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Förster Energy Transfer Measurements of Thiol 1 to Thiol 2 Distances in Myosin Subfragment 1†

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ABSTRACT: Förster energy transfer was used to measure the distance between reporter groups on the two reactive thiols of myosin, SH_1 and SH_2, and to detect changes in this distance upon binding of nucleotide. SH_1 was labeled with the fluorophore 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (1,5-IAEDANS) and SH_2 with the chromophoric acceptor *N*-[4-(dimethylamino)-3,5-dinitrophenyl]-maleimide (DDPM). Peptide studies verified that [^{3}H]-1,5-IAEDANS reacted specifically with SH_1, while [^{14}C]DDPM labeled both SH_2 and the alkali light chains. The [^{14}C]-DDPM-modified alkali light chains were replaced with unmodified light chains by the exchange procedure of Wagner and Weeds [Wagner, P. D., & Weeds, A. G. (1977) *J. Mol. Biol. 109*, 455-473]. Subfragment 1 labeled with 1,5-IAEDANS and then with DDPM exhibited two fluorescence lifetimes, 20.6 (AEDANS-SF_1, unquenched) and 9.3 ns (AEDANS-SF_1, quenched by DDPM). The latter lifetime decreased to an average of 2.85 ns after the addition of MgAMP-PNP, MgADP, or $MgPP_i$ (no change with MgAMP), indicating that the distance between the donor and acceptor decreased. An R_0 of 29 Å was calculated for the AEDANS/DDPM system assuming random orientation of the donor/acceptor pair. The decrease in the observed lifetimes upon the addition of Mg nucleotide corresponds to a change in the donor–acceptor distance from 28 to 21–22 Å. This observation is consistent with the proposal that nucleotide binding juxtaposes SH_1 and SH_2 to enhance their cross-linking with various bifunctional reagents [Burke, M., & Reisler, E. (1977) *Biochemistry 16*, 5559-5563; Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966-4970].

It is well established that myosin and its proteolytic fragment, myosin subfragment 1 (SF_1),[1] undergo spectroscopically sensitive conformational changes during ATP binding, hydrolysis, and product release [Morita, 1967; Seidel et al., 1970; Werber et al., 1972; Murphy, 1974; Mendelson et al., 1975; for reviews see Trentham et al. (1976) and Highsmith & Cooke (1983)]. Extrinsic spectroscopic probes used to monitor these changes are generally introduced by modification of SH_1, the most reactive cysteine in the heavy chain of myosin [see Reisler (1982) for a recent review]. A second activity critical thiol, SH_2, may also be modified rapidly if MgADP (or related compounds) is added (Sekine & Yamaguchi, 1963; Yama-

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[1] Abbreviations: SF_1, myosin chymotryptic subfragment 1; AL1, alkali light chain 1; AL2, alkali light chain 2; AMP-PNP, 5′-adenylyl imidodiphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; 1,5-IAEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid; DDPM, *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide; IAA, iodoacetamide; MalNET, *N*-ethylmaleimide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; 9-AA, 9-aminoacridine; DPH, 1,6-diphenyl-1,3,5-hexatriene; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene; AEDANS-SF_1, subfragment 1 modified at SH_1; SF_1-DDPM, subfragment 1 modified at SH_2; ATPase, adenosinetriphosphatase; DTE, dithioerythritol; Gdn·HCl, guanidine hydrochloride.

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guchi & Sekine, 1966; Schaub et al., 1975), suggesting that this thiol moves from a buried to an exposed position under these conditions.

Cross-linking studies have given us valuable information about the flexibility of the heavy chain region containing SH_1 and SH_2. Reisler et al. (1974) and Burke & Reisler (1977) found using the bifunctional reagents *p*-phenylenedimaleimide and 4,4′-difluoro-3,3′-dinitrodiphenyl sulfone that the rate of inactivation of myosin ATPase was markedly promoted by addition of MgADP. They suggested MgADP binding moved SH_1 and SH_2 closer together to enhance their cross-linking. Our studies have yielded similar results. We have proposed that these thiols can approach within 3–5 Å of each other because they bind to a single cobalt in an exchange inert Co(III) complex (Wells & Yount, 1979). Other evidence suggests these SH groups can be oxidized to form a disulfide bond (Wells & Yount, 1980; Togashi & Reisler, 1982). These results taken with other cross-linking studies (Wells et al., 1980) indicate that the sulfur–sulfur distance between SH_1 and SH_2 can vary from a minimum upper limit of 14 Å to a lower limit of 2 Å.

In the cases where Mg nucleotide stimulates the cross-linking of SF_1, the metal nucleotide becomes trapped at the active site of myosin [for review see Wells & Yount (1982)]. These studies suggest that the binding of nucleotide juxtaposes SH_1 and SH_2 to facilitate their cross-linking which in turn stabilizes a conformation favoring bound nucleotide. In the present study, we have tested this proposal using Förster energy transfer techniques to measure the distance between SH_1 and SH_2. This approach [for reviews see Stryer (1978) and Fairclough & Cantor (1978)] has proven to be an effective technique to map the topography of contractile proteins [Haugland, 1975; Miki & Mihashi, 1978; Takashi, 1979; Marsh & Lowey, 1980; Moss & Trentham, 1980; Tao & Lamkin, 1981; Takashi et al., 1982; for review see Morales et al. (1982)]. We have carried out energy transfer studies with the dansyl derivative 1,5-IAEDANS attached to SH_1 and the chromophoric acceptor *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide (DDPM) covalently linked to SH_2. Fluorescence lifetime measurements indicate that addition of Mg nucleotide changes the distance between the labeled sites from 28 to 21–22 Å. Thus, a 6–7 Å change in the distance between the reporter groups on SH_1 and SH_2 appears to occur upon binding nucleotide to SF_1. These results do not agree with recent similar measurements in which AEDANS and fluorescein were used as a donor/acceptor pair on SH_1 and SH_2 (Cheung et al., 1983). These studies gave an average SH_1 to SH_2 distance of 46 Å which did not change on addition of MgADP. Possible explanations for the large discrepancy in distance and the lack of effect on addition of nucleotides are discussed.

Materials and Methods

Materials

Na_2ATP, Li_3ADP, and AMP were from P-L Biochemicals, Schwarz/Mann, and Sigma, respectively. AMP-PNP was prepared according to Yount et al. (1971). Reagents used for gel electrophoresis were from Sigma. Ultrapure urea and $(NH_4)_2SO_4$ were from Schwarz/Mann while trypsin (bovine pancreas) and soybean trypsin inhibitor were from Sigma. DDPM, 1,5-IAEDANS, and IAA were purchased from Aldrich, Sigma, and Pierce, respectively. [^{3}H]-1,5-IAEDANS (55 000 cpm/nmol) was synthesized according to the procedure of Huang et al. (1975) and purified by using TLC as described by Hudson & Weber (1973). [^{14}C]DDPM (3000 cpm/nmol) was synthesized by coupling 4-(dimethylamino)-3,5-dinitroaniline with [1,4-^{14}C]maleic anhydride (Amersham) as described by Witter & Tuppy (1960) and purified by sublimation (mp 176–180 °C; reported 180 °C). All other chemicals were reagent grade, and water was twice deionized.

Methods

Protein Preparation. Rabbit skeletal myosin was prepared according to Wagner & Yount (1975) and was stored in 50% glycerol at −20 °C. Chymotryptic SF_1 was prepared as described by Weeds & Taylor (1975) and was assumed to have a molecular weight of 115 000 and an $\epsilon_{280}^{1\%} = 7.5$ cm^{-1} (Wagner & Weeds, 1977). Light chains were prepared by dissociating myosin in 5 M guanidine hydrochloride, followed by precipitation of the heavy chains by ethanol (Perrie & Perry, 1970). The light chains, in the supernatant, were then chromatographed on a DEAE-cellulose column after removal of guanidine hydrochloride (Holt & Lowey, 1975). The 19 000-dalton light chain (DTNB light chain) was removed by repeated precipitations from the pooled light chains with 18% ethanol (Perrie et al., 1973). Analysis by NaDodSO$_4$–polyacrylamide gel electrophoresis indicated that this light chain accounted for less than 5% of the remaining light chains. The concentration of alkali light chains was determined by absorption by using $\epsilon_{280}^{1\%} = 2.0$ cm^{-1} (Wagner & Weeds, 1977).

Enzyme Derivatives. SF_1 (17 μM) was treated with 17 μM 1,5-IAEDANS in 50 mM Tris, 0.1 M KCl, and 0.3 mM KN_3, pH 8.0 (Tris–KCl buffer). After the mixture was allowed to stand at 0 °C for 18–24 h in the dark, the reaction was terminated by adding a 100-fold molar excess of β-mercaptoethanol over 1,5-IAEDANS. The enzyme derivative was then purified by precipitation with 2.5 volumes of saturated $(NH_4)_2SO_4$, pH 8.0, followed by Sephadex G-25 gel filtration using Tris–KCl buffer at 4 °C. The AEDANS-SF_1 was subsequently filtered through an 0.8-μm Millex filter (Millipore Corp.) and stored on ice at concentrations of 2–4 mg/mL.

AEDANS-SF_1-DDPM was prepared by two different methods. In the first procedure, a 2-fold molar excess of DDPM (10 mM dissolved in acetone) was added to AEDANS-SF_1 (17 μM) in Tris–KCl buffer at 4 °C containing 0.17 mM ADP and 0.85 mM $MgCl_2$. After 5 min, the reaction was terminated by adding a 100-fold molar excess of β-mercaptoethanol over SF_1. In the second method, which was found to be more specific, an equivalent amount of DDPM was added to AEDANS-SF_1 (17 μM) in 50 mM Mes and 0.1 M KCl, pH 6.3 (Mes–KCl buffer), at 4 °C containing 0.17 mM ADP and 0.85 mM $MgCl_2$. The reaction was quenched after 20 min by adding a 100-fold molar excess of β-mercaptoethanol over DDPM. These enzyme derivatives were purified, filtered, and stored as described above for AEDANS-SF_1.

Reconstitution. AEDANS-SF_1-DDPM (20 μM) and purified alkali light chains (260 μM) were mixed together in 100 mM Tes, 2 mM DTT, and 2 mM EDTA, pH 7.0, and NH_4Cl was added to a final concentration of 4.7 M as described by Wagner & Weeds (1977). After gentle stirring for 15 min at 4 °C, the NH_4Cl was removed by dialyzing against 50 mM Tes, 1 mM DTE, and 1 mM EDTA, pH 7.0. The protein was then applied to a DEAE-cellulose (DE-52) column (30 cm × 1.5 cm) equilibrated in the same buffer, and modified SF_1 was eluted with a linear 200-mL NaCl gradient (0–0.12 M NaCl). Subsequently, the excess light chains were eluted with a 1.0 M NaCl step gradient (Wagner, 1982). The elution of protein was monitored by measuring the absorbance at 230 nm and the fluorescence intensity of AEDANS-SF_1; the various protein

fractions were pooled and analyzed by $NaDodSO_4$–polyacrylamide electrophoresis.

Peptide Studies. SF_1 (4 mg/mL) labeled with either [^{3}H]-1,5-IAEDANS (5000 cpm/nmol) or [^{14}C]DDPM (3000 cpm/nmol) was digested with trypsin (0.04 mg/mL) in Tris–KCl buffer, pH 8.0 at 25 °C. The reaction with trypsin was terminated after 30 min by addition of 0.12 mg/mL soybean trypsin inhibitor. Subsequent cleavage of trypsinized SF_1 at asparaginyl–glycine bonds by hydroxylamine was performed as described by Sutoh (1981). The enzyme derivative was dialyzed against 6 M Gdn·HCl, 1% β-mercaptoethanol, and 10 mM sodium acetate, pH 5.4. After dialysis, 4 mL of trypsinized SF_1 in Gdn·HCl buffer was added to an equal volume of 6 M Gdn·HCl and 2 M hydroxylamine, pH 9.0. The resulting solution was adjusted to pH 9.0 with 0.1 N NaOH. The reaction was terminated at various times by adding 0.1 mL of glacial acetic acid to 0.9 mL of the hydroxylamine reaction mixture. This solution was dialyzed against 1 L of distilled water with Spectropore 6 dialysis tubing (cutoff $M_r \simeq 2000$) to prevent the loss of small molecular weight peptides. Subsequently, the peptides were dialyzed vs. 1% $NaDodSO_4$, 20 mM Tris, and 0.1% β-mercaptoethanol, pH 8.3, in preparation for electrophoresis.

Polyacrylamide–$NaDodSO_4$ Gel Electrophoresis. The fragments produced by hydroxylamine cleavage were separated on a $NaDodSO_4$–16% acrylamide–0.43% bis(acrylamide) gel (Laemmli, 1970) containing 6 M urea. To minimize loss of the small peptides from the gel, 10% unbuffered glutaraldehyde was used to fix the hydroxylamine-treated peptides. All other peptides separations used a 7–14% polyacrylamide gradient gel in the presence of 0.1% $NaDodSO_4$. The peptides were fixed and stained overnight in 0.025% Coomassie Brilliant Blue G 250 in 45% methanol, 45% water, and 10% glacial acetic acid. After destaining, the gel was impregnated for 1 h with 22% 2,5-diphenyloxazole in glacial acetic acid for fluorography and dried in vacuo. Fluorography was performed as described by Bonner & Laskey (1974). Kodak XAR-5 X-O-Mat film was placed on top of the dried gel and exposed from 2 to 4 weeks at −80 °C.

Stoichiometry of Labeling. The amount of 1,5-IAEDANS bound to SF_1 was determined by absorption at 337 nm by using $\epsilon = 6100\ M^{-1}\ cm^{-1}$ (Hudson & Weber, 1973). Protein concentrations of AEDANS-SF_1 were determined spectrally at 280 nm by using $\epsilon_{280}^{1\%} = 7.5\ cm^{-1}$. The 280-nm absorption of AEDANS labeled stoichiometrically to SF_1 was less than 1.5% of that for the protein. The amount of DDPM bound to SF_1 was determined by the absorption at 442 nm by using $\epsilon = 2930\ M^{-1}\ cm^{-1}$ (Gold & Segal, 1964). Protein concentration of DDPM-modified SF_1 was determined by a dye binding method (see below) since DDPM has significant absorption at 280 nm.

Analytical Procedures. Ca^{2+}ATPase and K^+ATPase assays were performed as described previously (Wells et al., 1979b, 1980), except that release of inorganic phosphate was measured at 2 and 8 min after the addition of SF_1 to the assay mixture. Protein concentration measurements used a Coomassie Blue dye binding method (Bradford, 1976) with unmodified SF_1 as a standard as previously described (Wells et al., 1979a). Liquid scintillation counting was performed on a Beckman LS 7500 with aqueous counting scintillant (Amersham).

Absorption Measurements. Routine absorption measurements were made with a Beckman DU2 modified to produce a digital output of absorbance. All absorption spectra were taken with a Cary 14 that was modified to produce digital outputs for wavelength and slide wire response. The Cary 14 was interfaced to an HP 9825A computer and HP 7225A plotter (Hewlett Packard) such that spectra could be digitally stored on tape and mathematically manipulated. This was utilized for obtaining difference spectra and for doing the spectral overlap calculations needed for the determination of energy transfer parameters.

Steady-State Fluorescence. Steady-state fluorescence measurements and spectra were made with an SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL), which was interfaced with an HP 9825A computer and HP 7225A plotter. Corrected emission spectra were made with the excitation polarizer (Glan-Thompson) set a 55° from vertical and the emission polarizer set at 0°. All slits were adjusted to a 4-nm band-pass. The sample compartment was thermostatically controlled and permitted use of 1 cm × 1 cm cuvettes containing a minimum of 1 mL of solution.

Fluorescence Lifetime Measurements. The SLM 4800 spectrofluorometer was also used to determine fluorescence lifetimes. With this instrument, the excitation light passes through a monochromator and is intensity modulated at 6, 18, or 30 MHz by a Debye–Sears ultrasonic modulation tank. While in the lifetime mode, slit widths were adjusted as follows: 8 nm prior to the excitation monochromator grating, 0.5 nm prior to the modulation tank, and 0.5 nm prior to the sample chamber. Both emission monochromator slits were set at 16 nm. To eliminate the effects of anisotropic rotation on fluorescence lifetimes, the Glan-Thompson excitation polarizer was set at 35° from vertical, and there was no emission polarizer (Spencer & Weber, 1970).

A fluorescent molecule with a finite lifetime will introduce a phase lag in the emission beam and demodulate it with respect to the excitation beam (Spencer & Weber, 1969). At a given frequency, the magnitude of these effects is used to calculate a phase-derived and a modulation-derived fluorescence lifetime:

$$\tau_p = \frac{1}{\omega} \tan \phi \tag{1}$$

$$\tau_m = \left(\frac{1}{\omega}\right)\left(\frac{1}{m^2} - 1\right)^{1/2} \tag{2}$$

where ω is the circular modulation frequency, ϕ is the phase shift, and m is the modulation. In this study, lifetimes were determined by the phase shift and demodulation of fluorescence of a sample relative to a standard reference compound (Lakowicz et al., 1980, 1981). In this approach eq 1 and 2 are modified such that $\phi = \phi_{obsd} + \phi_R$ and $m = m_{obsd}(1 + \omega^2\tau_R^2)^{-1/2}$ where ϕ_R and τ_R are the phase and lifetime values of the reference compound. The reference compounds used in the present study were POPOP, DPH, and 9-AA which had lifetimes of 1.25, 8.7, and 15.7 ns, respectively, at 6 °C. These lifetime measurements were made with the excitation monochromator set at 375 nm and the emission monochromator set at 495 nm, the same settings used to measure AEDANS-SF_1 lifetimes. The lifetimes of AEDANS-SF_1 determined at 495 nm were >98% homogeneous as compared to 95–96% at 470 nm. This dependency of lifetime heterogeneity on emission wavelength was previously observed for 1,5-IAEDANS free in solution (Hudson & Weber, 1973).

The lifetimes were calculated with an HP 9825A computer that received the instrumental phase and modulation values. Alternate readings of the phase and modulation were taken with the reference and sample for a total of eight readings each. A computer program calculated phase (τ_p) and modulation (τ_m) lifetimes after one reference and one sample

reading. After each successive reading, lifetimes were recalculated by using the new phase and modulation values and the values from the immediately preceding reading. This resulted in 15 pairs of τ_p and τ_m values for the eight measurements each on the reference and the sample. The computer averaged the 15 values of τ_p and τ_m, determined the standard deviation in each, and printed the τ_p and τ_m values at each of the three modulation frequencies. Multiple data sets were obtained by making measurements at 6, 18, and 30 MHz for one set and then reversing the order for the second set etc. until the desired number of sets were collected. (One set required about 5 min to collect.) In most cases, particularly for heterogeneous systems, three sets of data were collected on each sample, and the average of these results was used for lifetime analyses.

Heterogeneity Analysis. If all of the lifetime values of a set are equal within experimental error, the sample is homogeneous, and the value of the fluorescence lifetime can be obtained with confidence. However, if the set exhibits heterogeneous character, indicated by τ_p values less than the τ_m values at any frequency and a decrease in both values with increasing frequency, multiple lifetime species are present. Weber (1981) presented theoretical equations for solving for lifetimes in heterogeneous systems. Using phase modulation methods, Jameson & Weber (1981) experimentally verified the equations by resolving two lifetimes in tryptophan-containing solutions.

In energy transfer experiments, it is necessary to solve for multiple lifetimes since in almost every case the donor will exhibit two lifetimes corresponding to the unquenched and quenched states. Several computer programs have been written and utilized to solve for the lifetimes (τ_i) and their relative fluorescence intensity contributions (α_i) in a heterogeneous system. These programs are iterative in nature and use a Monte Carlo approach to find the best fit to all of the input data. Each program calculates τ_i and α_i values plus gives the expected values for τ_p and τ_m at all three frequencies, based on the values that best fit the experimental data. In addition a χ^2 was calculated which is the sum of the squares of the differences between the expected and experimental τ_p and τ_m values at each frequency. FIT 1 requires one lifetime to remain fixed and determines τ_2, α_1, and α_2 as well as χ^2. This program is very useful when one lifetime is known with some confidence as it minimizes the error in the second lifetime. The FIT 2 routine determines the two lifetimes and the fractional fluorescence contribution of each component. In general, FIT 2 gives lower χ^2 values but less precise τ_2 values than the FIT 1 routine. The routine, FIT 3, will solve for a third lifetime and the fractional fluorescence contributions of the components of a ternary system *if* two of the three lifetimes are known. A modified FIT 3 routine, denoted FIT 3A, is used to calculate α_1, α_2, α_3, and χ^2 by using three known lifetimes. A computer program was also written to convert fractional fluorescence values (α_i) of each component to the corresponding mole fractions ($\bar{X}_i$) by assuming that the quantum yield of each component is proportional to its lifetime. A full description of the methodology used and computer programs will be given in a future publication (R. Dalbey, J. Weiel, W. Perkins, and R. G. Yount, unpublished results).

Results

A major consideration in doing Förster energy transfer studies is to label the donor and acceptor sites specifically (Stryer, 1978). Consequently the location of each modification has been verified by peptide studies with [^{3}H]-1,5-IAEDANS and [^{14}C]DDPM. Myosin treated with [^{3}H]-1,5-IAEDANS

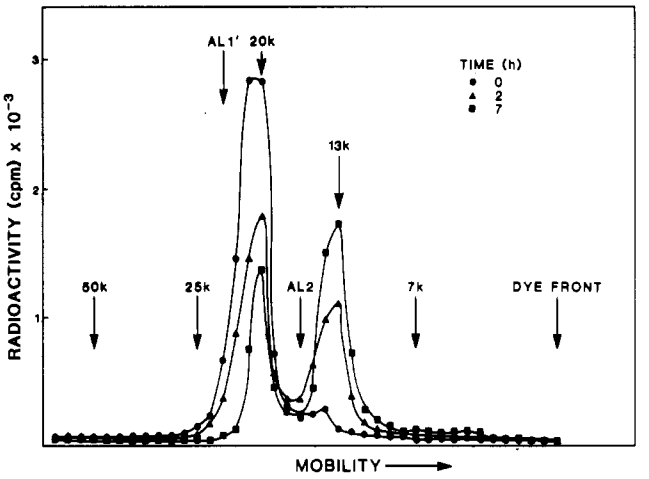

FIGURE 1: Gel electrophoresis separation after hydroxylamine fragmentation of trypsin-treated AEDANS-SF_1. SF_1 (17 μM) was treated with 13.6 μM [^{3}H]-1,5-IAEDANS (5000 cpm/nmol) in Tris–KCl buffer, pH 8.0, at 0 °C, for 18 h. The Ca^{2+}ATPase and K^+ATPase were 300% and 30%, respectively, relative to controls. AEDANS-SF_1 was then digested with trypsin and cleaved with 1 M hydroxylamine for various times (0–7 h) as described under Materials and Methods. The fragmented SF_1 was electrophoresed in $NaDodSO_4$–16% polyacrylamide slab gel in the presence of 6 M urea (see Materials and Methods). The radioactivity applied at times 0, 2, and 7 h were 6000, 5265, and 3674 cpm, respectively. Each lane of the gel was sliced in 1.5-mm pieces and solubilized in 0.5 mL of H_2O_2–NH_4OH (99:1) as described by Albanese & Goodman (1977) to determine the radioactivity. The counts in each lane were normalized to 6000 cpm for comparison purposes. The mobility of each fragment was determined by running parallel samples in adjacent lanes, and peptides were detected by Coomassie Blue staining.

has been shown previously by Takashi et al. (1976) to label only one tryptic peptide from myosin. Later Takashi (1979) and Marsh & Lowey (1980) showed that the 20K tryptic peptide known to contain SH_1 and SH_2 was the only peptide labeled by 1,5-IAEDANS treatment of SF_1. The location of the 1,5-IAEDANS labeling of SF_1 is defined further here by fragmenting the protein derivative with both trypsin and hydroxylamine (Bornstein & Balian, 1977) as described by Sutoh (1981). Hydroxylamine cleaves the 20K SF_1 tryptic peptide at the single Asn–Gly bond to produce a C-terminal 13K peptide containing SH_1 and a 7K peptide containing SH_2.

Gel electrophoretic separation of the limited trypsin digest of [^{3}H]AEDANS-SF_1 and subsequent Coomassie Blue staining showed the characteristic three major fragments 50K, 25K, and 20K from the 95K heavy chain fragment (Balint et al., 1978). Figure 1 shows that all radioactivity was localized in the 20K peptide. Subsequent hydroxylamine treatment of the trypsin digest produced the predicted 13K fragment and 7K fragment with concomitant loss of the 20K peptide. As can be seen in Figure 1, the radioactivity disappears from the 20K peptide with concomitant appearance in the 13K peptide. The trace amount of 13K peptide at "zero time" occurred because some time elapsed after the addition of hydroxylamine and during pH adjustment to 9.0 before the temperature reached 45 °C when timing started.

DDPM Labels both SH2 and the Alkali Light Chains. Yamashita et al. (1965) previously showed that DDPM could be used to modify SH_2. Figure 2A confirms this result and indicates that DDPM rapidly inactivates AEDANS-SF_1 in the presence of MgADP in Tris–KCl buffer, pH 8.0. The half-life ($t_{1/2}$) for the rate of inactivation was less than 14 s. The inactivation gave a linear incorporation of [^{14}C]DDPM into AEDANS-SF_1 which extrapolated to approximately one DDPM/AEDANS-SF_1 when all activity was lost (Figure 2B).

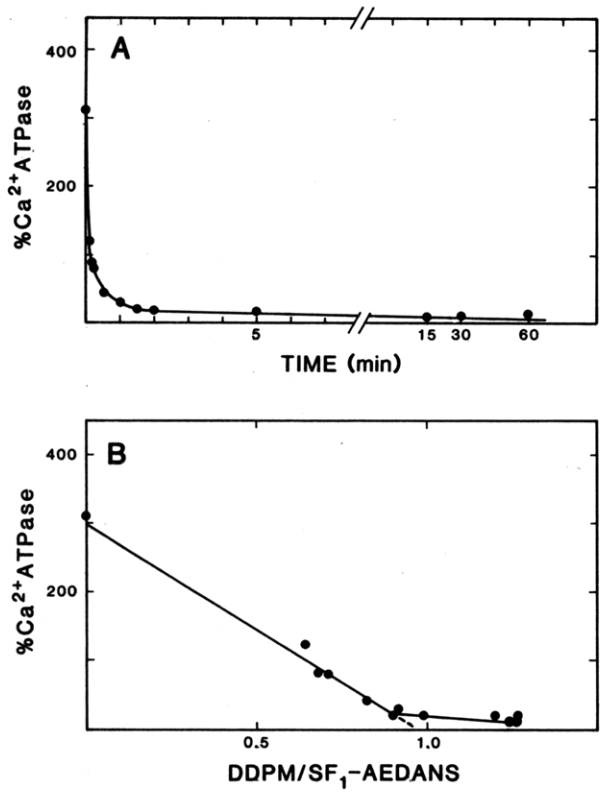

FIGURE 2: (Panel A) Inactivation of AEDANS-SF_1 Ca^{2+}ATPase by DDPM. SF_1 (15 μM) at 0 °C in Tris–KCl buffer, pH 8.0, was treated with 15 μM 1,5-IAEDANS for 18 h in the dark. The resulting Ca^{2+}ATPase and K^+ATPase were 311% and 20%, respectively, of untreated controls. AEDANS-SF_1 (15 μM) was treated with 30 μM DDPM (833 cpm/nmol) in the presence of 0.3 mM ADP and 0.6 mM $MgCl_2$. At indicated times, 1 mL of the reaction mixture was quenched with 100-fold molar excess DTT and subsequently assayed for Ca^{2+}ATPase activity (see Materials and Methods). (Panel B) Percent relative Ca^{2+}ATPase activity vs. [^{14}C]DDPM/AEDANS-SF_1 incorporated. At indicated times, 1 mL of the DTT quenched reaction mixture (14.5 μM protein) was treated with 0.5 mL of 12.5% trichloroacetic acid and the denatured protein pelleted by centrifugation (>95% protein recovered). The pellet was dissolved by adding 0.4 mL of Protosol (New England Nuclear) and heating at 61 °C for 4 h. The stoichiometry of [^{14}C]DDPM incorporation was determined by scintillation counting assuming 100% of the protein was recovered.

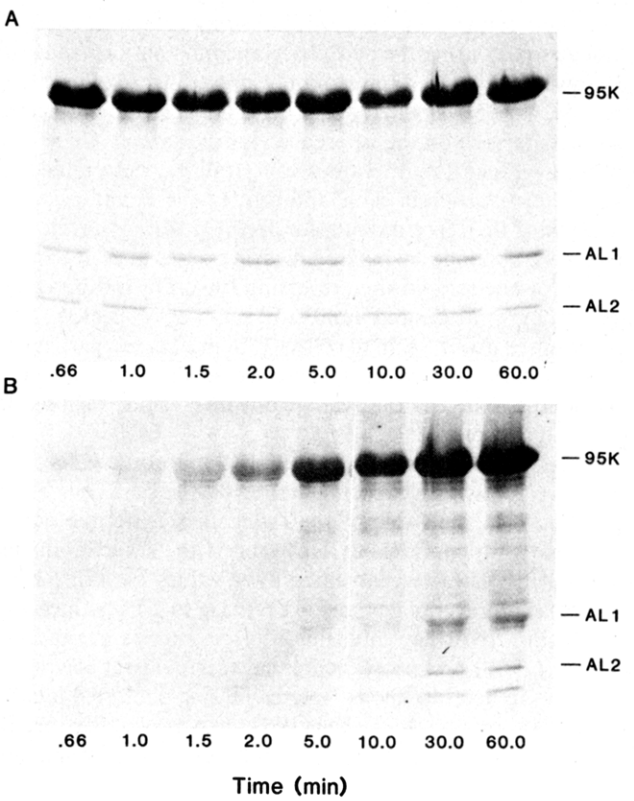

FIGURE 3: Time course of [^{14}C]DDPM labeling of AEDANS-SF_1. AEDANS-SF_1 was prepared as described in Figure 2. AEDANS-SF_1, 52 μM, in 50 mM Mes and 0.1 M KCl, pH 6.3, containing 1.2 mM ADP and 5 mM $MgCl_2$ was treated with 58 μM DDPM (3000 cpm/nmol). At various times, 150-μL aliquots were quenched by addition to a 1000-fold molar excess of β-mercaptoethanol. The samples were applied directly to $NaDodSO_4$–7–14% polyacrylamide gel. (Panel A) Coomassie Blue stained $NaDodSO_4$ slab gel. Each lane contained 129 μg of the derivatized protein and 3360 cpm of [^{14}C]DDPM. (Panel B) Fluorograph of the Coomassie Blue stained gel in panel A. The fluorograph was exposed for 14 days. The fluorograph detects only the [^{14}C]DDPM which is covalently bound to SF_1 since free [^{14}C]DDPM diffuses out of the gel.

Gel electrophoretic analysis at the 5-min reaction time showed the nonactivity related labeling occurred on the alkali light chains (data not shown). Similar analyses of the tryptic and hydroxylamine peptides showed [^{14}C]DPPM labeled only the 7K peptide containing SH_2 (Dalbey, 1983).

Removal of [^{14}C]DDPM-Labeled Alkali Light Chains. A more specific labeling of AEDANS-SF_1 is achieved by performing the DDPM reaction at pH 6.3 (see Materials and Methods). The rate of inactivation by DDPM in 50 mM Mes and 0.1 mM KCl at pH 6.3 is significantly slower than at pH 8.0 with a half-life of approximately 8 min (data not shown). Figure 3 shows that [^{14}C]DDPM is incorporated primarily in the 95K fragment and only slightly in the alkali light chains (less than 10% of the total [^{14}C]DDPM labeling). Nevertheless, to ensure labeling at only SH_2, we replaced the [^{14}C]DDPM-labeled light chains with unlabeled light chains using the exchange procedure of Wagner & Weeds (1977). After exchange was complete, SF_1 was purified on a DEAE-cellulose column (see Materials and Methods). The $NaDodSO_4$ slab gel in Figure 4A shows the Coomassie Blue stained protein before and after reconstitution. The corresponding fluorograph (Figure 4B) indicates that after reconstitution, there is no [^{14}C]DDPM on the alkali light chains. Similar analyses performed on the excess light chains isolated by DEAE-cellulose chromatography showed, as expected, that the alkali light chains were labeled with [^{14}C]DDPM (data not shown).

Spectral Overlap between Emission Bands of AEDANS-SF_1 and the Absorption Bands of SF_1-DDPM. The corrected fluorescence emission spectrum of AEDANS-SF_1 has good overlap with the absorption spectrum of SF_1 with DDPM attached to SH_2 (Figure 5). The shape of the spectrum for SF_1-DDPM is essentially identical with that of DDPM reacted with β-mercaptoethanol in Tris–KCl buffer, pH 8.0, indicating the protein does not affect the DDPM chromophore significantly. A molar absorptivity of 2930 M^{-1} cm^{-1} at 442 nm was determined for the DDPM–β-mercaptoethanol adduct, in good agreement with the value obtained previously by Gold & Segal (1964). When the DDPM–β-mercaptoethanol spectrum and the corrected emission spectrum of AEDANS-SF_1 were used, a spectral overlap integral (J) of 6.1×10^{13} M^{-1} cm^{-1} nm^4 was determined.[2]

[2] During the course of this work, it was observed that the spectrum of SF_1-DDPM changed as a function of time due to base-promoted hydrolysis of the maleimide ring (Yamamoto et al., 1977). This change in the absorption spectrum posed a potential problem since the distance determination depends on the spectral overlap integral. A careful lifetime study of AEDANS-SF_1-DDPM as a function of time indicated that there was no change as the maleimide ring opened. This occurred because the spectral overlap integral (J) for AEDANS and DDPM was the same for both the closed form and the ring opened form (6.1×10^{13} vs. 6.3×10^{13} M^{-1} cm^{-1} nm^4).

Table I: AEDANS-SF_1 Lifetime Determinations by Phase-Modulation Spectroscopy[a]

	experimental		predicted by FIT 2		predicted by FIT 1	
f (MHz)	τ_p (ns)	τ_m (ns)	τ_p (ns)	τ_m (ns)	τ_p (ns)	τ_m (ns)
6	20.52 ± 0.16	20.60 ± 0.39	20.48	20.62	20.46	20.55
18	20.11 ± 0.27	20.51 ± 0.16	20.22	20.49	20.25	20.47
30	20.16 ± 0.48	20.43 ± 0.21	20.10	20.43	20.10	20.42
computer-determined values: τ_1 (α_1)			20.75 (0.98)		20.60[b] (0.99)	
τ_2 (α_2)			10.01 (0.018)		7.35 (0.007)	
χ^2			0.019		0.031	

[a] AEDANS-SF_1 (34 μM in Tris-KCl buffer, pH 8.0, 6 °C) was prepared as described under Materials and Methods. f is the modulation frequency; τ_p and τ_m are the lifetimes based on phase and modulation, respectively. FIT 1 and FIT 2 are the names of computer program routines which solve for the best-fit lifetime and fractional fluorescence contribution of each component and yield τ_p and τ_m values expected at the three frequencies. [b] The underlined value, 20.60, is fixed in the FIT 1 routine.

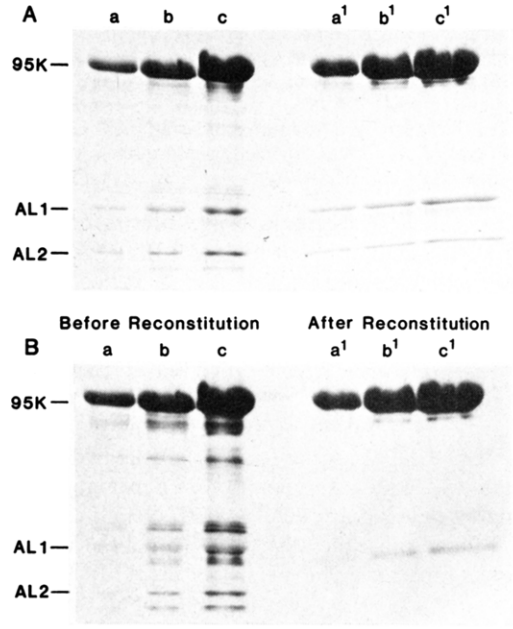

FIGURE 4: Exchange of the [^{14}C]DDPM-labeled alkali light chains with unmodified alkali light chains. AEDANS-SF_1-[^{14}C]DDPM was prepared as described in Figure 3. The light chains were exchanged as described under Materials and Methods for 15 min. The reconstituted protein was purified by DEAE-cellulose chromatography and analyzed by $NaDodSO_4$ gel electrophoresis (right side, panels A and B) with 7–14% polyacrylamide. Control samples showing AL1 and AL2 labeled with [^{14}C]DDPM were run on the left (panels A and B). (Panel A) Coomassie Blue stained $NaDodSO_4$ slab gel; 38, 76, and 153 μg of protein were added to lanes a, b, and c, respectively. The same amount of protein was added on the right side of the gel. (Panel B) Fluorograph of Coomassie Blue stained gel in panel A. The radioactivity applied to lanes a, b, and c was 700, 1400, and 2800 cpm, respectively. Lanes a¹, b¹, and c¹ contained 613, 1227, and 2452 cpm, respectively.

AEDANS-SF_1 Lifetime Measurements. The fluorescence lifetime determination of a representative AEDANS-SF_1 preparation is shown in Table I. As can be seen, the sample is essentially homogeneous as indicated by the close correspondence in τ_p and τ_m values at all three frequencies. Analysis of these data by the FIT 2 and FIT 1 computer routines indicated that less than 2% of a component with a lifetime other than approximately 20.6 ns was present. The average lifetime of AEDANS-SF_1 for nine preparations was found to be 20.6 ● 0.15 ns at 6 °C. This value is in excellent agreement with the lifetime of 20.6 ns as determined by Tao & Lamkin (1981) using pulse fluorometry. Similar lifetime values have previously been reported for AEDANS-labeled SF_1 and myosin by Mendelson et al. (1973) and Botts et al. (1979).

Energy Transfer between AEDANS (SH_1) and DDPM (SH_2). Fluorescence lifetime measurements performed with

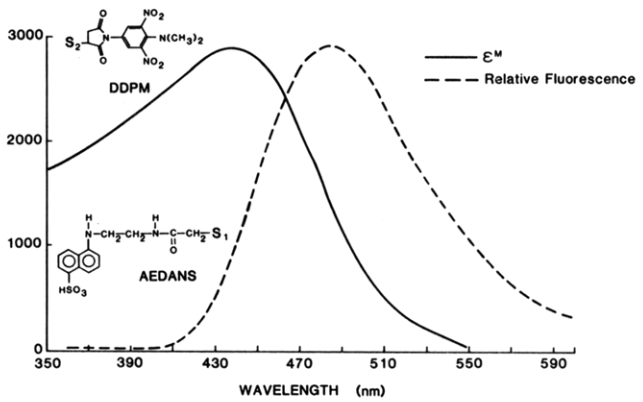

FIGURE 5: Overlap between the absorption spectrum of SF_1-DDPM and the corrected emission spectrum of AEDANS-SF_1. The corrected emission spectrum (---) of AEDANS-SF_1 was measured at 6 °C on an SLM 4800 spectrofluorometer. The absorption spectrum (—) of IAA-SF_1-DDPM was determined at 1 °C on a Cary 14 spectrophotometer interfaced to a Hewlett Packard 9825 desktop computer. Determination of the molar extinction coefficient at various wavelengths is described under Results. SH_1 was blocked by treating SF_1 (20 μM) in Tris-KCl buffer, pH 8.0, with 1 mM iodoacetamide for 30 min at 0 °C. The Ca^{2+}ATPase and K^+ATPase were 394% and 9%, respectively, relative to untreated controls. This derivative was purified by $(NH_4)_2SO_4$ precipitation followed by Sephadex G-25 gel filtration in 50 mM Mes and 0.1 M KCl, pH 6.3. The IAA-SF_1 (17 μM) was treated with 17 μM DDPM in the presence of 0.17 mM ADP and 1.7 mM $MgCl_2$ and purified in the same manner as AEDANS-SF_1-DDPM (see Materials and Methods).

SF_1 labeled with 1,5-IAEDANS and DDPM indicated that energy transfer was occurring (Table II). Analysis of the fluorescence data with the FIT 1 routine indicated that two lifetimes were present, one at 20.6 ns (AEDANS-SF_1, unquenched) and another at 8.1 ns (AEDANS-SF_1, quenched by DDPM) (χ^2 = 0.13). Analysis by the FIT 2 routine gave lifetimes of 20.3 and 7.5 (±0.9) ns, respectively, with χ^2 values of 0.04. Two lifetimes were expected since the stoichiometry of labeling with 1,5-IAEDANS was 0.9 and with DDPM, 0.4. In addition, all DDPM-labeled alkali light chains were replaced with unlabeled light chains. Similar lifetime measurements with nonreconstituted AEDANS-SF_1-DDPM (Table II) gave essentially the same lifetime results. In this case, analysis by FIT 1 indicated the second lifetime was 9.3 (±0.2) ns with χ^2 = 0.13. Analysis by FIT 2 gave values of 20.3 (±0.35) and 8.8 (±0.12) ns with χ^2 = 0.017.

Table II lists the parameters used to determine the distance between the AEDANS- and DDPM-labeled sites. The distance at which energy transfer is 50% for this system is 29 Å assuming $\kappa^2 = {}^2/_3$. By use of the lifetime of the reconstituted AEDANS-SF_1-DDPM system, 8.1 ns, an efficiency of transfer of 60% was calculated which corresponds to a distance of 27

Table II: Energy Transfer Parameters for AEDANS-SF_1-DDPM[a]

n	1.4
J (M^{-1} cm^{-1} nm^4)	6.1×10^{13}
Q_D	0.63
κ^2	0.667
R_0 (Å)	29
τ_d (ns)[b]	20.6
τ_{da} (ns),[b] LC exchanged	8.1 (±0.8)
τ_{da} (ns),[b] LC not exchanged	9.3 (±0.22)
r[c] (Å), LC exchanged	27
r[c] (Å), LC not exchanged	28

[a] $R_0 = [(8.79 \times 10^{-5})k^2n^{-4}Q_DJ]^{-6}$. R_0 is the distance at which the transfer efficiency is 50%. n is the refractive index of the medium between acceptor and donor and is taken to be 1.4 (Fairclough & Cantor, 1978). $J = \int_0^\infty F(\lambda)\epsilon(\lambda)\lambda^4\, d\lambda / \int_0^\infty F(\lambda)\, d\lambda$ is the spectral overlap integral, where F is the corrected fluorescence intensity of the donor and ϵ is the molar extinction coefficient of the acceptor. A computer program was written to calculate the overlap integral from the digitalized absorption and fluorescence spectra with 0.2-nm resolution. $Q_D = 0.63$ is the quantum yield of the donor in the absence of acceptor (Takashi, 1979). κ^2, the orientation factor, was assumed to be $^2/_3$. LC = alkali light chains. [b] τ_d is the fluorescence lifetime of the donor, AEDANS-SF_1, in the absence of an acceptor (see Table I). τ_{da} is the fluorescence lifetime of AEDANS-SF_1 in the presence of acceptor DDPM determined by using the FIT 1 routine with AEDANS-SF_1 lifetime set at 20.6 ns. The "LC exchanged" value was based on one sample measured on three different days. The "LC not exchanged" value was based on three different enzyme preparations. The values in parentheses are the calculated standard deviations. [c] r is the distance between SH_1 and SH_2. The distance $r = (E^{-1} - 1)^{1/6}R_0$ ($\kappa^2 = ^2/_3$) where E is the efficiency of energy transfer, $E = 1 - \tau_{da}/\tau_d$.

Table III: Effect of AMP-PNP Addition on Energy Transfer between AEDANS and DDPM on SF_1[a]

AMP-PNP/SF_1*	τ_3	α_1	α_2	α_3	χ^2
0		0.69	0.31		0.218
0.2	(−0.92)	0.69	0.31	0.006	0.109
0.4	1.29	0.69	0.30	0.016	0.0051
0.6	2.71	0.74	0.21	0.050	0.014
0.8	2.68	0.77	0.16	0.064	0.0032
1.0	3.37	0.80	0.11	0.089	0.059
1.5	2.76	0.81	0.11	0.080	0.024
5.0	2.73	0.81	0.11	0.080	0.016

[a] AEDANS-SF_1-DDPM was prepared by method two at pH 6.3 as described under Materials and Methods. AMP-PNP was added in increasing amounts to 50 μM AEDANS-SF_1-DDPM containing 2.4 mM $MgCl_2$. SF_1* = all forms of SF_1. This preparation contained 20% SF_1 and 80% AEDANS-SF_1 of which 48% was modified at SH_2 by DDPM. The lifetime data for τ_3 were solved by a FIT 3 routine by using τ_1 = 20.6 ns and τ_2 = 9.29 ns. α_1, α_2, and α_3 correspond to the fractional fluorescence contribution of the 20.6-ns, 9.29-ns, and τ_3 species, respectively. χ^2 is the goodness-of-fit parameter.

Å. Similar calculations using the 9.3-ns lifetime of the nonreconstituted AEDANS-SF_1-DDPM system gave a distance of 28 Å.

Effect of Nucleotides on Energy Transfer between AEDANS (SH_1) and DDPM (SH_2). The ability to label SH_1 and SH_2 specifically with a donor/acceptor pair allowed us to ask the question as to whether the distance between these two thiols changes on addition of nucleotides or nucleotide analogues. Table III shows that there are dramatic changes in the AEDANS lifetime values as AMP-PNP is added to nonreconstituted AEDANS-SF_1-DDPM (SF_1*). Three AEDANS lifetimes were observed: (i) τ_1, 20.6 ns, AEDANS-SF_1 alone (in our hands, this lifetime is unaffected by addition of nucleotides);[3] (ii) τ_2, 9.3 ns, AEDANS-SF_1-DDPM; (iii) τ_3, 2.85 ns, the new shorter average lifetime of AEDANS-SF_1-DDPM brought about by AMP-PNP binding. The χ^2 values were consistently low for all ratios of MgAMP-PNP/SF_1* assuming a ternary but not a binary system was present. As can be seen in Table III, at AMP-PNP/AEDANS-SF_1-DDPM ratios 0.6 or greater, the τ_3 values calculated were essentially constant. At lower ratios the small fractional contribution of fluorescence (α_3) by the AEDANS-SF_1-DDPM species containing bound AMP-PNP gives τ_3 values which are less reliable or even meaningless (e.g., the negative τ_3 value at a ratio of AMP-PNP/SF_1* of 0.2). The relative fluorescence intensity values (α_i) of the lifetime species change as expected. Thus, as increasing AMP-PNP is added, the relative fluorescence contribution of the AEDANS-SF_1-DDPM component (α_2) decreases from 0.31 to 0.11 while the α_3 value (AEDANS-SF_1-DDPM with bound AMP-PNP) increases from 0 to 0.08. The AEDANS-SF_1 component (τ_1) is unaffected by AMP-PNP addition, and hence its *relative* fluorescence contribution increases from 0.69 to 0.81 even though its mole fraction remains constant (see below).

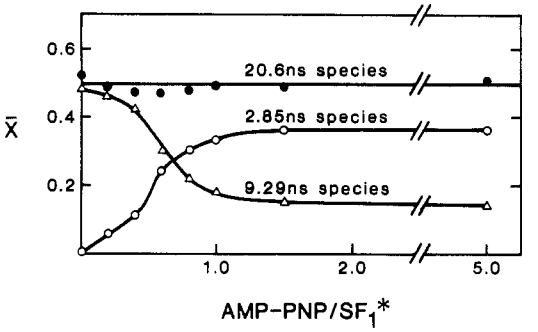

FIGURE 6: Effect of MgAMP-PNP on the mole fraction of the AEDANS lifetime species exhibited by AEDANS-SF_1-DDPM. AEDANS-SF_1-DDPM was prepared and measurements were made as described in Table III. SF_1* is the same as in Table III. $\bar{X}_1$, $\bar{X}_2$, and $\bar{X}_3$ correspond to the mole fraction of the τ_1, τ_2, and τ_3 species, respectively. A modified FIT 3 program was used to calculate α_1, α_2, α_3, and χ^2 using τ_1, τ_2, and τ_3 values of 20.6, 9.29, and 2.85 ns, respectively. The τ_3 value of 2.85 was the average of five lifetimes determined at AMP-PNP/SF_1* ratios of 0.6 or greater (see Table III). The $\bar{X}_i$ values were calculated by using these new α_i values and the corresponding τ_i values by using a computer program described under Materials and Methods.

The relative fluorescence contributions (α_i) and the average lifetimes (τ_i) can be used to calculate the mole fractions ($\bar{X}_i$) of each fluorescent species. Figure 6 shows the results of such calculations for the AMP-PNP titration data from Table III. As expected, the mole fraction of AEDANS-SF_1 ($\bar{X}_1$) does not change (0.49 ± 0.015) as the AMP-PNP/SF_1* ratio goes from 0 to 5.0. The mole fraction of AEDANS-SF_1-DDPM ($\bar{X}_2$), however, decreases from 0.48 to 0.14 as the mole fraction of the AMP-PNP bound species ($\bar{X}_3$) increases from 0 to 0.36. The small effect of nucleotide at low ratios of AMP-PNP/SF_1* on $\bar{X}_2$ and $\bar{X}_3$ likely reflects the probability that unmodified SF_1 and AEDANS-SF_1 bind nucleotides more tightly than AEDANS-SF_1-DDPM.

[3] Tao & Lamkin (1981) observed a 1-ns decrease in the lifetime of AEDANS upon addition of MgADP to AEDANS-SF_1. Other workers (Mendelson et al., 1975; Botts et al., 1979) have found a 0.3-ns decrease or less in the AEDANS lifetime with nucleotide bound to AEDANS-SF_1. Our measurements indicate there is no apparent change in the AEDANS lifetime when MgADP is added (<0.1 ns), but there is a 6.7% decrease in the fluorescence intensity of AEDANS, a result consistent with the findings of Takashi et al. (1982). The discrepancy between the lifetime and quantum yield of AEDANS on SF_1 suggests either that static quenching is occurring or that the absorption spectrum of AEDANS-SF_1 changes on addition of nucleotide.

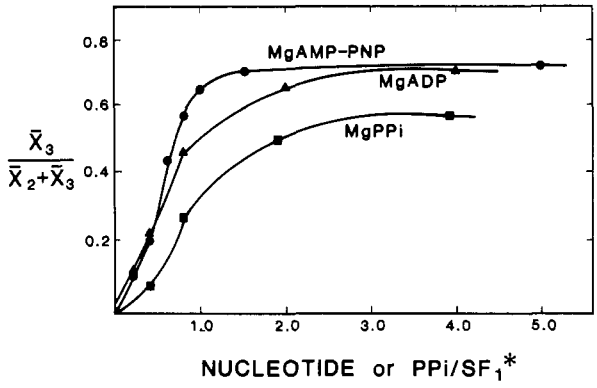

FIGURE 7: Binding of nucleotide or PP_i to AEDANS-SF_1-DDPM (SF_1*). The ratio $\bar{X}_3$ to $\bar{X}_2 + \bar{X}_3$ reflects the amount of nucleotide bound to the AEDANS-SF_1-DDPM protein species (see Results). The mole fractions were determined as described in Figure 6 by using a τ_1 and τ_2 of 20.6 and 9.29 ns, respectively. τ_3 for AMP-PNP, ADP, and PP_i was 2.85, 3.22, and 2.5 ns, respectively. The titration studies with MgADP and $MgPP_i$ were performed with the same enzyme preparation while a different preparation was used for the AMP-PNP titration.

Table IV: Summary of the Effects of Nucleotides or PP_i on the Distance between SH_1 and SH_2

additions	τ (ns)[a]	r (Å)[b]	Δr (Å)[c]
none	9.29 (±0.22)	28.1	
AMP-PNP	2.85 (±0.29)	21.4	6.7
ADP	3.22 (±0.25)	21.9	6.2
PP_i	2.50 (±0.36)	20.9	7.2

[a] The lifetimes of AEDANS-SF_1 quenched by DDPM. The lifetime with no addition (9.29 ns) was the average of three different AEDANS-SF_1-DDPM preparations (Table II). The lifetime after AMP-PNP additions was the average determined by using a FIT 3 routine setting τ_1 = 20.6 ns and τ_2 = 9.29 ns (Table III). The τ values after ADP or PP_i additions were averaged by using τ_3 values obtained at the three highest PP_i or ADP/SF_1* ratios (see Figure 7). [b] These distances were calculated by using an R_0 equal to 29 Å (see Table II) where Q_D = 0.63. As pointed out in footnote 3, the addition of MgADP to AEDANS-SF_1 results in a 6.7% decrease in the fluorescence intensity of AEDANS. A decrease of this magnitude could be explained by a change in the quantum yield from 0.63 to 0.59. However, such a change decreases R_0 only 0.35 Å (29 to 28.65 Å) and hence is negligible. [c] The calculated change in the distance between SH_1 and SH_2 when nucleotide or PP_i was added.

It was of interest to determine the effects of ADP and PP_i binding to AEDANS-SF_1-DDPM in comparison with AMP-PNP. Data similar to that in Table III and Figure 6 were obtained for both PP_i and ADP. In each case, $\bar{X}_1$ (AEDANS-SF_1) did not change upon PP_i or ADP addition. As observed with AMP-PNP, $\bar{X}_2$ decreases and $\bar{X}_3$ increases as expected with an increasing amount of ligand added. Figure 7 shows the ratio of AEDANS-SF_1-DDPM containing bound AMP-PNP, ADP, or PP_i to total AEDANS-SF_1-DDPM species vs. the amount of added ligand. The ratio, $\bar{X}_3/(\bar{X}_2 + \bar{X}_3)$, reflecting the amount of nucleotide bound to AEDANS-SF_1-DDPM, changes from 0 to a saturation value of 0.72 under these conditions. Thus, a portion of the AEDANS-SF_1-DDPM is refractory to nucleotide addition since the ratio does not approach one and does not change significantly at AMP-PNP/SF_1* ratios greater than one. PP_i binds more weakly and does not appear to reach saturation even at a PP_i/SF_1* ratio of 4. Again the apparent sigmoid character of ligand binding likely reflects the fact that the AEDANS-SF_1 and SF_1 bind nucleotides or PP_i more tightly than AEDANS-SF_1-DDPM.

The titration studies indicated that there was increased Förster energy transfer between AEDANS and DDPM upon binding of AMP-PNP, ADP, or PP_i. Table IV summarizes the average lifetimes that were calculated from nucleotide or PP_i titration studies. The τ_3 values calculated for ADP (3.2 ns) or PP_i (2.5 ns) are within experimental error of the AMP-PNP value of 2.85 ns given in Table III. These τ_3 values correspond to a distance between SH_1 (AEDANS) and SH_2 (DDPM) of about 21 Å. This is to be compared to an SH_1 to SH_2 distance of 28 Å determined in the absence of added ligand. Thus, the addition of AMP-PNP, ADP, or PP_i all appear to move SH_1 and SH_2 approximately 7 Å closer together.

Discussion

The aim of the present study was to measure the distance between SH_1 and SH_2 and to see if this distance was influenced by the binding of nucleotide. The peptide results presented established that SH_1 had been specifically labeled with 1,5-IAEDANS (Figure 1). In agreement with this finding, the lifetime of AEDANS-SF_1 is >98% homogeneous with a value of 20.6 ns (Table I). The stoichiometry of labeling at SH_1 was between 0.75 and 0.9 AEDANS/SF_1. DDPM was found to label predominantly SH_2, but small amounts of the alkali light chains were also modified (Figure 3). However, it was possible to remove the DDPM-modified alkali light chains by using the exchange procedure described by Wagner & Weeds (1977) (Figure 4). The stoichiometry of labeling at SH_2 with DDPM in the fluorescence experiments was between 0.4 and 0.6 DDPM/AEDANS-SF_1. Excellent agreement was found in determining the extent of DDPM modification by using either [^{14}C]DDPM or the visible absorption spectra.

In our fluorescence measurements the donor exhibited two lifetimes corresponding to the unquenched (acceptor absent) and the quenched (acceptor present) states. It was necessary to solve for multiple lifetimes in order to determine the quenched lifetime, the value needed to determine the extent of energy transfer between AEDANS and DDPM. Several computer programs based on the equations of Weber (1981) were written to solve for lifetimes in a heterogeneous system. In order to establish our ability to resolve lifetimes in a heterogeneous system using phase-modulation techniques (Materials and Methods), noninteracting binary and ternary systems consisting of pyrene and carbazole in ethanol, or pyrene, carbazole, and POPOP in ethanol, were examined. These studies indicated that the technique and computer program routines were capable of solving for the lifetimes and the fractional fluorescence contribution of each component (R. Dalbey and J. Weiel, unpublished results).

Analysis of the fluorescence lifetime data indicated as expected two lifetimes were present when SF_1 was labeled with both 1,5-IAEDANS and DDPM. The two lifetimes were 20.6 (AEDANS-SF_1, unquenched) and 8.1 ns (reconstituted AEDANS-SF_1, quenched by DDPM). The low χ^2 (goodness-of-fit parameter) indicates that a binary system is present. When these lifetime results were used, a distance of 27 Å (R_0 = 29 Å) between SH_1 and SH_2 was calculated. The lifetimes present were essentially the same even when the DDPM-modified light chains were not replaced with unmodified light chains. In this case, the second lifetime was 9.3 (±0.2) ns, an average lifetime determined from three different protein preparations. On the basis of this quenched lifetime, a distance of 28 Å was calculated. It was shown previously by Marsh & Lowey (1980) that the distance between SH_1 and the thiol located on the alkali light chains is approximately 40 Å. Thus, the effect of DDPM on the alkali light chains on AEDANS-SF_1 lifetimes was negligible for two reasons: (i) less than 10%

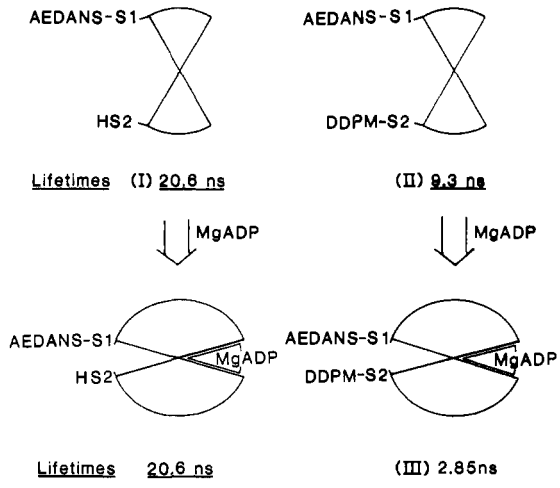

FIGURE 8: Schematic model showing three AEDANS lifetime species that result when MgADP is added to SF_1*.

of the total DDPM labeling was on the alkali light chains and (ii) the distance separating the SH group on the alkali light chains and SH_1 (40 Å) is large compared to the R_0 (29 Å) for the DDPM/AEDANS donor/acceptor pair.

The addition of nucleotide to AEDANS-SF_1-DDPM resulted in a significant change in the AEDANS lifetime values. This effect was due to an increase in energy transfer between AEDANS and DDPM since the addition of nucleotide to AEDANS-SF_1 had no effect on the lifetime or shape of the emission spectrum of AEDANS. Likewise, addition of nucleotide had no effect on the absorption spectrum of IAA-SF_1-DDPM. A small change in the lifetime of AEDANS did occur on addition of MgADP to AEDANS-SF_1-MalNEt (20.6 to 21.0 ns). This change had a negligible effect on the calculated SH_1 to SH_2 distance where a value of 20.6 ns was used.

A schematic model of how Mg nucleotide binding affects the position of SH_1 and SH_2 is shown in Figure 8.[4] According to this model, as nucleotide is added to SF_1*, the mole fraction of the 9.3-ns species ($\bar{X}_2$) should decrease while the mole fraction of the third species ($\bar{X}_3$) should increase. The 20.6-ns species, AEDANS-SF_1, should be unaffected since in our hands the addition of MgADP does not change the AEDANS-SF_1 lifetime. This correlation is exactly what is observed for each titration. Figure 6 shows that the mole fraction of the 20.6-ns species (average $\bar{X}_1 = 0.49$) stays constant throughout the titration with AMP-PNP. As expected, the mole fraction of the 9.3-ns species ($\bar{X}_2$) decreases from 0.48 to 0.14 as AMP-PNP is added, while the mole fraction of the 2.85-ns species ($\bar{X}_3$) increases from 0 to 0.36. The ratio of $\bar{X}_3$ to $\bar{X}_3 + \bar{X}_2$, which is equal to the amount of nucleotide or pyrophosphate bound to SF_1*, should approach 1 at saturating levels of nucleotide. For reasons that are not clear some 25% of our AEDANS-SF_1-DDPM preparations did not respond to Mg nucleotide addition. This could result if the linkage between the active site and the SH_1, SH_2 region was altered in part of the enzyme during the preparation of our SF_1 derivatives. In this case the average SH_1 to SH_2 distance would remain the same but part of the preparation would be refractory to Mg nucleotide binding at the active site. It may be relevant to point out that, in general, only 70% of most SF_1 preparations trap MgADP by cross-linking SH_1 and SH_2.

The binding of nucleotide to AEDANS-SF_1-DDPM increased the efficiency of energy transfer between AEDANS and DDPM. Titrations with AMP-PNP, ADP, or PP_i gave a new average lifetime of 2.85 ns. This lifetime indicates that binding of nucleotide or PP_i changes the distance between SH_1 and SH_2 from 27–28 to 21 Å. This apparent 6–7 Å decrease in the distance suggests a large conformational change results when nucleotide is added. The fact that PP_i induces the same apparent movement of SH_1 and SH_2 as ADP and AMP-PNP indicates that binding of the polyphosphate portion to the active site is sufficient to promote the conformational changes observed. This observation is also consistent with trapping studies (Wells et al., 1980) which show that $MgPP_i$ is the minimal substrate analogue which will be trapped by cross-linking SH_1 and SH_2. No change in the SH_1 to SH_2 distance was observed upon addition of a 20-fold molar excess of MgAMP to AEDANS-SF_1-DDPM, indicating the effects of the nucleotides or PP_i are specific.

Earlier cross-linking studies (Burke & Reisler, 1977; Wells & Yount, 1979) have suggested SH_1 and SH_2 can be brought closer together by binding of MgADP to the active site. A variety of cross-linking reagents which give sulfur–sulfur distances of 2–14 Å appear to specifically cross-link SH_1 and SH_2 (Wells & Yount, 1982). These results indicate that the peptide region containing SH_1 and SH_2 must be highly flexible. The distances inferred from cross-linking studies (2–14 Å) are much shorter than the distance calculated from Förster energy transfer experiments (21–22 Å). However, chemical cross-linking experiments can trap conformations which occur rarely whereas energy transfer experiments reflect time-averaged conformations. Moreover, uncertainty in the distance measurements is introduced because of the size of the AEDANS and DDPM moieties. When CPK molecular models are used, the distance between the center of the dansyl group to the sulfhydryl attached to AEDANS is about 10 Å. Similarly, the distance between the center of the aniline ring and the sulfhydryl attached to DDPM is about 6 Å. Thus, if these groups were pointed away from each other, an additional 16 Å would be introduced into the SH_1 to SH_2 distance measurements. Because of this uncertainty the apparent absolute distances are less important than the relative distances determined in the absence (28 Å) and in the presence (21 Å) of magnesium nucleotide.

The size of the donor/acceptor pair suggests an alternative explanation for the effect of Mg nucleotide binding on the AEDANS-SF_1-DDPM lifetime. That is, it is possible Mg nucleotide binding simply promotes the rotation of AEDANS relative to DDPM to bring them closer together without affecting the SH_1 to SH_2 distance. This explanation, while feasible, does not explain the markedly enhanced rates of cross-linking of SH_1 and SH_2 by bifunctional reagents in the presence of MgADP.

A potential problem in these studies arises from the fact that the relative orientation of the donor and acceptor pair is not known. κ^2, the orientation factor, was taken as $^2/_3$, which assumes that both the donor and acceptor groups have a

[4] Our original concept had Mg nucleotide being trapped by cross-linking SH_1 and SH_2 across a jawlike active site (Wells & Yount, 1979). However, several lines of evidence argue against this model. One, SH_1 and SH_2 are separated by only nine amino acids (Elzinga & Collins, 1977) and thus can only be 38 Å apart even in the most extended form of the polypeptide chain. CPK models of this chain show that a binding pocket can be formed, but it is so small that all parts of ATP must be within 10–15 Å of SH_1 or SH_2. Förster energy transfer measurements between SH_1 and the active site (Tao & Lamkin, 1981; J. Weiel, W. J. Perkins, and R. G. Yount, unpublished results) put SH_1 (and SH_2) some 25–39 Å away, too far to be directly involved in binding or cleavage of ATP. Thus the cross-linking agents appear to trap Mg nucleotide indirectly by stabilizing a conformation which favors tight nucleotide binding. The fact that actin addition rapidly releases bound Mg nucleotide from SF_1 (Perkins et al., 1981) also argues against a simple jaw model.

random orientation. This may not be the case since Mendelson et al. (1973) have shown that the AEDANS on SH_1 is immobilized to a large extent. However, Takashi (1979) showed that AEDANS on SH_1 does have some motion but is more restricted than free 1,5-IAEDANS. The emission anisotropy of 1,5-AEDANS bound to SH_1 of SF_1 is 0.25 (Mendelson et al., 1973) compared to a value of 0.32 determined for free 1,5-IAEDANS in propylene glycol at −55 °C (Hudson & Weber, 1973). This decrease in the limiting anisotropy can be attributed to the donor rotating very rapidly within a volume of a cone having a semiangle of 23° (Kawato et al., 1977). If one assumes AEDANS bound to SH_1 has this property and that DDPM on SH_2 is fixed, then using the approach of Stryer (1978) there is an 80% probability the distance between SH_1 and SH_2 (no nucleotide present) falls in the range 20–32 Å. The range in the presence of nucleotide decreases to 15–24 Å. Similarly, Hillel & Wu (1976) have developed a statistical approach to determine the significant range of the donor–acceptor distances in energy transfer experiments. When this method is used, it can be determined that there is 90% probability that the SH_1 to SH_2 distance in the absence of nucleotide will fall in the range between 22 and 35 Å. (Here it is assumed the donor is free to rotate within a semiangle of 30° and the acceptor is fixed.) These ranges in the distance are unrealistically large since both approaches assume the acceptor is fixed, a condition unlikely to exist in most cases. In addition, the underlying assumption in both of these approaches was that the donor and acceptor have only one transition dipole moment. As pointed out by Haas et al. (1978), most molecules exhibit multiple absorption or emission transition dipole moments. A consequence of this fact is that the range in κ^2 is greatly reduced. IAEDANS itself is known to have more than one transition dipole moment (Hudson & Weber, 1973), and thus, κ^2 is more likely to have a value close to $^2/_3$.

Another potential problem arises in these studies if binding of nucleotide affects κ^2. A change in κ^2 could explain the apparent 6–7-Å decrease in the SH_1 to SH_2 distance that is observed when nucleotide binds. It is known from nitroxide spin-label studies that probes on both SH_1 and SH_2 become slightly more mobile when nucleotide or PP_i is added (Seidel et al., 1970; Seidel, 1972). However, calculations using the Förster equation indicate that κ^2 would have to increase by a factor of 5 in order to explain the changes in lifetimes observed. Such a large change seems extremely unlikely.

Attempts were made to switch the donor/acceptor pairs on SH_1 and SH_2. Such an experiment might yield different lifetime values if κ^2 was not equal to $^2/_3$. All attempts to modify SH_2 specifically with 1,5-IAEDANS failed. The rate of reaction of 1,5-IAEDANS with SH_2 in the presence of MgADP was very slow. To obtain a significant loss in the Ca^{2+}ATPase activity, a 20-fold molar excess of 1,5-IAEDANS over DDPM on SH_1 of SF_1 was needed ($t_{1/2} \simeq 20$ h). Na-DodSO$_4$–polyacrylamide gel electrophoresis using [^{3}H]-1,5-IAEDANS indicated that the alkali light chains were labeled in addition to SH_2. This significant nonspecific labeling precluded definitive lifetime measurements from being performed on this system.

Cheung and co-workers (Cheung et al., 1983) have reported that Förster energy transfer measurements indicate SH_1 and SH_2 are 46 Å apart in the presence or absence of nucleotide. In these studies the donor/acceptor pair was 1,5-IAEDANS and (iodoacetamido)fluorescein, respectively. The calculated distance, 46 Å, is surprisingly large since the two cysteines modified are separated by only nine amino acids in the primary sequence (Elzinga & Collins, 1977). In an extended β-sheet structure (3.3 Å per residue) the thiol groups would be approximately 33 Å apart. Measurement using appropriate CPK models in which the SH_1 and SH_2 cysteines were rotated away from each other gave a maximum sulfur–sulfur distance of 38 Å. Even allowing for the bulk of the 1,5-IAEDANS and fluorescein groups, it seems unlikely that the two groups are pointing away from each other to give a 46-Å separation.

The reason for the large discrepancy between the SH_1 to SH_2 distances reported here and the Cheung and co-workers' measurements is not clear. Some differences in the experimental protocols should be noted. Cheung et al. used papain SF_1 (this work used chymotryptic SF_1) and made their fluorescence measurements at 20 °C rather than at 6 °C. In addition, their efficiency of energy transfer values were determined by using quantum yields rather than by lifetimes as were determined here. An inherent problem with the former approach is that the specificity and stoichiometry of labeling with donor/acceptor pairs must be determined with great accuracy which is difficult to do experimentally in complex systems. On the other hand, lifetime measurements give directly the relative amounts of donor/acceptor pairs and a direct indication of heterogeneity of labeling. Another difference was in the acceptor molecules used in the two studies. Fluorescein as used by Cheung et al. is a bulky polyaromatic chromophore which is likely to give larger anisotropy values than DDPM. Thus, as Cheung and co-workers suggest, κ^2 may be larger than $^2/_3$ which could account in part for the larger SH_1 to SH_2 distance they measured. The large bulk of the fluorescein molecule may also prevent nucleotide-induced conformational changes.

In conclusion, the distance between SH_1 and SH_2, in the absence of nucleotide, has been determined to be about 28 Å. The binding of nucleotide results in an apparent 6–7-Å decrease in the distance between SH_1 and SH_2. This decrease confirms the change in the distance between these two key thiols predicted by earlier cross-linking studies. Finally, the use of phase-modulation fluorescence lifetime measurements has proven to be a powerful approach in Förster energy transfer studies of complex systems.

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Registry No. AMP-PNP, 25612-73-1; ADP, 58-64-0; PP_i, 14000-31-8.

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