Spatial Relationship between a Fast-Reacting Thiol and a Reactive Lysine Residue of Myosin Subfragment 1†

Reiji Takashi,* Andras Muhlrad,‡ and Jean Botts

ABSTRACT: Fluorescence energy transfer was used to examine the spatial proximity between two key side chains in myosin subfragment 1 (S-1), viz., the reactive thiol (SH_1) located on the C-terminal 20K tryptic fragment and the reactive lysyl (RLR) on the N-terminal 27K tryptic fragment of S-1 heavy chain. S-1 was specifically labeled at SH_1 with an energy donor, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (AEDANS), and at RLR with an energy acceptor, 2,4,6-trinitrobenzenesulfonate (TNBS). Prior blocking of SH_1 with AEDANS increased the p*K* of RLR from 9.04 to 9.42. Trinitrophenylation of SH_1-blocked S-1 was about 50% slower and sharply reduced the Ca^{2+}ATPase activity. Reciprocally, blocking of RLR with TNBS slowed the rate of reaction of SH_1 and AEDANS by 40–60%. Addition of the second label does not grossly alter the conformation resulting from the first label. S-1 labeled at RLR with TNBS and at SH_1 with optically inert iodoacetamide shows the same TNP difference spectrum ± MgADP (λ_{min} 365 nm) as S-1 with SH_1 free. Also, S-1 labeled at SH_1 with AEDANS and at RLR with an optically inert methyl group shows the same AEDANS emission spectrum (λ_{em}^{max} 475 nm), excited-state lifetime (τ = 20.3 ns), and rotational correlation time (Φ = 106 ns) as S-1 with RLR free. When the decrease of either the quantum yield or the excited-state lifetime of the donor in the absence and presence of the acceptor was measured, the energy transfer efficiency was found to be 70%. The apparent interchromophore distance was calculated to be 2.6 nm through the use of the Förster equation with an uncertainty of less than 12%.

Subfragment 1 (S-1)[1] is the domain of myosin in which energy transduction is thought to occur by virtue of the interactive binding of actin and nucleotide to this domain. The detailed spatial arrangement of defined residues on S-1 is thus manifestly important. Because crystallographic information about S-1 is nonexistent, other means of discerning arrangement have to be used. Two well-defined residues of S-1 are the reactive thiol ("SH_1") and the reactive lysyl residue ("RLR"). Blocking of either group with group-specific reagents profoundly affects ATPase at the nucleotide binding site [e.g., modification of either the SH_1 group or RLR quenches K^+(EDTA)ATPase activity] (Kielley & Bradley, 1956; Takashi et al., 1976; Kitgawa et al., 1960; Fabian & Muhlrad, 1968). It is nonetheless unlikely that either SH_1 or RLR participates directly in ATPase catalysis; indeed there is good evidence that SH_1 does not (Wiedner et al., 1978; Botts et al., 1979). As established by Balint et al. (1978), the heavy chain fragment of S-1 is cut by trypsin into three fragments "20K" (C terminus), "50K", and "27K" (N terminus). SH_1 is located in the 20K tryptic fragment (Balint et al., 1978), and RLR is in the 27K tryptic fragment (Miyanishi & Tonomura, 1981; Mornet et al., 1980; Hozumi & Muhlrad, 1981). Collectively the foregoing information suggests that, although SH_1 and RLR reside on different fragments, both are near the nucleotide binding site, and their ligands affect it similarly, and also that perhaps they are near to one another. This paper shows that the two residues are within Förster energy transfer distance and thus contributes another element to our knowledge of the functional arrangement of S-1.

Materials and Methods

Chemicals. [^{3}H]AEDANS was synthesized by the method of Huang et al. (1975). 2,4,6-Trinitrobenzenesulfonate (TNBS) and AEDANS were purchased from Aldrich Chemical Co. [^{3}H]Formaldehyde was purchased from New England Nuclear, α-chymotrypsin was from Worthington Biochemical Corp., and ATP was from Sigma Chemical Co. All other chemicals were of analytical grade.

Proteins. Myosin was prepared from the back and leg fast-twitch muscles of rabbit (Tonomura et al., 1966). S-1 was prepared from such myosin by digestion with α-chymotrypsin in the presence of EDTA (Weeds & Taylor, 1975) and was purified by filtration through a Sephacryl S-200 column equilibrated with 0.15 M KCl and 10 mM Tes, pH 7.0. The purified S-1 was concentrated to about 10 mg/mL with an Amicon MX-50 at 0 °C, and was dialyzed against 50 mM ammonium acetate and 1 mM DTT, pH 7.0, at 4 °C. The S-1 solution was made 0.2 M in sucrose (special enzyme grade, Schwarz/Mann) and lyophilized. The ATPase properties before and after lyophilization were unaltered by the foregoing procedure. Actin was prepared from acetone powder of rabbit skeletal muscles by the method of Spudich & Watt (1971).

Protein Concentration. Myosin ($A^{1\%}_{280nm}$ = 5.70) and S-1 ($A^{1\%}_{280nm}$ = 7.50) (Wagner & Weeds, 1977) concentrations were estimated from optical density measurements, while G-actin concentrations were measured by absorbance at 290 nm ($A^{1\%}_{290nm}$ = 6.30) (Houk & Ue, 1974). In each case an appropriate correction for light scattering was made. Concentration of S-1 labeled with either TNP or AEDANS was measured by the Folin–phenol method (Lowry et al., 1951) with bovine serum albumin as a standard. Molecular weights of S-1 and G-actin were assumed to be 1.10×10^5 (Margossian et al., 1981) and 4.2×10^4 (Elzinga et al., 1973).

Labeling of S-1. For preparation of TNP(S-1), S-1 (40–60 μM) was allowed to react with 0.1 mM TNBS in 0.1 M

† From the Cardiovascular Research Institute, University of California at San Francisco, San Francisco, California 94143. *Received March 19, 1982.* This research was supported by HL-16683, PCM-79-22174, and AHA CI-8.

‡ Senior Fellow of the MDA. Permanent address: Department of Oral Biology, Hebrew University—Hadassah School of Dental Medicine, Jerusalem, Israel.

[1] Abbreviations: S-1, myosin subfragment 1; RLR, reactive lysine residue in S-1; TNP, trinitrophenyl; AEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; DTT, dithiothreitol; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; IAA, iodoacetamide; IAA(S-1), S-1 labeled with IAA; AEDANS(S-1), S-1 labeled with AEDANS; TNP(S-1), trinitrophenylated S-1; TNP[^{14}C]IAA(S-1), S-1 labeled with both TNP and [^{14}C]IAA; AEDANS-TNP(S-1), S-1 labeled with both AEDANS and TNP; PP_i, pyrophosphate; EDTA, ethylenediaminetetraacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonate.

0006-2960/82/0421-5661$01.25/0

Tris-HCl at pH 7.7 and 25 °C for 5 min and was then dialyzed against 10 mM potassium maleate, pH 6.2, at 4 °C for 4–5 h. This was followed by dialysis against the desired buffer. The amount of TNP bound to S-1 was monitored by the increase in absorbance at 345 nm (A_{345}), assuming $\epsilon_{345} = 1.45 \times 10^4$ (Okuyama & Satake, 1960). Labeling of S-1 with AEDANS at SH_1 was performed as described elsewhere (Takashi et al., 1976; Takashi, 1979) except that incubations were carried out for 60 min rather than 30 min. AEDANS attached to S-1 in this way has been shown to reside in the 20K tryptic fragment (Hozumi & Muhlrad, 1981). The amount of AEDANS bound to S-1 was estimated in two ways. In the primary method we used the radioactivity of [^{3}H]AEDANS and the known concentration of S-1. In the secondary method we measured absorbance at 338 nm ($\epsilon = 6.3 \times 10^3$) of both the labeled S-1 and unlabeled S-1 under identical conditions. An apparent absorbance due to unlabeled S-1 itself was subtracted from that of labeled S-1 to estimate the concentration of bound AEDANS.

Labeling of S-1 by Reductive Alkylation with [^{3}H]Formaldehyde and Sodium Cyanoborohydride. Reductive alkylation of S-1 was carried out according to Dottavio-Martin & Ravel (1978) and Jentoft & Dearborn (1979), with slight modifications: 20 μM S-1 was incubated in a solution containing 10 mM $NaCNBH_3$, 5 mM ^{3}HCHO, 50 mM Hepes, and 80 mM KCl, 0 °C, at pH 7.5. At given time intervals, an aliquot was withdrawn and filtered through a Sephadex G-25 column in 0.15 M KCl and 10 mM Tes, pH 7.0, at 4 °C. The amount of ^{3}HCHO incorporated into protein was measured in a Beckman scintillation counter.

Steady-State Fluorescence Measurements. Steady-state fluorescence measurements were made at 15 °C in a Perkin-Elmer MPF-4 fluorescence spectrophotometer. We used a comparative method (Parker, 1968) to measure the quantum yield of AEDANS bound to S-1 in 0.15 M KCl, 20 mM Tes, and 5 mM $MgCl_2$, pH 7.0, at 15 °C; this measurement has a limitation discussed elsewhere (Takashi, 1979). Protein concentrations ranged from 0.4 to 1.3 μM (0.8–0.9 mol of AEDANS/mol of S-1), and these samples had an absorbance ranging from 2.6×10^{-3} to 8.4×10^{-3} at the excitation wavelength, 340 nm.

In the experiments on the effects of nucleotide upon the extrinsic fluorescence of S-1, the fluorescence of the labeled S-1 solution in the cell was measured under the conditions given above. Subsequently a small volume of stock nucleotide solution was added to the cell containing the labeled S-1, and the fluorescence was remeasured. Although the fluorescence intensity was corrected for dilution effects caused by the addition of small aliquots of stock solution, these corrections generally did not exceed 0.5%. Protein concentrations ranged from 0.3 to 1.1 μM S-1 (0.86–0.99 mol of AEDANS/mol of S-1) with 0.4 mM ATP and were 1.0 μM S-1 (0.74 or 0.86 mol of AEDANS/mol of S-1) with either 0.4 mM ADP or 0.4 mM PP_i.

We also studied the effects of F-actin on the fluorescence of the labeled S-1 in two ways. In the first method the fluorescence was measured on the same solution before and after addition of F-actin. In the second method, two kinds of labeled S-1 solution ($\simeq$10 μM) were prepared under identical conditions: one containing the S-1 alone and the other, S-1 with F-actin. Just prior to fluorescence measurements an aliquot of solution (with or without F-actin) was added to the cell containing 0.15 M KCl, 20 mM Tes, and 5 mM $MgCl_2$, pH 7.0, and the fluorescence of the labeled S-1 with or without F-actin was measured. Protein concentrations employed were 0.45–0.48 μM S-1 (0.86–0.88 mol of AEDANS/mol of S-1), and the ratio of actin to S-1 was between 2.5 and 5.4.

Fluorescence Lifetime and Polarization Anisotropy Measurements. These measurements were made on a "double-beam" time-resolving apparatus (Mendelson et al., 1975; Botts et al., 1979).

In the excited-state lifetime experiments we are concerned with the superposed response of the species of S-1 in which the SH_1 is ligated with AEDANS but RLR is free and with the species in which SH_1 is ligated with AEDANS and RLR is ligated with TNP; the donor emissions are assumed to have excited-state lifetimes τ_0 and τ_Q respectively, so the expected response from an infinitely sharp excitation curve is

$$\log [I(t)/I(0)] = \log [(1 - P) \exp(-t/\tau_0) + P \exp(-t/\tau_Q)] \quad (1)$$

where P is the fraction of the RLR's that bear TNP. However, the response curve actually observed results from an envelope of excitation pulses; i.e., the observed response curve is "convolved" with the intensity–time curve of the exciting lamp. In our work the mathematical process of obtaining what the theoretical response curve would be if the excitation were infinitely sharp involved an iterative reconvultion program developed by Professor Ludwig Brand (Badea & Brand, 1979) and was entered into our Eclipse 230-S computer by Dr. Peter Torgerson.

ATPase Measurements. ATPase activity of S-1 was measured in 0.6 M KCl and 50 mM Tris-HCl, pH 8.0 and 25 °C, unless otherwise stated. The P_i liberated was determined by the method of Fiske & Subbarow (1925).

Efficiency of Fluorescence Energy Transfer. In energy transfer experiments, two systems were studied under indentical conditions, i.e., AEDANS(S-1) with and without bound TNP. Protein concentrations ranged from 0.5 to 0.7 μM. The corresponding absorbances at the excitation wavelength depended upon how many moles of chromophores are attached to 1 mol of S-1, but such absorbances never exceeded 0.02. In steady-state fluorescence measurements, fluorescence energy transfer is indicated by a decrease in the fluorescence of the donor in S-1 doubly labeled with both AEDANS and TNP compared to the fluorescence of S-1 labeled only with donor. Usually two excitation wavelengths, 360 and 380 nm, were selected, and the fluorescence was observed at either 460 or 480 nm. In the time-resolved measurements, lifetimes of two kinds of samples, i.e., AEDANS attached to S-1 and both AEDANS and TNP attached to S-1, were measured under the same conditions used in steady-state experiments. Excitation wavelengths were selected by Corning 7-60 and 0-52 glass filters, and emission wavelengths were selected by a Corning 0-51 glass filter and by two specially made Corion Corp. (serial no. 644980) filters.

The efficiency (E) of fluorescence energy transfer was computed as

$$E = 1 - Q_{DA}/Q_D = 1 - \tau_{DA}/\tau_D \quad (2)$$

where Q and τ are respectively the quantum yield and the lifetime of the donor in the presence of the acceptor (DA) and in its absence (D). Q was measured as the fluorescence intensity in the steady-state experiments, and τ was obtained from the time-resolved measurements.

The overlap integral was approximated by the following equation where the summation was carried out over 2-nm intervals:

$$J = \sum_{\Delta\lambda} F_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda / \sum_{\Delta\lambda} F_D(\lambda)\Delta\lambda$$

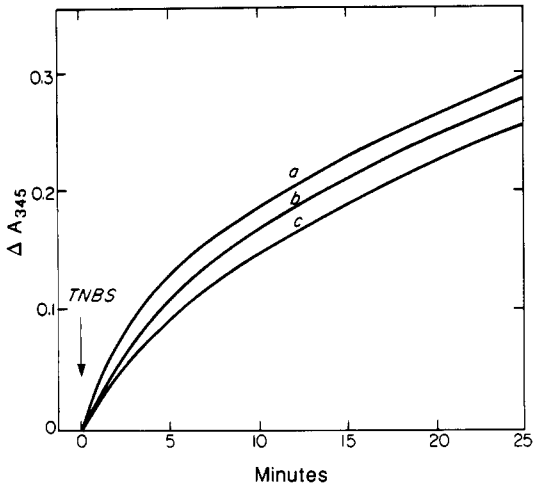

FIGURE 1: Time course of the reaction of TNBS with lysine residues of S-1, IAA(S-1), and AEDANS(S-1). 7.8 μM S-1 or modified S-1 was incubated in a solution containing 100 mM imidazole, and 0.1 mM TNBS, pH 8.0, at 25 °C, and the reaction was followed by an increase in A_{345}. (a) S-1; (b) IAA(S-1) (1.03 mol of [^{14}C]IAA/mol of S-1); (c) AEDANS(S-1) (0.92 mol of AEDANS/mol of S-1).

$F_D(\lambda)$ is the corrected fluorescence emission of the donor and $\epsilon_A(\lambda)$ is the extinction coefficient of the acceptor. The computation was performed on a KIM-1 microcomputer.

Results

Reactivity of TNBS toward S-1, AEDANS(S-1), and IAA(S-1). It was early noted (Kubo et al., 1960) that the reaction of myosin with TNBS is slowed by prior treatment with the thiol reagent *p*-(chloromercuri)benzoate. Using S-1 and reagents of higher specificity for SH_1, we have found that when this reactive thiol is first blocked, then the reactivity of TNBS toward the RLR is indeed markedly diminished (Figure 1). Also, the Ca^{2+}ATPase, which is elevated by SH_1 blocking with AEDANS or IAA, is decreased on trinitrophenylation (see also Figure 5). The time course of trinitrophenylation followed spectrophotometrically is biphasic—first fast and then slow. Previously (Muhlrad & Takashi, 1981) we have successfully modeled this time course by assuming it to be a second-order reaction with one very reactive lysyl (the RLR) and with the remaining 82 lysyls considered to react more slowly and all at the same rate. The two differential equations of the model are thus characterized by two second-order rate constants k_f and k_s, which we have measured by applying the equations to data of various kinds. In the present work we applied this analysis to spectrophotometric measurements on unlabeled S-1, AEDANS(S-1), and IAA(S-1) (Table I). It is noteworthy that the rate of trinitrophenylation of RLR is reduced by 52% when AEDANS(S-1) is used, and by 24% when IAA(S-1) is used, compared to the rate when unlabeled S-1 is used. On the other hand the rate of trinitrophenylation of all the other lysyls is essentially unaffected by prelabeling at SH_1. These conclusions were further checked by studying the aforementioned rate of losing enzymatic activity. Using this method we found that the second-order rate constant, k_{enz}, is reduced by 59% using AEDANS(S-1), and by 29% using IAA(S-1), compared to the rate using unlabeled S-1. Finally, we measured the trinitrophenylation rate (k_f) as a function of pH, for both unlabeled S-1 and AEDANS(S-1), so as to estimate the influence of prealkylation of SH_1 on the p*K* of RLR (Muhlrad & Takashi, 1981). Preblocking of SH_1 increases the apparent p*K* of the RLR from 9.04 to 9.42 (Figure 2).

Reactivity of AEDANS toward TNP(S-1). The upper curves of Figure 3 show that, at equal times, less AEDANS reacts with ca. 50% labeled TNP(S-1) than with unlabeled S-1. Since AEDANS is highly specific for SH_1, this behavior shows that the reactivity of SH_1 toward AEDANS is diminished if the RLR of the S-1 had been reacted with TNBS. The lower curves of Figure 3 illustrate that both reaction of RLR with TNBS (see activities at zero time) and reaction of SH_1 with AEDANS quench the K^+(EDTA)ATPase activity of S-1. Although it is not immediately obvious from the figure, it can nevertheless also be calculated from the data therein that, for equal increments in AEDANS bound, the decrease in the activity (expressed as a fraction of the zero-time activity) of TNP(S-1) is greater than that of unlabeled S-1. Analogous results were obtained (but are not shown) when [^{14}C]IAA was used instead of [^{3}H]AEDANS.

Change in the Absorption Spectrum of TNP(S-1). As noted previously (Muhlrad, 1977), marked changes were found in the UV difference spectrum of TNP bound to RLR upon addition of ATP or its analogues, indicating that localized conformational changes take place in the vicinity of the RLR.

Table I: Rate Constants of Trinitrophenylation of S-1, AEDANS(S-1), and IAA(S-1)

enzyme preparation	k_f (min^{-1} M^{-1})[a]	k_s (min^{-1} M^{-1})[b]	k_{enz} (min^{-1} M^{-1})[c]
S-1	2.60×10^3	4.5	2.80×10^3
AEDANS(S-1)[d]	1.24×10^3	3.6	1.35×10^3
IAA(S-1)[e]	1.38×10^3	4.1	2.25×10^3

[a] k_f is the rate constant of the fast reaction, followed by an increase in A_{345}. [b] k_s is the rate constant of the slow reaction, followed by an increase in A_{345}. [c] k_{enz} is the rate constant calculated from decay of enzymatic activity [K^+(EDTA)ATPase for S-1 and Ca^{2+} ATPase for the labeled S-1]. [d] 0.83 mol of AEDANS/mol of S-1. [e] 1.03 mol of [^{14}C]IAA/mol of S-1. Trinitrophenylation of S-1 was performed in a solution containing 7.8 μM S-1 or labeled S-1, 100 mM imidazole, pH 8.0, and 0.1 mM TNBS, at 25 °C.

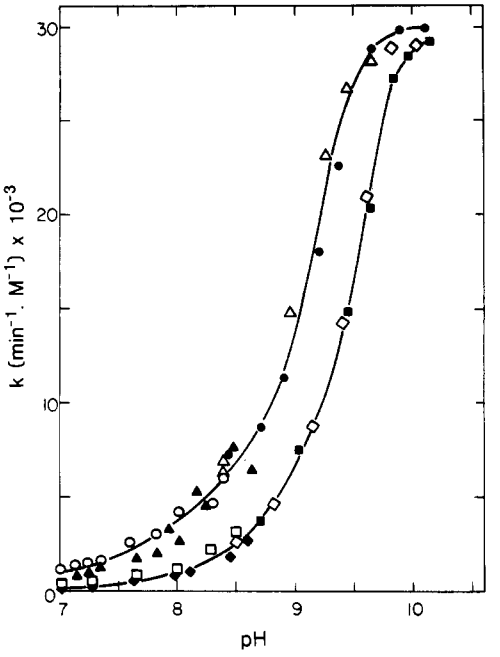

FIGURE 2: pH dependence of the rate of trinitrophenylating the RLR of S-1 or of AEDANS(S-1). Rate constants were assessed by either computer analysis of the kinetic curves of A_{345} (○, ●, □, ■) or a decrease in either K^+(EDTA)ATPase activity (△, ▲) or Ca^{2+} ATPase activity (◇, ◆) in the presence of 100 mM imidazole buffer (○, ▲, □, ◆) or 100 mM borate buffer (△, ●, ◇, ■) at 25 °C. S-1 (○, ●, △, ▲); AEDANS(S-1) (0.92 mol of AEDANS/mol of S-1) (□, ■, ◇, ◆). ATPase assay was performed as described under Materials and Methods, reported elsewhere (Muhlrad & Takashi, 1981).

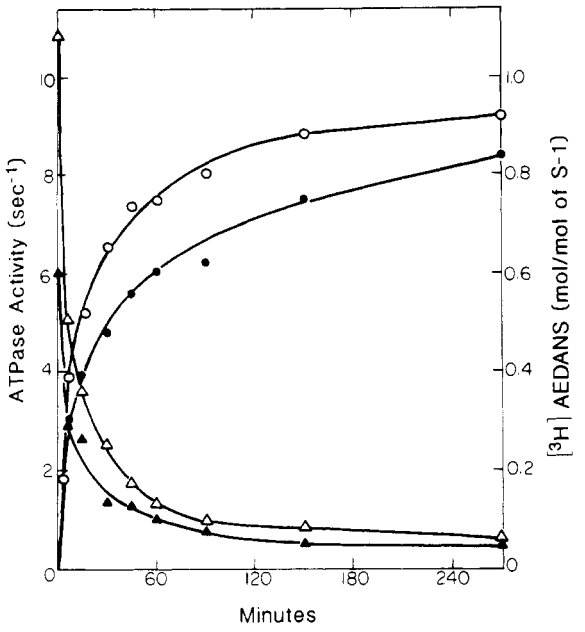

FIGURE 3: Time course of the reaction of the SH_1 of S-1, or of TNP(S-1), with AEDANS. 40 μM S-1 (○, △) or TNP(S-1) (0.46 mol of TNP/mol of S-1) (●, ▲) was incubated in a solution containing 0.15 M KCl, 50 mM Tes, and 0.4 mM [^{3}H]AEDANS, pH 7.0, at 0 °C in the dark. At given time intervals, an aliquot of 0.45 mL was placed in 50 μL of 1.4 M 2-mercaptoethanol to quench the excess AEDANS, and the solution was then dialyzed against 0.15 M KCl and 10 mM Tes, pH 7.0, at 4 °C. ATPase activity was measured in 0.6 M KCl, 50 mM Tris-HCl, 5 mM EDTA (△, ▲), and 1 mM ATP, pH 8.0, at 25 °C. The amount of [^{3}H]AEDANS bound to S-1 (○) or TNP(S-1) (●) was calculated from the radioactivity of each sample.

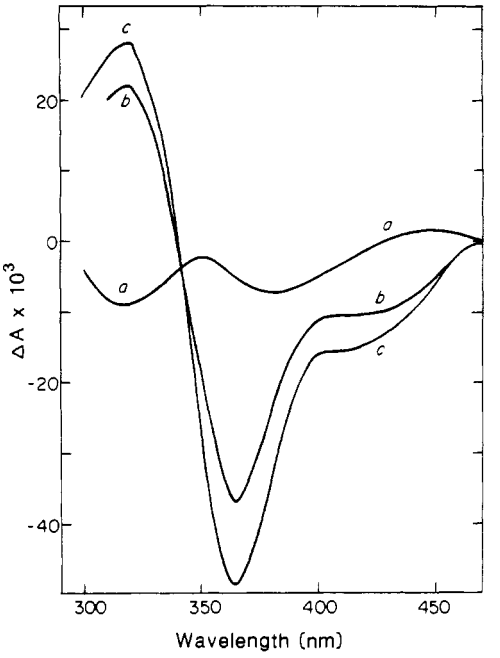

FIGURE 4: Difference spectra of TNP(S-1) and TNP[^{14}C]IAA(S-1). 31.5 μM TNP(S-1) or TNP[^{14}C]IAA(S-1), both containing 0.91 mol of TNP/mol of S-1 and the latter also containing 1.08 mol of [^{14}C]IAA/mol of S-1. Optical density was recorded on a Cary 118 spectrophotometer in 0.15 M KCl and 50 mM Tes, pH 7.0, at 0 °C between 300 and 470 nm. Matched 10-mm silica thermostated cells were used. (a) TNP[^{14}C]IAA(S-1) in the sample cell and TNP(S-1) in the reference cell. Either (b) TNP[^{14}C]IAA(S-1) or (c) TNP(S-1) in the presence of 0.8 mM MgADP in the sample cell was measured against the corresponding S-1 in the absence of nucleotide in the reference cell.

Here we compare spectroscopic properties of TNP(S-1) with those of TNP[^{14}C]IAA(S-1) to understand how alkylating SH_1 affects the environment of the TNP moiety. In Figure 4, curve a shows the UV difference spectrum between TNP(S-1) and TNP[^{14}C]IAA(S-1). Only very small differences were observed in the spectrum betwee 300 and 470 nm. However, addition of nucleotide induced a well-defined difference spectrum with a minimum at 365 nm not only in TNP(S-1) (curve c) but also in TNP[^{14}C]IAA(S-1) (curve b). The change in the UV difference spectrum of TNP[^{14}C]IAA(S-1) ($-\Delta\epsilon_{365} = 1.30 \times 10^3$ M^{-1} cm^{-1}) appears to be slightly smaller than that in TNP(S-1) ($-\Delta\epsilon_{365} = 1.70 \times 10^3$ M^{-1} cm^{-1}). The results show that the environment of TNP-RLR is little changed by labeling of the SH_1 thiol with IAA. (However, as shown above, the rate of reacting RLR with TNBS *is* changed by alkylation of SH_1.)

Spectral Overlap Integral between the Absorption Spectrum of TNP Bound to S-1 and the Emission Spectrum of AEDANS Bound to S-1. Since there is a useful overlap between the emission spectrum of AEDANS bound to S-1 and the absorption spectrum of TNP bound to S-1, i.e., $J = 8.21 \times 10^{-15}$ cm^3 M^{-1} (refer to Table III), one would expect that the fluorescence emission of AEDANS at SH_1 could be quenched by TNP at RLR, provided that SH_1 and RLR are close enough to permit resonance energy transfer between the two chromophores. These expectations are fulfilled, as the quantum yield of AEDANS in the doubly labeled S-1 is reduced (as described below) even though its spectral distribution appears to be unchanged.

Relative Fluorescence Intensity and Ca^{2+}ATPase of AEDANS(S-1) as a Function of TNP Incorporated per RLR. In intraparticle energy transfer calculations it is not straightforward to calculate the concentrations of doubly labeled, singly labeled, etc., particles if the chemical reactivities of the two labeling sites are interactive. To overcome this difficulty we have first isolated S-1 in which the SH_1 group is labeled almost exactly 1 mol of label/1 mol of SH_1. Then we have titrated this material with TNBS, using an assay (enzymatic effect) which, as we have shown above, is highly specific to the trinitrophenylation of RLR. The actual sequence of procedures is as follows. We prepare S-1 with α-chymotrypsin (thus LC_2 is degraded), and then by ion-exchange chromatography on DE-52 (Weeds & Taylor, 1975) we resolve the isozyme mixture and isolate S-1(LC_1) (which if rechromatographed elutes as a single component). Now we label this material with AEDANS to a degree (ca 0.8) up to which the labeling remains quite specific (Takashi et al., 1976; Takashi, 1979). Since the label moiety that is bound confers an extra negative charge on the particle, labeled S-1 can be separated from unlabeled S-1 by rechromatography on DE-52. The elution was monitored at both 280 and 340 nm and the trailing fractions of the first peak were pooled as fluorescently labeled S-1 (LC_1). In this way we were able to obtain labeled S-1 which contains 1 mol of AEDANS per S-1. This AEDANS(S-1) was titrated with TNBS, and at each extent of titration we measured the percent reduction in Ca^{2+}ATPase activity and the percent quenching of AEDANS fluorescence (Figure 5). The former appeared to decrease to 1.6% activity when an average of 2.3 mol of TNP was attached per mol of S-1; the latter increased rather rapidly to 70% quenching when 1.3 mol of TNP was attached per mol of S-1, and thereafter very slowly to 75–77%. Elsewhere one of us (Muhlrad et al., 1975) measured, as a function of incubation time with TNBS, the fraction of surviving K^+(EDTA)ATPase, $y_V(t)$, and of unligated RLR, $y_T(t)$ (judged by the aforementioned kinetic analysis). We found that the plots of log $y_v(t)$ vs. t and log

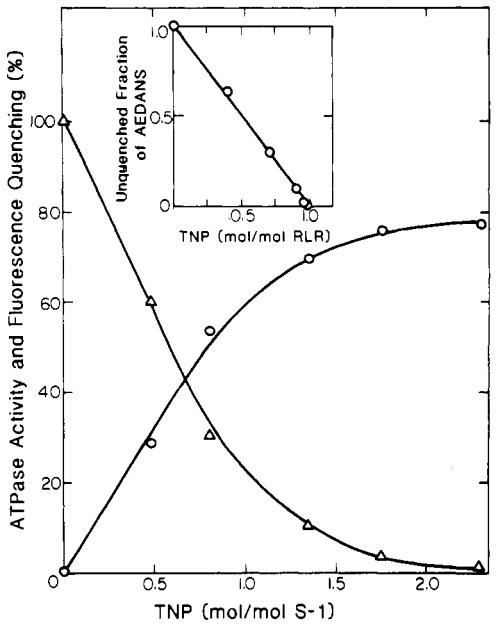

FIGURE 5: Fluorescence quenching and the reduction in ATPase activity of AEDANS(S-1) as a function of TNP incorporated per S-1. Fluorescence was measured in a solution containing 0.15 M KCl, 20 mM Tes, 5 mM $MgCl_2$, and 0.5–0.7 μM labeled S-1 (1.0 mol of AEDANS/mol of S-1), pH 7.0, at 15 °C. An appropriate correction was made for the fluorescence quenching due to the absorption of TNP at the exciting wavelength. The Ca^{2+}ATPase activity was measured under the conditions described in Figure 3, except that 10 mM $CaCl_2$ was used instead of 5 mM EDTA; the activity of the AEDANS(S-1) was 8.96 s^{-1}. The percent of ATPase activity (Δ) and percent quenching of AEDANS fluorescence (O). The inset represents the fraction of unquenched fluorescence of AEDANS bound to S-1 ($1 - y_Q$) as a function of TNP bound to RLR.

$y_T(t)$ vs. t were essentially superimposable and therefore concluded that $y_V = y_T$. Data for such a comparison measuring Ca^{2+}ATPase were again obtained in the present work (Table II). Figure 5 (main plot) shows y_Q, the fraction of quenched bound AEDANS emission, and y_V as parametric functions of "total TNP bound per mole of S-1". Numerically total TNP bound per mole of S-1 can be eliminated, and one parameter can be plotted against the other. But since according to our previous work $y_V = y_T$, this amounts to plotting $1 - y_Q$ vs. y_T (Figure 5, inset). This plot is linear, with intercepts at (1, 0) and (0, 1), and is excellent evidence that energy from ligated AEDANS is transferred exclusively to the TNP attached to the RLR.

There is another way to test whether a significant energy transfer occurs between AEDANS at SH_1 and trinitrophenylated lysines other than RLR. If transfer occurs *only* between labeled SH_1 and labeled RLR, then the quotient, (fraction of energy transferred)/(concentration of bilabeled pairs) should be independent of the concentration of bilabeled pairs. However, if the premise is false, or if our analysis is faulty, one may be unable to verify this independence of the quotient.[2]

[2] A caveat may arise if the number of non-RLR lysines reacted were proportional to the number of RLR lysines, but this is unlikely for the following reasons. Suppose n_s is the number of slowly reacting lysyl residues and n_f the number of fast-reacting lysyl residues that have reacted. We expect that $dn_s/dt = k_s(82S_0 - n_s)(T_0 - n_s - n_f)$ and $dn_f/dt = k_f(S_0 - n_f)(T_0 - n_s - n_f)$, where the k's are the second-order rate constants of the two classes of residues, 82 is the number of slowly reacting lysyl residues per S-1, S_0 is the total concentration of S-1, and T_0 is the total concentration of TNBS. Dividing the first by the second equation, we get $dn_s/dn_f = (k_s/k_f)(82S_0 - n_s)/(S_0 - n_f)$, showing that n_s can never be proportional to n_f throughout the course of the reaction.

Table II: Efficiency of Fluorescence Energy Transfer between Chromophores at SH_1 and RLR

expt	total bound TNP[a] (mol/mol of S-1)	y_V[b]	y_T[c]	E/C (%)[d]
1	0	1.00	1.00	0
2	0.48	0.60	0.59	71
3	0.81	0.30	0.34	76
4	1.35	0.09	0.06	69
5	1.75	0.02	0.01	77
6	2.31	0.01	0.00	78

[a] These values were taken from data presented in Figure 5. [b] The fraction of intact active site was calculated from the Ca^{2+}ATPase data. [c] The fraction of nonreacted RLR was calculated by using the rate constants obtained from the computer analysis, where two cases were compared; one was $k_f = 2.4 \times 10^3$ min^{-1} M^{-1} and $k_s = 4.2$ min^{-1} M^{-1}, and the other was $k_f = 2.25 \times 10^3$ min^{-1} M^{-1} and $k_s = 3.9$ min^{-1} M^{-1}. Both cases gave approximately the same results. [d] The efficiency was calculated according to an equation in the present paper, with the appropriate correction being made for the absorption of TNP at the excitation wavelength.

We start with S_0 molar S-1 that has been labeled exactly 1:1 with AEDANS at its SH_1 and measure its AEDANS emissions, F_D. Then we partly trinitrophenylate the material, measure its emission again, and find that it has decreased to F_{DA}. From these two measurements we find $1 - F_{DA}/F_D = y_Q$, and using Figure 5 (inset) we ascertain that to obtain y_Q a fraction, α, of the RLR's must have been trinitrophenylated, that is, the concentration of S-1 that is labeled at the SH_1 but not at the RLR is $S_0(1 - \alpha)$, and the concentration of bilabeled pairs, C, is $S_0\alpha$. The measurements of unquenched and quenched samples can be used to calculate the molar fluorescences, f_D and f_{DA}, of a bound AEDANS alone and one near a labeled RLR, since $f_D = F_D/S_0$ and $F_{DA} = S_0[f_D(1 - \alpha) + f_{DA}\alpha]$. The fraction of energy that is transferred, E, is $1 - F_{DA}/F_D$, which is, of course, entirely experimental; however, using the interpretations involving α, we find that also $E = \alpha(1 - f_{DA}/f_D)$. Thus $E/C = (1 - f_{DA}/f_D)/S_0$ is, as expected, a quotient independent of α. We emphasize that the E values obtained for different extents of trinitrophenylation are experimental; on the other hand, all deductions involving α, including the constancy of the quotient, depend on being able to deduce α correctly. Therefore, whether E/C remains constant despite differences in extent of trinitrophenylation is a legitimate test of the analysis and its premises. Evidently there is no significant difference in E/C (Table II); as the degree of labeling varies from 0.40 TNP/RLR to 0.99 TNP/RLR, the values of E/C are all in the range 69–76%. However, as the degree of labeling of TNP to RLR approached unity, E/C seems to increase slightly. This may indicate that at higher levels of trinitrophenylation, slight fluorescence quenching also occurs between AEDANS and TNP bound to the other lysine residues in addition to RLR. In general, these results support the idea that the fluorescence of AEDANS at SH_1 is almost exclusively transferred to the TNP that is bound to the RLR and not to other lysyls.

Because the labeling sites are interactive, we also measured E using doubly labeled S-1 that was labeled in the reverse order, i.e., we first labeled S-1 (in this case the unresolved isozyme mixture) with TNP, and then subsequently with AEDANS. In this order of labeling we could not easily isolate 100% TNP-labeled S-1 by chromatography, so we were forced to ignore site–site interaction and assumed that the two labelings were independent. However, in two different preparations we obtained E/C as 72% (0.61 mol of TNP/mol of RLR, 0.80 mol of AEDANS/mol of S-1) and 69% (0.64 mol

Table III: Energy Transfer Parameters

λ_{em} (nm)[a]	475	$J \times 10^{15}$ ($cm^3 M^{-1}$)	8.21
λ_{ab} (nm)[a]	420	R_0 (nm)	3.05
τ_0 (ns)[b]	20.3	E (%)	70
τ_Q (ns)[b]	6.8	R (nm)	2.6
Q_D[c]	0.64		

[a] λ_{em} is the fluorescence emission maximum of donor, and λ_{ab} is the absorption maximum of the acceptor. [b] τ_0 and τ_Q are the excited-state lifetimes of the donor in the absence and presence of energy acceptor, respectively. [c] Q_D is the quantum yield of the donor in the absence of the acceptor. All other parameters were calculated as described under Results.

of TNP/mol of RLR, 1.0 mol of AEDANS/mol of S-1). These results suggest that the independence approximation is fairly good and that the sequence of labeling is not very important.

As an alternative way to obtain the efficiency of fluoresence energy transfer, we measured the excited-state lifetime of AEDANS bound to S-1 and of AEDANS bound to TNP(S-1) using a time-resolving fluorometer (Figure 6). For the AEDANS(S-1) we obtained a single exponential fluorescence intensity decay curve with a lifetime of 20.3 ns which is good agreement with that reported elsewhere (Wadzinski et al., 1979). The emission kinetics approximated two exponential decays with lifetimes of 6.8 and 20.3 ns (Table III) when both AEDANS and TNP are bound to S-1. On the basis of these values, the efficiency of fluorescence energy transfer was calculated to be ca. 67%. Although this value is slightly smaller than that obtained from steady-state fluorescence measurements as described earlier, it is likely that the difference is within experimental error. Thus we concluded that the value of transfer efficiency measured by steady-state fluorescence and that obtained by the lifetime measurements are in satisfactory agreement. In summary, we use 0.70 as the value for E/C in the following calculations.

Estimates of the Distance between AEDANS at SH_1 and TNP at RLR. The theory of fluorescence resonance energy transfer (Förster, 1948, 1965) relates E (the fraction of energy absorbed by the donor which is transferred to the acceptor), R (the donor–acceptor distance), and R_0 (the "critical transfer distance"):

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

The numerical value of R_0 is decided by a cluster of universal constants, 9.79×10^2, quantities characteristic of the molecules chosen as donor and acceptor, J (the "overlap integral" expressing overlap between donor emission and acceptor absorption), Q_D (the quantum yield of the donor in the absence of the acceptor), κ^2 (a function of three angles defining the relative orientation of donor and acceptor dipoles), and n (the refractive index of the chosen medium), viz.

$$R_0 = (9.79 \times 10^2)(JQ_D\kappa^2)^{1/6}(n^{-4})^{1/6} \text{ (nm)} \quad (4)$$

In principle, therefore, R_0 can be manipulated experimentally, and of course E is measured. It follows from (3) that $E(R_0)$ is a sigmoidal curve, which is zero and has zero slope at $R_0 = 0$ and which approaches unity and zero slope as $R_0 \to \infty$. From the methodological viewpoint it is best to work in the neighborhood of the inflection point of this curve, since there the slope is maximal, and its location is sensitive both to variations in measuring E and to choice (and measurement) of R_0. The inflection is always at $E = 5/12 \simeq 0.42$ and at a value of R_0 which is $(5/7)^{1/6}R \simeq 0.94R$; the slope at the inflection is $(35/24)/(5/7)^{1/6}R \simeq 1.55/R$ and thus varies inversely with R. In principle, one measures E and chooses

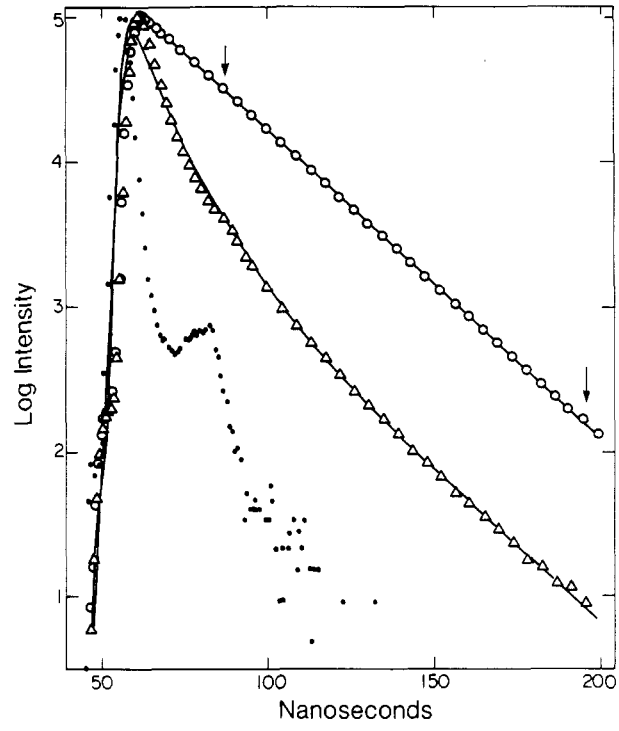

FIGURE 6: Time-resolved fluorescence anisotropy decay curves. Experimental points are normalized to a common peak height: AEDANS (S-1) (○); AEDANS-TNP (S-1) (△); lamp (●). By a nonlinear least-squares program, lines are computed to fit data over the time span indicated by the two arrows. Control data (○) are fit by a single exponential, giving $\tau_0 = 20.28$ ns. Application of eq 1 to the data when TNP is bound to a fraction, P, of the RLR's yields $\tau_0 = 20.26$ ns, $\tau_Q = 6.78$ ns, and $P = 0.946$, this last figure being in good agreement with the 95% loss in enzymatic activity measured for the same protein (exposure ratio [TNP]/[S-1] = 2.17). With a lower exposure ratio of 0.56, analysis of the corresponding fluorescence data (not illustrated here but fit, in this case, over a range of 70-174 ns) gave $\tau_0 = 20.18$ ns, $\tau_Q = 6.90$ ns, and $P = 0.423$, in reasonable agreement with the τ values above and the measured 40% loss in enzymatic activity for the same protein. Conditions: 0.8 μM S-1, 0.15 M KCl, 20 mM Tes, and 5 mM $MgCl_2$, 15 °C, pH 7.0.

R_0 and then uses (3) to calculate R. Knowledge of R gives the approximate location of the inflection point at (0.42, 0.92R). Were it possible, one would then use a new $R_0 = 0.94R$, measure E, and recompute R. In practice one rarely can choose a desired R_0, and these considerations are only of conceptual value. In the calculation to follow κ^2 was assumed to have a value of $^2/_3$ which is appropriate if the donor and acceptor dipoles rotate rapidly and extensively enough to randomize their relative orientation (Förster, 1959). The validity of this assumption will be discussed later. The quantum yield of the donor was measured to be $Q_D = 0.63$ (see Materials and Methods). The value n was taken as the refractive index of the buffer used, viz., 1.4. Furthermore, experimentally we measured $J = 8.21 \times 10^{-15}$ cm^3 M^{-1} and $E = 0.7$. From these parameters (Table III) we can estimate the intramolecular distance R between AEDANS and TNP, both bound to the specific sites of S-1, as ca. 2.6 nm.

Effect of MgATP, Its Analogues, and F-Actin on the Fluorescence of AEDANS Bound to S-1. The fluorescence emission intensity of AEDANS bound to SH_1 is diminished by 7.7 ± 1.28% (4), 6.9 ± 0.56% (2), or 4.7 ± 1.52% (2) when MgATP, MgADP, or $MgPP_i$ is added, respectively. In contrast, when F-actin is added, the intensity is increased by 3.5 ± 1.96% (8). These numbers are expressed as the mean value ± SD, with the number of experiments using different protein preparations given in parentheses. The shape of the spectrum seems unchanged within experimental resolution. Following

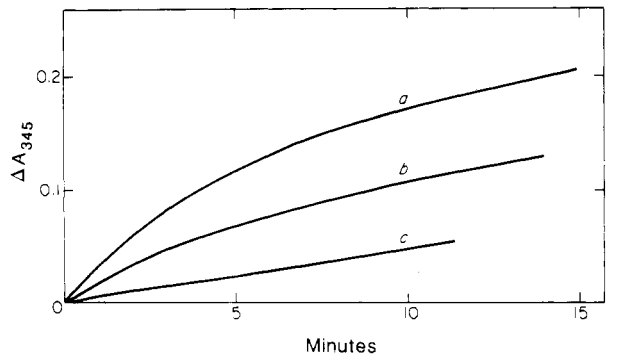

FIGURE 7: Trinitrophenylation of formaldehyde-treated and control (untreated) AEDANS(S-1). 10 μM AEDANS(S-1) or reductive-alkylated AEDANS(S-1) was incubated in a solution containing 100 mM Tris-HCl and 0.125 mM TNBS, pH 7.7, at 25 °C. The reaction was followed by an increase in A_{345}. (a) AEDANS(S-1) (0.88 mol of AEDANS/mol of S-1); (b) reductive-alkylated AEDANS(S-1) (3.02 mol of ^{3}HCHO and 0.88 mol of AEDANS/mol of S-1); (c) reductive-alkylated AEDANS(S-1) (15.67 mol of ^{3}HCHO and 0.88 mol of AEDANS/mol of S-1). The amount of TNP bound to RLR (mol/mol of RLR): (a) 0.91; (b) 0.40; (c) 0.00.

these observations we tested whether nucleotide or actin additions changed the distance (as measured by fluoresence resonance energy transfer) between AEDANS at SH_1 and TNP at the RLR. Experiments show that neither MgADP nor MgATP addition changes E; however, these additions do change certain factors of R_0, viz., reduce Q_D, the quantum yield of bound AEDANS, alter the absorption spectrum of bound TNP, and reduce J (7.42×10^{-15} cm^3 M^{-1}). These changes in turn reduce R from 2.64 (no MgATP) to 2.55 nm (with MgATP). Analogously, F-actin addition increased R to 2.66 nm but did not change E. Due to the uncertainty in κ^2 (to be described under Discussion), these differences in the distance may or may not be significant.

Effect of Reductive Alkylation of Lysine Residues on the Fluorescence of AEDANS Bound to SH_1. Since, as shown above, the binding of TNBS at RLR causes chemical changes (e.g., in reactivity) at SH_1, it would not be inconceivable that changes in SH_1-bound AEDANS fluorescence when RLR reacts with TNP result from this purely "chemical" influence, rather than from the energy acceptor quality of TNP. Such a circumstance would invalidate our interpretation in terms of fluorescence resonance energy transfer. A control experiment in which RLR is ligated but an acceptor is not attached is therefore imperative. Unfortunately, we could not perform this control by attaching a nonabsorptive group which strongly resembles TNP; the best that we could do was to alkylate RLR reductively with formaldehyde in the presence of sodium cyanoborohydride. First, we examined whether formaldehyde could also react with RLR. Figure 7 shows the time course of trinitrophenylation of AEDANS(S-1) and formaldehyde-treated AEDANS(S-1) with TNBS. An initial rapid binding phase becomes smaller as AEDANS(S-1) is alkylated at the lysine residues with formaldehyde, indicating that formaldehyde does bind to RLR of S-1 as well as to other lysine residues by a reductive alkylation, although the specificity of this chemical modification toward RLR is less than that of TNBS.

Knowing that formaldehyde binds to the RLR, we tested the effect of reductive alkylation of the lysine of S-1 on the fluorescence of AEDANS(S-1). In no sample did we observe any significant change in the emission spectrum (λ_{em}^{max} 475), the fluorescence intensity, and the lifetime (τ = 20.1–20.2 ns) of AEDANS(S-1) or the rotational correlation time (Φ = 101–106 ns) of S-1. Therefore, we concluded that chemical modification of some lysine residues of S-1, particularly RLR per se, does not seem to affect the fluorescence characteristics of AEDANS bound to SH_1 of S-1. Changes in the emission of bound AEDANS when RLR ligates TNP must be ascribed to the energy acceptor properties of TNP.

Discussion

In this paper it was established that some sort of influence can be transmitted from the SH_1 of S-1 to the RLR of S-1, and vice versa, since the blocking of either site affects the chemical reactivity of the other. That SH_1 and RLR are interactive suggests but does not prove that the two sites are separated by a distance which is considerably less than 12 nm, the longest chord in S-1 (Mendelson & Kretzschmar, 1980). Proof came from estimating the intersite distance by applying Förster's equation to data on transfer of energy between AEDANS ligated at SH_1 and TNP ligated at RLR. This distance turned out to be 2.6 nm. Although distance estimates of this kind have received some general validation, each specific instance has uncertainties to overcome. In this instance we investigated (1) whether both labelings were specific, (2) whether quenching of the donor fluorescence was due solely to the energy transfer of the acceptor, and not to ligation of the acceptor group, and (3) whether the assumption of a specific value for κ^2 was justified.

The high specificity of the AEDANS-SH_1 reaction was previously established (Takashi et al., 1976), but that of the TNBS-RLR reaction was not. Our analysis showed that when half a mole of TNP is bound to S-1, 85% of it is on RLR, and that when one mole is bound, 70% of it is on RLR, so the specificity is very good (considering that there are 82 other lysines besides RLR) but by no means absolute. Our approach was saved, however, because we were able to show that the quenching of the donor fluorescence is independent of TNP bound to lysyls other than RLR. Consistent with this independence was the additional finding that the fraction of energy transferred per doubly labeled (i.e., SH_1 and RLR) S-1 remains constant as the total TNP per S-1 varies from 0.5 to 2.3. In other words, although RLR could not be labeled with absolute specificity, it turned out that the labeling of other lysyls was immaterial to the quenching of the donor emission.

That the observed change in AEDANS emission when TNP is on RLR is due soley to the energy transfer to TNP and not to consequences of ligating RLR per se was assured by two facts: Only the quantum yield of the donor AEDANS is affected by the RLR-bound TNP, not the distribution of energies in the emission spectrum. Furthermore, ligation of RLR by a nonchromophore does not alter spectral shape, donor lifetime, or rotational correlation time of S-1.

In connection with a previous use of SH_1-AEDANS in energy transfer (Takashi, 1979; Morales & Botts, 1980), we adduced evidence that this fluorophore may indeed execute the high-speed wobble required to randomize its emission dipole: The emission anisotropy of S-1 immobilized in a high-viscosity solvent is significantly less than the emission anisotropy of AEDANS immobilized in propylene glycol at −55 °C. In the aforementioned previous work (Takashi, 1979), we studied energy transfer between (iodoacetamido)fluorescein (IAF) at SH_1 and AEDANS at Cys-373 of actin complexed with S-1. We found that neither exchange of fluorophores (which are quite different in structure) nor surface structure disruption of S-1 altered the fraction of energy transferred—results best explained by assuming that before either maneuver the donor AEDANS dipole was already in randomizing motion. The TNP group, however, is not fluorescent, and we cannot make an analogous assessment of its motion. If the

SH_1-bound AEDANS is in randomizing motion and the RLR-bound TNP group is not, then $^1/_3 \leq \kappa^2 \leq {}^4/_3$. However, the corresponding extremes in R_0 would differ by only 12% (this is because one takes a sixth root). If the RLR-bound TNP is also in randomizing motion, the $\kappa^2 = {}^2/_3$, which is the assumption that we made in our calculations. Thus we feel that the uncertainty in our distance estimate of 2.6 nm (due to this cause only) is less than 10%.

The heavy chain of S-1 is cut by trypsin into three major fragments, (C terminus)-20K-50K-27K-(N terminus) (Balint et al., 1978). Balint et al. showed SH_1 to reside on 20K; we (Hozumi & Muhlrad, 1981) and others (Miyanishi & Tonomura, 1981; Mornet et al., 1980) showed RLR to reside on 27K. Thus, although the groups are far from one another in terms of the primary sequence, they are actually rather near in the three-dimensional arrangement of the heavy chain.

Interactions between groups on S-1 may be of importance in energy transduction (Morales & Botts, 1979). It was already known that blocking of SH_1 and blocking of RLR have very similar effects on events at the nucleotide-binding (ATPase) site, for example, on K^+(EDTA)ATPase. It has been suggested (Wiedner et al., 1978; Botts et al., 1979) that these effects do not arise because these groups participate in catalysis, but because introducing bulk at such sites induces a *regional* change in conformation. Finding in this work that SH_1 and RLR interact with each other is consistent with these ideas.

References

Badea, M. G., & Brand, L. (1979) *Methods Enyzmol. 61*, 378–425.

Balint, M., Wolf, I., Tracsafalvi, A., Gergely, J., & Sreter, F. A. (1978) *Arch. Biochem. Biophys. 190*, 793.

Botts, J., Ue, K., Hozumi, T., & Samet, J. (1979) *Biochemistry 18*, 5157.

Dottavio-Martin, D., & Ravel, J. M. (1978) *Anal. Biochem. 87*, 562.

Elzinga, M., Collins, J. H., Kuehl, W. M., & Adelstein, R. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2687.

Fabian, F., & Muhlrad, A. (1968) *Biochim. Biophys. Acta 162*, 596.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375.

Förster, T. (1948) *Ann. Phys. (Leipzig) 2*, 55.

Förster, T. (1959) *Discuss. Faraday Soc. 27*, 7.

Förster, T. (1965) in *Modern Quantum Chemistry* (Sinanoglu, O., Ed.) Part III, pp 93–137, Academic Press, New York.

Houk, W., & Ue, K. (1974) *Anal. Biochem. 62*, 66.

Hozumi, T., & Muhlrad, A. (1981) *Biochemistry 20*, 2945.

Huang, K.-H., Fairclough, R. H., & Cantor, C. R. (1975) *J. Mol. Biol. 97*, 443.

Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4359.

Kielley, W. W., & Bradley, L. B. (1956) *J. Biol. Chem. 218*, 653.

Kitagawa, S., Yoshimura, J., & Tonomura, Y. (1960) *J. Biol. Chem. 233*, 2835.

Kubo, S., Tokura, S., & Tonomura, Y. (1960) *J. Biol. Chem. 235*, 2835.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randell, R. J. (1951) *J. Biol. Chem. 193*, 265.

Margossian, S. S., Stafford, W. F., & Lowey, S. (1981) *Biochemistry 20*, 2151.

Mendelson, R., & Kretzschmar, K. M. (1980) *Biochemistry 19*, 4108.

Mendelson, R., Putnam, S., & Morales, M. F. (1975) *J. Supramol. Struct. 3*, 162.

Miyanishi, T., & Tonomura, Y. (1981) *J. Biochem. (Tokyo) 89*, 831.

Morales, M. F., & Botts, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3857.

Morales, M. F., & Botts, J. (1980) *Muscle Contraction—Its Regulatory Mechanism*, p 133, Japan Scientific Societies Press, Tokyo, Springer-Verlag, West Berlin.

Mornet, D., Pantel, P., Bertrand, R., Audemard, E., & Kassab, R. (1980) *FEBS Lett. 117*, 183.

Muhlrad, A. (1977) *Biochim. Biophys. Acta 216*, 422.

Muhlrad, A., & Takashi, R. (1981) *Biochemistry 20*, 6749.

Muhlrad, A., Lamed, R., & Oplatka, A. (1975) *J. Biol. Chem. 250*, 175.

Okuyama, T., & Satake, K. (1960) *J. Biochem. (Tokyo) 47*, 454.

Parker, C. A. (1968) in *Photoluminescence of Solutions*, p 262, Elsevier, Amsterdam.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866.

Takashi, R. (1979) *Biochemistry 18*, 5164.

Takashi, R., Duke, J., Ue, K., & Morales, M. F. (1976) *Arch. Biochem. Biophys. 175*, 279.

Tonomura, Y., Appel, P., & Morales, M. F. (1966) *Biochemistry 5*, 515.

Wadzinski, L., Botts, J., Wang, A., Woodard, J., & Highsmith, S. (1979) *Arch. Biochem. Biophys. 198*, 397.

Wagner, P. D., & Weeds, A. G. (1977) *J. Mol. Biol. 109*, 455.

Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54.

Wiedner, H., Wetzel, R., & Eckstein, F. (1978) *J. Biol. Chem. 253*, 2763.