds, 1974) gave the degree of alkylation of the single cysteine. ND = no determination made.

Subfragment-1. A total of five exchange experiments were performed. These are numbered 1–5 in Tables I–III. In two of these, alkali 1 labeled with 1,5-IAEDANS was incorporated into subfragment-1; in the other three experiments, the label on the incorporated alkali 1 was IAF. Measurement of the extent of incorporation, by the methods of extinction and fluorescence described above, yielded 80 and 100% incorporation of the two experiments utilizing 1,5-IAEDANS-labeled alkali 1. In one case, the 1,5-IAEDANS was tritiated and radioactive counting confirmed the 80% value derived from other methods.

In the three cases where subfragment-1 contained alkali 1 labeled with IAF, this fluorophore induced a significant change in the ionic strength at which the protein was eluted from the ion-exchange column (see Figure 1). It was therefore possible, as indicated by the gels of Figure 1, to resolve the labeled subfragment-1 from the other components of the exchange mixture. In the first experiment employing IAF as the label on the alkali 1, it was suspected, from the degree of incorporation, that the isolated subfragment-1 contained ~0.5 mol of free labeled alkali 1. Limited material did not permit further chromatography to be performed. As the measurement of the efficiency of energy transfer will not be affected by the presence of free labeled alkali 1 (because the singly and doubly labeled subfragment-1 species both contain 0.5 mol of free labeled light chain), it seemed justified to use the subfragment-1 despite the impurity. The extent of incorporation was taken to be 100%. Conservative pooling of the labeled subfragment-1 peak from the exchange column avoided a repetition of this problem in the other experiments involving IAF-labeled alkali 1, and the extent of incorporation was found to be 100% in these cases.

ATPase Activity of Subfragment-1. Subfragment-1 containing a fluorescently labeled alkali 1 light chain exhibited the same Ca^{2+}-, K^+-, and actin-activated ATPase activities as subfragment-1 containing unmodified alkali 1. In the case of the actin-activated ATPase, the quantity of material available limited measurements to a single set of assays in all five exchange experiments. That is, each point in the double-reciprocal plot (6–10 points/plot) was the result of a single measurement. Extrapolation to V_{max} and K_m was, therefore, subject to some uncertainty. The data are given in Table II. The introduction of the second fluorophore of the donor–acceptor pair, at the SH_1 thiol, was monitored by extinction and ATPase measurements. It was found, by both methods, that the reaction had generally reached completion during the 24-h reaction period. That is, the addition of 0.7 mol of fluorophore to 1.0 mol of subfragment-1 resulted in the labeling of ~70% of the SH_1 thiol groups. In such a situation, one would expect to find a concomitant loss of ~70% of the K^+-ATPase activity. The data presented in Table II confirm this expectation.

Table II: ATPase Activities of Labeled Subfragment-1 Species[a]

expt no.	label on alkali 1	K^+-ATPase (s^{-1})	Ca^{2+}-ATPase (s^{-1})	actin-activated ATPase V_{max} (s^{-1})	actin-activated ATPase K_m (μM)
		Control			
S1(A1)		11.3	1.1	20.0	5.0
S1(A2)		12.0	1.0	25.0	10.0
		Subfragment-1 Labeled at the Alkali 1			
1	1,5-IAEDANS	12.8	1.6	38.5	15.9
2	IAF	11.5	1.2	38.5	12.9
3	1,5-IAEDANS	7.8	1.0	16.7	5.8
4	IAF	12.3	1.1	15.0	7.6
5	IAF	10.0	1.4	20.8	6.9

expt no.	label on alkali 1	label on SH_1 thiol	K^+-ATPase (s^{-1}) alkylation before	K^+-ATPase (s^{-1}) alkylation after	Ca^{2+}-ATPase (s^{-1}) alkylation before	Ca^{2+}-ATPase (s^{-1}) alkylation after
		Doubly Labeled Subfragment-1				
1	1,5-IAEDANS	IAF	18.3	8.2	3.7	9.3
2	IAF	1,5-IAEDANS	19.2	6.2	2.6	8.4
3	1,5-IAEDANS	IAF	17.1	5.8	3.4	5.0
4	IAF	1,5-IAEDANS	24.0	6.8	3.2	6.4
5	IAF	1,5-IAEDANS	17.6	6.2	3.4	8.3

[a] For solvent conditions of all ATPases, see Materials and Methods. Note that in the experiments with the doubly labeled subfragment-1 the ATPase assays were performed at 37 °C. All rates derive from single determinations except for the actin-activated rates where a double-reciprocal plot yielded values of V_{max} and K_m. The values for the control subfragment-1 species are fairly typical although some variation in V_{max} and K_m was observed between different preparations [see also Wagner & Weeds (1977)]. No change in ATPase activities was observed after lyophilization of control subfragment-1.

Fluorescence of Subfragment-1 Labeled on the Alkali 1 Light Chain. The fluorescence characteristics of subfragment-1, possessing an alkali 1 light chain labeled with either 1,5-IAEDANS or IAF, were studied in the presence and absence of nucleotides and F-actin.

It was found that the addition of ATP, liganded by Mg^{2+}, Mn^{2+}, or Ca^{2+}, induced an 8–10% decrease in the fluorescence intensity of the extrinsic chromophore IAF. When the extrinsic chromophore was 1,5-IAEDANS, the change in intensity was generally 6–8%. This intensity decrease was sustained until the ATP was fully hydrolyzed, at which point the fluorescence intensity increased to a value ~4% less than that of unliganded subfragment-1 (Figure 4). The direct addition of ADP liganded by various cations induced approximately the same 4% decrease. The ATP analogue AMPPNP produced an intensity decrease of ~7%. The fluorescence intensity decreased further, to ~10%, upon subsequent displacement of the AMPPNP from the active site by the addition of ATP. A similar effect was observed upon the addition of ATP to subfragment-1 liganded by ADP. It was not possible, within experimental resolution (2 nm), to detect any wavelength shifts accompanying these intensity changes.

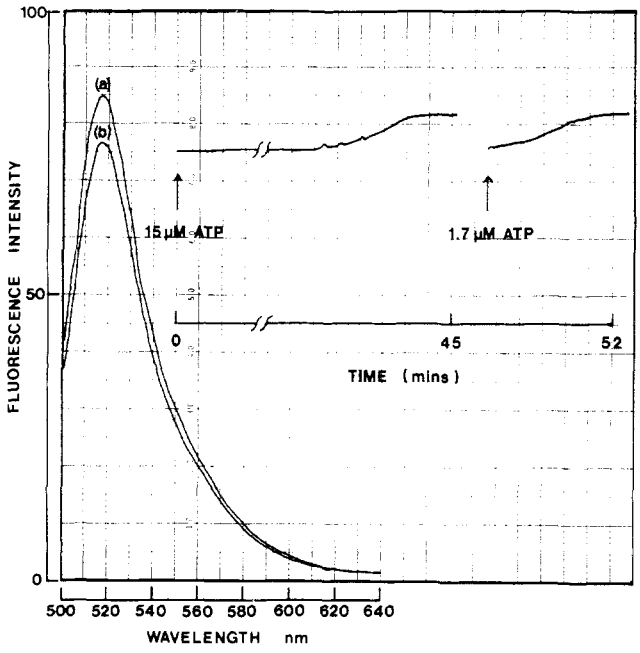

FIGURE 4: Emission spectra of subfragment-1 containing IAF-labeled alkali 1 light chain upon excitation at 495 nm (a) and after the addition of an approximately 35-fold molar excess of ATP (viz., ~6.7 μM ATP) (b). After this ATP addition the fluorescence emission was monitored at 517 nm. The traces shown indicate the changes in fluorescence intensity upon the addition and subsequent hydrolysis of ATP. The same effect was observed upon two further additions of ATP (not shown here).

The addition of F-actin (10-fold molar excess) to subfragment-1 in the presence of ATP accelerated the rate of hydrolysis but did not itself induce any obvious change in the fluorescence characteristics. Upon development of the rigor condition, the extrinsic fluorescence intensity decreased monotonically over a long period of time (30 min). Turbidity changes probably account for this phenomenon. Subsequent addition of ATP induced the dissociation of the rigor complex and restored the fluorescence intensity to the level observed during steady-state ATP hydrolysis.

Fluorescence of Subfragment-1 Labeled on the Alkali 1 Light Chain and at the SH_1 Thiol. The phenomenon of singlet–singlet nonradiative energy transfer, Förster transfer, has been described and reviewed many times [see, for example, Fairclough & Cantor (1978) and Stryer (1978)]. Expressed simply, it involves the nonradiative transfer of light energy from a donor (fluorophore) to an acceptor (fluorophore or quencher). This generally results in a decrease in intensity of the donor fluorescence and an intensity increase in the acceptor fluorescence. Figures 5 and 6 illustrate the practical effect of this phenomenon as observed in these studies. In Figure 5c, two fluorescence emission spectra are shown. One (broken line) is derived from an algebraic addition of the emission spectra of two subfragment-1 species: one labeled on the alkali 1 light chain with IAF (acceptor) (Figure 5b) and the other labeled at the SH_1 thiol with 1,5-IAEDANS (donor) (Figure 5a). The second emission spectrum in Figure 5c (solid line) is that of a single subfragment-1 species, labeled with both IAF (on the alkali 1 light chain) and 1,5-IAEDANS (on the SH_1 thiol). Figure 6 illustrates the same effect upon reversal of donor and acceptor attachment sites. The degree of fluorescence enhancement exhibited by the two systems varies partly because the donor stoichiometries are essentially the same in both cases, but the acceptor stoichiometry in Figure 5 is 1.0 and that in Figure 6 is 0.5. The degree of acceptor enhancement was found to vary somewhat between

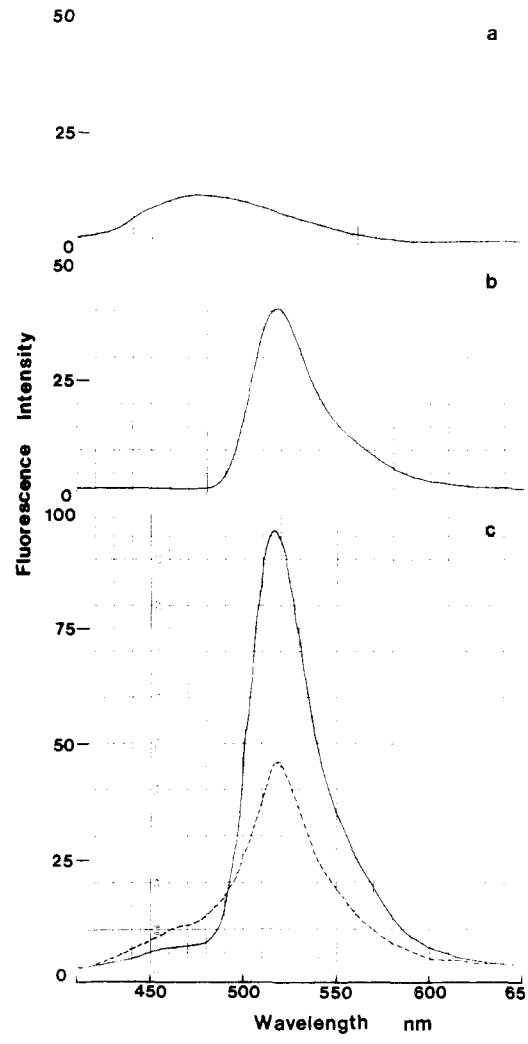

FIGURE 5: Fluorescence emission spectra of singly and doubly labeled subfragment-1. (a) Emission spectra of subfragment-1 labeled with 1,5-IAEDANS at the SH_1 thiol; (b) emission spectrum of subfragment-1 labeled with IAF on the alkali 1 light chain; (c) (---) addition of spectra a and b and (—) emission spectrum of subfragment-1 labeled with 1,5-IAEDANS at the SH_1 thiol and with IAF on the alkali 1 light chain. Excitation wavelength for all spectra was 330 nm. See text for additional details.

the different exchange experiments whereas the degree of donor quenching was more consistent. The reason for this is unclear. The concentrations of the subfragment-1 species, the stoichiometries of the labels, and the optical conditions under which the spectra were obtained were identical for the three spectra of Figure 5 and also for the three spectra of Figure 6.

Comparison of the two spectra of Figure 5c and the two spectra of Figure 6c shows clearly a significant (~40%) decrease in fluorescence intensity of the donor, 1,5-IAEDANS, in the 420–460-nm region when the subfragment-1 also possesses the acceptor, IAF. When these two labels are present, but on different moieties, and energy transfer cannot occur, the fluorescence intensity in this wavelength range is significantly greater. Similarly, when donor and acceptor are on the same molecule, the acceptor fluorescence intensity is increased or sensitized in the 500–520-nm range in comparison with the intensity obtained from a spectral addition of the two singly labeled species.

The same effect is also observed if a comparison is made of an emission spectrum of the doubly labeled subfragment-1 (solid line) and an emission spectrum recorded for a solution

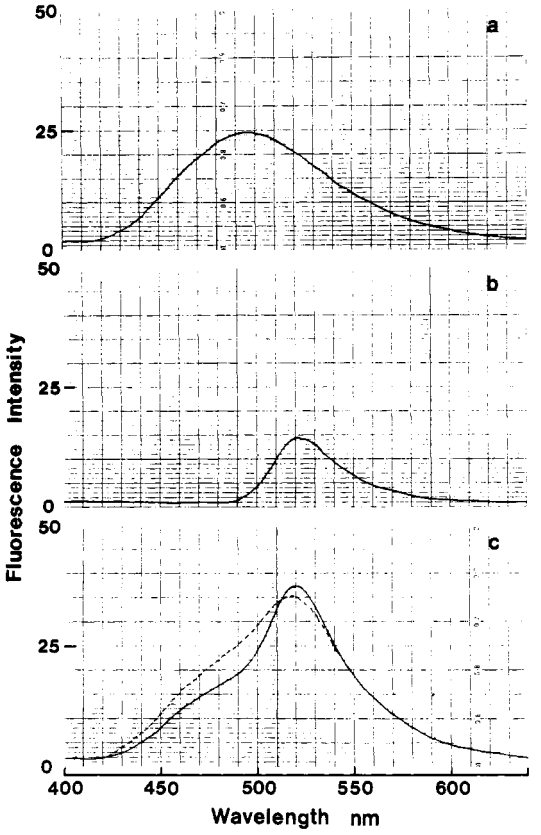

FIGURE 6: Fluorescence emission spectra of singly and doubly labeled subfragment-1. (a) Emission spectrum of subfragment-1 labeled with 1,5-IAEDANS on the alkali 1 light chain; (b) emission spectrum of subfragment-1 labeled with IAF at the SH_1 thiol; (c) (---) addition of spectra a and b and (—) emission spectrum of subfragment-1 labeled with 1,5-IAEDANS on the alkali 1 light chain and with IAF at the SH_1 thiol. Excitation wavelength for all spectra was 330 nm. See text for additional details.

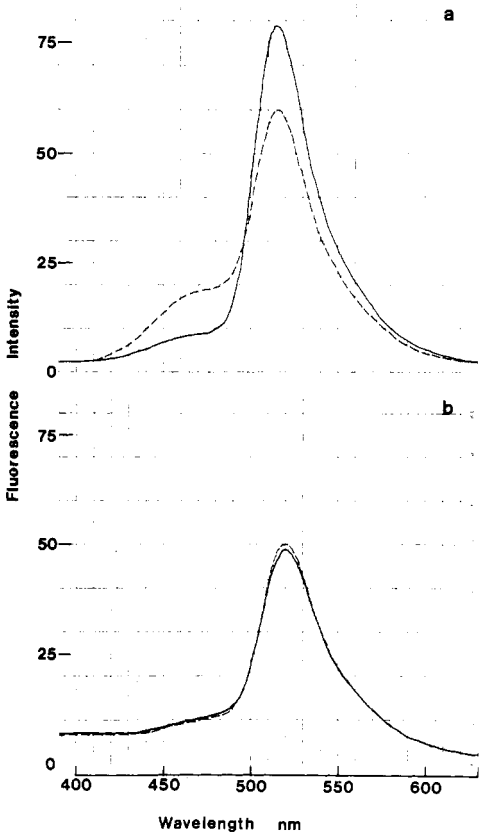

FIGURE 7: Fluorescence emission spectra of singly and doubly labeled subfragment-1. (a) Emission spectrum of doubly labeled subfragment-1 (solid line) and an emission spectrum recorded for a solution containing a mixture of the two singly labeled species (broken line); (b) emission spectra recorded for the same species as in (a) but in a denaturing solvent. Excitation wavelength for all spectra was 330 nm. See text for additional details.

containing a mixture of the two singly labeled subfragment-1 species, one containing donor only and the other acceptor only (broken line) (Figure 7a).

In all experiments emission and excitation spectra were recorded for the various subfragment-1 species in a denaturing solvent (5 M guanidine hydrochloride and 0.05 M Tris, pH 7.5, at 25 °C). This gave a direct measure of the relative fluorophore concentrations, thus enabling normalization of the spectra recorded in nondenaturing conditions to be made if necessary. Figure 7b shows emission spectra recorded for the same subfragment-1 species as in Figure 7a, but in the denaturing solvent. Under these conditions it can be seen that little difference was found between the spectrum of the mixture of singly labeled subfragment-1 species (broken line) and the spectrum of the doubly labeled subfragment-1 species (solid line). This spectral superimposition confirms that the fluorophore concentrations are appropriately balanced in the two systems and that the donor quenching and acceptor enhancement observed do not derive from trivial reabsorption of light or from other artefactual processes.

Measurement of Energy Transfer Efficiency, E, and the Distance, R_o. Measurement of either donor fluorescence quenching or acceptor fluorescence enhancement allows the efficiency of energy transfer, E, to be determined. It is from the value of E that the donor–acceptor distance is calculated. The method used here for the determination of E employed measurements of donor quenching as described under Materials and Methods. The equation

$$E = 1 - (F_{DA}/F_D) \quad (1)$$

where F_{DA} = the fluorescence intensity, under a given set of optical conditions, of the doubly labeled subfragment-1 and F_D = the fluorescence intensity, under the same optical conditions, of subfragment-1 labeled only with donor, applies only to a system in which each subfragment-1 is labeled with both 1 mol of donor and 1 mol of acceptor. This situation did not obtain in these studies, and so it was necessary to make corrections for the observed labeling stoichiometries. This was achieved by the following method, which assumes that the reactivity of the SH_1 thiol is unaltered by the presence of a fluorophore on the alkali 1 light chain. That is, alkylation of the SH_1 thiol, in a mixed population of labeled and unlabeled subfragment-1, is random.

Consider a subfragment-1 species into which A mol of acceptor have been incorporated on the alkali 1 light chain. This moiety is then reacted with donor at the SH_1 thiol. If D mol of donor are incorporated in this manner, then the subfragment-1 population will contain four different labeled species.

The mole fractions of these four possible species in 1 mol of total subfragment-1 will be the following: (1) AD mol of subfragment-1 labeled with both donor and acceptor; (2) $(1 - A)D$ mol of subfragment-1 labeled only with donor; (3) $(1 - D)A$ mol of subfragment-1 labeled only with acceptor; (4) $(1 - A)(1 - D)$ mol of unlabeled subfragment-1. These considerations apply only to a system, such as the one described here, in which there is 1 mol of unique attachment sites for donor and 1 mol of unique attachment sites for acceptor per mol of subfragment-1; viz., $D \leq 1$ and $A \leq 1$. As described under Materials and Methods, donor quenching was measured at wavelengths where the contributions of species (3) and (4)

Table III: Values of Efficiency of Energy Transfer, R_o, and R[a]

expt no.	location of donor (1,5-IAEDANS)	location of acceptor (IAF)	stoichiometry of donor	stoichiometry of acceptor	efficiency of energy transfer	R_o (Å)	R (Å)
1	thiol of alkali 1	SH_1 thiol	0.80	0.50	0.63	41	37
2	SH_1 thiol	thiol of alkali 1	0.76	1.00	0.70	44	38
3	thiol of alkali 1	SH_1 thiol	1.00	0.80	0.48	40	41
4	SH_1 thiol	thiol of alkali 1	0.75	1.00	0.65	44	39
5	SH_1 thiol	thiol of alkali 1	0.65	1.00	0.57	44	42

[a] These data refer to the five exchange experiments performed. Stoichiometries were measured as described under Materials and Methods, and transfer efficiences, R_o, and R were calculated as indicated under Results.

to the fluorescence intensity were zero. Therefore, species (1) and (2) only need be considered in the calculation of E. Both species (1) and (2) will contribute to the observed value of F_{DA}, and a separation of these contributions is necessary. It is important to recognize that the contribution of species (2), to the value of F_{DA}, must be subtracted because the donor emission has no chance of being quenched by an acceptor in this species. This contribution was determined from the value F_D, itself measured on a population of subfragment-1 molecules containing D mol of donor. Given that, in the mixture of 1 mol of labeled subfragment-1 species described above, $D(1 - A)$ mol is labeled only with donor, the equation describing E becomes

$$E = 1 - \frac{F_{DA} - F_D[D(1 - A)]}{F_D} \quad (2)$$

This modified equation was used to calculate E.

The equation relating E to the distance between donor and acceptor, R, is

$$E = R_o^6/(R_o^6 + R^6) \quad (3)$$

where R_o is the "Förster critical distance".

R_o is defined by

$$R_o^6 = (8.79 \times 10^{-5})n^{-4}\phi J\kappa^2 \text{ Å}^6 \quad (4)$$

where n is the refractive index of the solvent, ϕ is the quantum efficiency of the donor, J is the overlap integral, and κ^2 is an orientation factor relating the donor emission transition dipole and the acceptor absorption transition dipole. It can be seen that if $R = R_o$ the $E = 0.5$, and so R_o is the distance at which the efficiency of energy transfer between the donor and acceptor is 0.5.

Calculation of ϕ and J, as described under Materials and Methods, yielded the following values. For subfragment-1 with 1,5-IAEDANS on the alkali 1 light chain and IAF on the SH_1 thiol, $\phi = 0.30$ and $J = 1.1081 \times 10^{14}$ cm^{-1} M^{-1} nm^4. For subfragment-1 with IAF on the alkali 1 light chain and 1,5-IAEDANS on the SH_1 thiol, $\phi = 0.48$ and $J = 1.0524 \times 10^{14}$ cm^{-1} M^{-1} nm^4. n was taken as 1.4, and κ^2 was initially given the value 2/3. R, the distance between the donor and acceptor, was calculated from eq 3.

In some experiments the transfer efficiency was derived from measurements of acceptor fluorescence enhancement as described by Schiller (1975). The values of R calculated from this transfer efficiency agreed quite well with the values derived from donor quenching measurements.

The results of the different experiments are summarized in Table III.

Effects of Nucleotides and F-actin upon the Distance R. The addition of MgATP to the doubly labeled subfragment-1 induced a small (~3%) change in the measured transfer efficiency E. These changes were not sufficiently consistent to justify any confidence being placed in them. A 3% change in transfer efficiency would result only in a 1-Å change in R, if it is assumed that κ^2 is unchanged, and this cannot be considered significant. The addition of F-actin also failed to elicit any significant change in E and hence in R (assuming κ^2 is unchanged).

Discussion

The results presented in this paper show that it is possible, using the exchange procedure of Wagner & Weeds (1977), to incorporate a chemically modified alkali 1 light chain into skeletal muscle myosin subfragment-1 without incurring any loss in ATPase activity.

Properties of Labeled Subfragment-1. The introduction of a labeled light chain into myosin or its subfragments was first reported by Okamoto & Yagi (1976). They introduced spin-labeled DTNB light chains into skeletal muscle myosin by using a dissociating medium of 0.6 M KSCN or 4 M LiCl. Some loss of ATPase was observed in the treated myosin. It was found that the EPR spectrum of the spin-labeled myosin was altered by the presence of calcium ions. Later work of Okamoto & Yagi (1977) employed similar exchange experiments to produce a myosin molecule containing alkali light chains labeled with a spin-label at their single thiol and possessing unperturbed K^+- and Ca^{2+}-ATPase but only 50% of control actin-activated ATPase. The addition of Ca^{2+} and ATP produced no change in the spectrum of the spin-label. Additional experiments were reported in which myosin containing alkali light chains alkylated with *N*-ethylmaleimide exhibited a 60% loss of K^+-ATPase. The authors did not comment upon the very different effects of the two alkylating agents, and this apparent discrepancy makes comparisons with the present data difficult.

It is well established that during the hydrolysis of ATP by myosin, changes occur in the UV and fluorescence spectral characteristics of the protein (Morita, 1967; Werber et al., 1972). In the present study, it was found that during ATP hydrolysis by subfragment-1 labeled on the alkali 1 light chain, the fluorescence intensity of the fluorophore decreased by 6–10%. This effect may derive from a change in the microenvironment of the fluorophore and is perhaps another manifestation of the conformational change that perturbs the UV and tryptophan fluorescence spectra of myosin during ATP hydrolysis. A fast reaction kinetics study of labeled subfragment-1 may allow the conformational changes associated with this spectral change to be related to the myosin ATPase cycle and, in particular, to the sequence of changes in the intrinsic fluorescence of myosin (Bagshaw & Trentham, 1974; Bagshaw et al., 1974).

The observation reported here that subfragment-1 containing alkali 1 light chains modified at their single thiol retains full ATPase activity appears to be at variance with the work of Wagner & Yount (1976). They reported that heavy meromyosin lost ATPase activity upon reaction with a purine disulfide analogue of ATP at the single cysteine of the alkali light chains. Subsequent work, however, has suggested that disulfide exchange may have occurred on the heavy chain and

that the ATPase loss derives from this rather than from the alkali light-chain modification (R. Yount, personal communication).

A nucleotide-dependent conformational change in the alkali light chains of myosin has also been observed by Yamamoto & Sekine (1977a,b). They found that the alkali light chains of skeletal muscle myosin exhibited changes in their reactivities toward a thiol-specific fluorophore upon the addition of nucleotides. Similar findings, using 1,5-IAEDANS as the probe, were reported by Nihei (1978). It should be noted, however, that both the heavy and the light chains are labeled in these studies, thus making an unambiguous interpretation of the data difficult.

Energy-Transfer Measurements on Subfragment-1. Förster energy-transfer studies on myosin appear thus far to be restricted to the work of Haugland (1975) and Takashi et al. (1977). Haugland (1975) employed a wide variety of donor–acceptor pairs in a study of the active-site region of myosin. However, the conclusions drawn from this study were of a preliminary nature and did not include any experimental details regarding distances between specific donor–acceptor pairs. Takashi et al. (1977) have given a preliminary report of their energy-transfer studies aimed at determining the distance between the SH_1 thiol of subfragment-1 and the cysteine-373 residue of G-actin when these two proteins form a rigor complex. [The full report has since been published: Takashi (1979).] The only other known distance within the myosin "head", that between the SH_1 and SH_2 thiols, has been provided by chemical cross-linking experiments (Reisler et al., 1974; Burke et al., 1976).

During the course of this work an additional report has appeared which describes the measurement of Förster transfer between the single cysteine of the alkali light chain and the active site of subfragment-1 (Moss & Trentham, 1979).

A major aim of the present work was to determine the distance between the single cysteine residue of the alkali 1 light chain and the SH_1 thiol on the heavy chain of subfragment-1. The accuracy of the final distance obtained from energy-transfer efficiency measurements will clearly depend not only upon the accuracy of measurement of the various experimental parameters but also upon the choice of the theoretical parameters, such as κ^2, required for the calculation.

The problems associated with uncertain and low extinction coefficients for bound fluorophores have been discussed above and in detail by Fairclough & Cantor (1978). The potential problem of concentration imbalance of the fluorophores has been avoided by employing excitation and emission spectra recorded in a denaturing solvent to normalize the concentrations. In this manner it was possible to match concentrations very closely.

The only assumed theoretical value subject to significant ambiguity is κ^2. By setting $\kappa^2 = 2/3$, it has been assumed that both donor and acceptor are tumbling rapidly and are free to assume any orientation. In practice, this situation is rarely met. Theoretically, κ^2 can vary between 0 and 4, thus ultimately limiting the accuracy with which R_o, and hence R, can be determined. Upper and lower limits can be placed upon the value of κ^2, and hence R, by utilizing time-resolved fluorescence depolarization measurements to derive information regarding donor and acceptor motional freedom (Dale & Eisinger, 1975). Mendelson et al. (1973) have performed such measurements on 1,5-IAEDANS bound at the SH_1 thiol of subfragment-1 and have shown that it is immobilized to a large extent. Thomas et al. (1975) also observed strong immobilization of a spin-label attached at the SH_1 thiol. These observations suggest that the use of $\kappa^2 = 2/3$ may be inappropriate for the present investigation. Recently, however, Takashi (1979) has found that the emission anisotropy of 1,5-IAEDANS bound to the SH_1 thiol of subfragment-1 in a sucrose solution of high viscosity was significantly less than the emission anisotropy of 1,5-IAEDANS in propylene glycol at −55 °C measured by Hudson & Weber (1973). This suggests that some motion of the label attached to subfragment-1 occurs even though subfragment-1 as a whole is immobile.

Even in the absence of definitive information regarding an appropriate value for κ^2, it is possible to limit the range of values it can assume. Hillel & Wu (1976) have developed an interpretation of energy-transfer measurements which enables a statistically significant range of the donor–acceptor distance, R, to be defined. They have calculated probability distribution functions for the separation between donor–acceptor pairs based upon the experimental efficiency of energy transfer and a parameter defined by R and κ^2. Examination of the graphical representation of a distribution function of Hillel & Wu (1976), in which one transition moment is fixed and the other is free to orient on the surface of a cone of 30° half-angle, showed that there is a 90% probability that R falls in the range 28–46 Å.

Experimental and theoretical considerations suggest that this range may be reduced further. Foremost among these is the experimental observation that the calculated value of R is insensitive to a reversal of the points of attachment of donor and acceptor. It might be expected that, if one or both of the fluorophores depart from the assumed state of rapid tumbling and unconstrained motion, a reversal of the labels would induce a change in their relative dipole geometries and hence in κ^2 and R. No such change in R was observed, thus suggesting that the choice of 2/3 for κ^2 may be a valid approximation. Further support for this suggestion comes from the theoretical considerations of Haas et al. (1978). The mathematical expression for κ^2 is based upon the assumption that the emission of donor and the absorption of acceptor are each characterized by a single transition dipole moment. Haas et al. (1978) have pointed out, however, that many chromophores possess electronic transitions with mixed polarizations and thus the range of values that κ^2 can assume is greatly reduced. In summary, as pointed out by Stryer (1978), employing an orientation factor of 2/3 probably introduces an uncertainty in the calculated distance of less than 20%.

Steady-state measurements on doubly labeled subfragment-1 revealed no change in this distance upon the addition of MgATP. It is possible that fast reaction kinetic studies would reveal a transitional change in this distance, but it must be recognized that the ATPase characteristics of subfragment-1 are significantly altered by alkylation of the SH_1 thiol (Sekine & Kielley, 1964). A further potential problem arises because the binding of nucleotides to subfragment-1 may alter κ^2, thus complicating the extraction of distance information from observed changes in transfer efficiency.

In conclusion, the distance between the SH_1 thiol on the heavy chain and the single cysteine of the alkali 1 light chain has been calculated as ~40 Å. Further application of Förster energy-transfer measurements coupled with immune electron microscopy (Strycharz et al., 1978) of antibody-labeled myosin will be used to define additional structural features of the myosin head.

Acknowledgments

The authors express their thanks to Drs. P. A. Benfield and L. E. Cannon for assistance with the amino acid analyses and

peptide maps and to Dr. W. B. Gratzer for helpful discussions.

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