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An investigation of the SH_1-SH_2 and SH_1-ATPase distances in myosin subfragment-1 by resonance energy transfer using nanosecond fluorimetry

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The separation between the two reactive thiols SH_1 (Cys-704) and SH_2 (Cys-694) and that between SH_1 and the active site of myosin subfragment-1 were further investigated by Förster energy transfer techniques. The SH_1-SH_2 distance was determined with the probe 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (AEDANS) attached to SH_1 as the energy donor and 5-(iodoacetamido)fluorescein (IAF) attached to SH_2 as energy acceptor. The results derived from measurements of donor lifetimes yielded a donor-acceptor separation in the range 26–52 Å, with the distance $R(2/3)$ based on rapid and isotropic probe motions being 40 Å. These parameters were not sensitive to added MgADP, in agreement with previous results obtained by using the steady-state method. The SH_1-SH_2 distance was also determined with AEDANS attached to SH_1 and *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM) attached to SH_2. The range in R for the AEDANS/DDPM pair was 12–36 Å, with $R(2/3)$ equal to 27 Å. The transfer efficiency between these two probes increased by an average of 38% upon addition of MgADP. These results are in agreement with those previously reported (Dalbey, R.E., Weiel, J. and Yount, R.G. (1983) Biochemistry 22, 4696–4706), but the uncertainty in choosing an appropriate value of the orientation factor to describe the AEDANS-DDPM separation does not allow a unique interpretation of the observed increase in energy transfer because it could reflect either an increase in the average orientation factor or a decrease in the donor-acceptor separation. Nevertheless, the results are consistent with the notion that nucleotide binding induces structural perturbations that can be sensed by SH_1 and SH_2. The distance between SH_1 and the ATPase site was determined with AEDANS linked to SH_1 and the nucleotide analogue 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate (TNP-ADP) noncovalently bound to the active site as energy acceptor. The bound TNP-ADP was highly immobilized, with a depolarization factor approaching unity. The separation between AEDANS at SH_1 and TNP-ADP at the active site was in the range 15–44 Å. The actual minimal separation between SH_1 and the active site is probably less than 15 Å, which suggests that direct interaction between the two sites cannot be ruled out from energy transfer results.

Abbreviations: S-1, chymotryptic subfragment-1 of myosin; AEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; IAF, 5-(iodoacetamido)fluorescein; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; TNP-ADP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; IAA, iodoacetamide; S-1(ADEANS, SH_2), subfragment-1 modified with AEDANS at SH_1 and with SH_2 unmodified; S-1-(AEDANS,IAF), subfragment-1 modified with AEDANS at SH_1 and with IAF at SH_2; S-1(AEDANS,DDPM), subfragment-1 modified at SH_1 with AEDANS and at SH_2 with DDPM.

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Introduction

Resonance energy transfer has been increasingly used to determine distances between specific sites in individual contractile proteins and their complexes. The measurement is relatively easy to make and, if both donor and acceptor can be shown to be selectively located, this approach to estimate molecular distances can yield invaluable information on proximity relationships. In the absence of crystallographic data, distances derived from energy transfer results can provide an initial estimate of the overall shape of the macromolecular system. This has been one of the motivations in such studies with muscle proteins.

Several donor-acceptor distances have been determined within the heavy chain of skeletal myosin subfragment-1. These distances [1–4], together with those between heavy chain and light chain [5–7], are useful parameters not only for establishing the geometric relationships among the several sites within S-1, but also for understanding the dynamic nature of the myosin head under various physiological states. We recently investigated the distance between the two fast-reacting sulfhydryl groups of myosin S-1 heavy chain located at Cys-704 (SH_1) and Cys-694 (SH_2). This was done by measuring the quenching of the steady-state fluorescence intensity of a donor (AEDANS) attached to one sulfhydryl group or the sensitized intensity of an acceptor (IAF) covalently linked to the other sulfhydryl group [3]. Based on these energy transfer results and the anisotropy values of the attached donor and acceptor, we concluded that the donor-acceptor distance is in the range 29–76 Å. If the arbitrary assumption of rapid and isotropic motions were made for the two attached fluorophores ($\kappa^2 = 2/3$), the distance $R(2/3)$ would be 43–47 Å. This distance and the distance range were insensitive to added MgADP, but were slightly perturbed when the doubly labeled S-1 was complexed with actin. While the minimal distance and $R(2/3)$ were considerably greater than the separation between the two sulfhydryl groups inferred from chemical crosslinking studies, the energy transfer distance was interpreted in terms of a time-averaged value which reflects a range of instantaneous distances. These transient distances, which arise from the highly flexible nature of the region of the heavy chain between Cys-694 and Cys-704, can be trapped by appropriate crosslinking reagents [8], but cannot be individually sensed by the optical method. A similar investigation of the SH_1-SH_2 separation was subsequently reported by Dalbey et al. [4], who measured by phase fluorometry the lifetime of the donor AEDANS attached to SH_1 in the presence and absence of the acceptor DDPM covalently attached to SH_2. Their study yielded a distance of 28 Å for the donor-acceptor separation based on $\kappa^2 = 2/3$, and showed a decrease of this distance by 6–7 Å in the presece of Mg^{2+} and nucleotides. No lower and upper limits of the separation were estimated. It was not immediately clear whether the discrepancies between the two reports were due to the different acceptors that were linked to SH_2, or to the different fluorescence methods (steady state vs. lifetime) that were used in determining transfer efficiency.

In an early study, Tao and Lamkin [1] determined the separation between AEDANS attached to SH_1 and the ATPase site of S-1 by using TNP-ADP as the energy acceptor. Their results indicated a distance of 39 Å based on the assumption of $\kappa^2 = 2/3$. Because of this relatively large separation, it was concluded that in native subfragment-1, SH_1 is unlikely to interact directly with the ATPase site.

We have reinvestigated the SH_1-SH_2 distance by using the pulse nanosecond method to measure donor lifetime and the same donor and acceptor that were used in the original study. The present results confirm the previous observations obtained from steady-state measurements [3]. We also determined the SH_1-SH_2 separation by the pulse method with the same donor-acceptor pair that were used by Dalbey et al. [4]. Our results are in general agreement with those of the previous investigators. It appears that structural information derived from energy transfer with extrinsic probes may be probe-dependent. In the present work we also determined the limiting fluorescence anisotropy of TNP-ADP bound to S-1. This result indicated a highly immobilized bound nucleotide and enabled us to determine the lower and upper limits of the separation between SH_1 and the ATPase site.

Materials and Methods

Protein preparation and protein labeling

Myosin was prepared from rabbit skeletal muscle as previously described [3]. Subfragment-1 was prepared by digesting insoluble myosin with chymotrypsin according to Weeds and Taylor [9]. After separation on a DEAE-cellulose (DE-52) column, the two isozymes S-1(A1) and S-1(A2) were pooled for subsequent labeling with fluorescent probes. The concentration of S-1 was estimated from a molecular weight of 115000 and an extinction coefficient of $E_{280}^{1\%} = 7.5$ cm^{-1} [10]. Alkali light chains were prepared by the method of Perrie and Perry [11] by dissociating heavy chains from light chains in 5 M guanidine hydrochloride, followed by ethanol fractionation to remove heavy chains. The DTNB light chains were subsequently separated from alkali light chains by precipitation in 18% ethanol at 4°C over a period of 20–24 h [5]. The separated alkali light chains were then chromatographed on a DEAE-cellulose column [12] and subsequently lyophilized. The concentration of light chains was determined from absorbance by using $E_{280}^{1\%} = 2.0$ cm^{-1} [10]. Ca^{2+}-ATPase and K^+-ATPase assays were carried out as described previously [3].

The sulfhydryl group of Cys-704 (SH_1) of S-1 heavy chain was labeled with AEDANS and of Cys-694 (SH_2) was labeled with IAF in a medium comprising 30 mM Tes/60 mM KCl (pH 7.5) [3]. When DDPM was used as energy acceptor, it was attached to SH_2 [4]. Briefly, S-1 in which SH_1 was already modified by AEDANS or IAA was incubated with a stoichiometric amount of DDPM dissolved in acetone (10 mM) at 4°C in a medium comprising 50 mM Mes/100 mM KCl (pH 6.3) and in the presence of a 10-fold molar excess of ADP and 50-fold molar excess of $MgCl_2$. The reaction was stopped after 20 min by addition of a 100-fold excess of β-mercaptoethanol and unreacted probe was removed by exhaustive dialysis. The extent of probe incorporation into SH_1 or SH_2 was determined by absorbance measurements and qualitatively corroborated by measurements of Ca^{2+}-ATPase and K^+-ATPase activities. The following molar extinction coefficients were used: AEDANS, 6000 cm^{-1} at 337 nm; IAF, $7.7 \cdot 10^4$ cm^{-1} at 496 nm; and DDPM, 2930 cm^{-1} at 442 nm [13].

Prior to fluorescence measurements, the light chains of labeled S-1 (both singly and doubly labeled) were exchanged with previously isolated alkali light chains. This was performed by incubating S-1 (10–20 μM) with a 10-fold molar excess of light chains for 20 min at 4°C in the presence of 2 mM dithiothreitol/2 mM EDTA/4.7 M NH_4Cl/30 mM Tes/60 mM KCl at pH 7.0, according to the general procedure of Wagner and Weeds [10]. The NH_4Cl was then removed by dialysis against 30 mM Tes/1 mM dithiothreitol/1 mM EDTA (pH 7.0). The dialyzed protein was chromatographed in a DEAE-cellulose column equilibrated in the same buffer by using a linear gradient of 0–0.15 M NaCl to elute the exchanged S-1, followed by a step gradient of 1.0 M NaCl to elute the excess light chains. The S-1 fractions were pooled, concentrated by precipitation with $(NH_4)_2SO_4$, and redissolved in 30 mM Tes/60 mM KCl (pH 7.5) and subsequently dialyzed against the same buffer. The exchanged S-1 was then analyzed by SDS-polycrylamide electrophoresis. All fluorescence results were obtained with S-1 preparations in which light chains had been exchanged.

Labeled S-1 was tryptic digested for 10 min at room temperature by using a ratio of S-1 : trypsin equal to 100. The reaction was terminated with a 3-fold excess of trypsin inhibitor. Under these conditions, the heavy chains were cleaved into three segments with molecular weight 20000, 27000 and 50000 as demonstrated on 12% SDS-polyacrylamide gels.

AEDANS, IAF and TNP-ADP were purchased from Molecular Probes, Inc. (Junction City, OR), and were used without further purification. Ultrapure $(NH_4)_2SO_4$ was from Schwarz/Mann, bovine pancreas trypsin and soybean trypsin inhibitor were from Worthington. All other chemicals were of reagent grade.

Fluorescence measurements

Fluorescence lifetimes of AEDANS attached to S-1 were determined by a PRA pulse nanosecond fluorescence spectrometer, with the exciting light polarized at 54.7° from the horizontal. The detailed procedures of the measurements and data reduction have been described elsewhere [3]. A Ditric 3-cavity 340 nm interference filter (half-

maximum bandwidth 8 nm) was used for excitation of the probe and a 3-cavity 470 nm interference filter (bandwidth 6 nm) was used to isolate its emission in the presence and absence of the acceptors IAF and TNP-ADP. For the experiments in which DDPM was the energy acceptor, the emission was isolated by either a 470 nm or a 490 nm 3-cavity interference filters. These measurements were carried out in 30 mM Tes/60 mM KCl (pH 7.5), at at 10°C.

The statistics used to evlauate the goodness of fit between observed data and the chosen decay function were the weighted residual plot, the autocorrelation function of the residuals, and the chi-square ratio (χ^2_R) which is defined by

$$\chi^2_R = \frac{\Sigma \Delta F_i^2/\sigma_i^2}{N-P}$$

ΔF_i is the difference between observed and fitted values of the ith data point, σ_i^2 is the variance of the ith data point. N is the total number of data points (usually 300–800 points), and P is the number of parameters being fitted. For the present experiments, a value of χ^2_R of 1.2 corresponds to a 95% confidence limit. A value significantly greater than 1.5 indicates a poor fit between the chosen decay function and the observed decay data.

Steady-state fluorescence measurements were performed on a Perkin-Elmer 650-40 ratio spectrofluorimeter equipped with a semi-automated polarization asembly and a magnetic stirrer (Woods Manufacturing Co., Newton, PA). Quantum yields were determined by the comparative method as described previously [3] and with the exciting light polarized at 54.7° from the horizontal. Quinine bisulfate was used as the standard [14]. The fluorescence anisotropy spectrum of TNP-ADP was determined in glycerol and the anisotropy spectrum in the presence of a large excess of S-1 was determined in the same aqueous medium used for lifetime measurements. The limiting anisotropy of TNP-ADP bound to S-1 was determined at 10°C and in several concentrations of sucrose. The data were extrapolated to zero temperature/viscosity to yield the limiting anisotropy value.

The efficiency of energy transfer, E, was determined from the ratio of donor lifetime observed in the absence of acceptor (τ_0) and the lifetime observed in the presence of acceptor (τ):

$$E = 1 - \tau/\tau_0$$

The calculations of the overlap integral between donor and acceptor and of the donor-acceptor separation have been detailed previously [3] and need not be repeated here. The depolarization factors of attached fluorophores were calculated in the usual manner from the fundamental and limiting anisotropies of the probes [3,15].

Results

Energy transfer in S-1(AEDANS, IAF)

We previously showed that the single lifetime of AEDANS attached to S-1 in which light chains were not exchanged was in the range 19–20 ns, dependent upon the S-1 preparation. This value remained essentially unaltered when SH_2 was modified with IAA. In the present work with labeled S-1 in which light chains were exchanged, the decay pattern of the attached probe was also monoexponential, in the range 19–20 ns (data not shown). The exchange did not produce any dif-

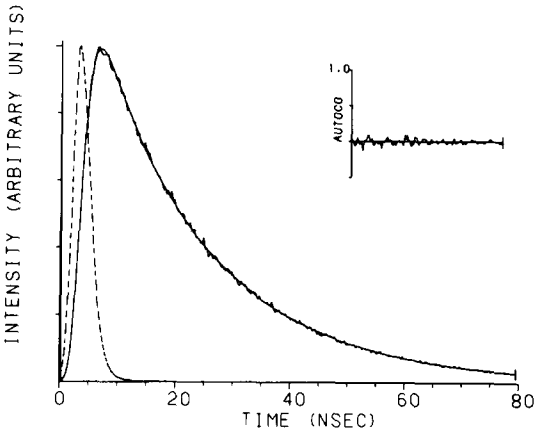

Fig. 1. Emission decay of AEDANS attached to the SH_1 of myosin subfragment-1 in which SH_2 was modified with IAF, 10°C. Protein concentration was 5 μM. Donor (AEDANS) concentration was 4.5 μM and acceptor concentration was 1.1 μM. Solid line is the best fit to the observed decay and the dashed curve is lamp profile. $\tau_1 = 20.4 \pm 0.3$ ns, $\tau_2 = 5.0 \pm 0.3$ ns, $\chi^2_R = 1.24$. Amplitudes $\alpha_1 = 0.80$ and $\alpha_2 = 0.20$. Inset is the autocorrelating function of the weighted residuals between the decay data and the chosen biexponential decay function.

ference in the probe lifetime. If heterogeneous labeling of light chains occurred when S-1 was modified at SH_1, it was not detectable from lifetime measurements. This was our previous conclusion based on analysis of SDS-polyacrylamide gels of tryptic digested S-1.

In the presence of IAF attached to SH_2, the decay pattern of AEDANS attached to SH_1 was biexponential. In a typical experiment (Fig. 1), $\tau_1 = 20.4 \pm 0.3$ ns and $\tau_2 = 5.0 \pm 0.3$ ns. The optical properties of the three-cavity interference filter used to isolate the donor emission and the spectral properties of the attached IAF [3] suggest that little or no acceptor emission would be transmitted by the filter. Control experiments carried out under identical optical conditions showed that no photon counts were detected with a sample of IAF-modified S-1 which contained the same IAF concentration as in the doubly labeled S-1. We also found that no photon counts were accummulated over a period of 30 min even when the IAF concentration was increased by a factor of 7. These results show that the acceptor emission in the doubly labeled sample was completely blocked and not detected, and the two lifetimes observed with this sample originated from donor emission. The long component was the lifetime of the fraction of probes which were not involved in energy transfer to IAF, while the short component arose from the fraction of probes which transferred excitation energy to IAF. The corresponding fractional amplitudes of the lifetimes were in good agreement with the proportions of the two populations of probes. The parameters characterizing the donor-acceptor separation derived from the lifetime measurements are listed in Table I. The present distance parameters are somewhat smaller than those previously obtained by the steady-state method and without light chain exchange. The $R(2/3)$ value (40 Å) is about 10% shorter than the previous estimate, but still considerably longer than the value 28 Å sensed by the AEDANS/DDPM pair [4] (and see next section). Qualitatively, these results are in accord with our previous observations. They also confirm the previous finding that energy transfer from AEDANS to IAF in S-1 is not affected by added MgADP, since the lifetime data were within experimental error. In the prior study, we did observe a second fluorescent band on SDS-polyacrylamide gels when SH_2 was extensively modified with IAF. When the labeling was kept below 50%, the gels showed no evidence of a second fluorescent band. This was the condition under which SH_2 was labeled with IAF in the previous energy transfer study by steady-state techniques. In the present work the doubly labeled S-1(AEDANS, IAF) did show a second faint fluorescent band on polyacrylamide gels, corresponding to 21 000 in molecular weight prior to light chain exchange. After exchange the gels showed a single fluorescent band corresponding to the heavy chain, and a single fluorescent band corresponding to the M_r 20 000 heavy chain fragment after controlled tryptic digestion. Insofar as the gel patten was concerned, the light chain exchanged preparations used in the present work were similar to those previously used without exchange.

TABLE I

ENERGY TRANSFER PARAMETERS OF S-1-(AEDANS,IAF)

τ_0 is the lifetime of donor (AEDANS) attached to SH_1 in the absence of acceptor (IAF), τ is donor lifetime in the presence of IAF attached to SH_2. E is the measured transfer efficiency. The quantum yield, Q, of the donor was determined with subfragment-1 in which SH_1 was modified with AEDANS and SH_2 with IAA. R was obtained from the forster critical transfer distance R_0: $R = R_0(E^{-1} - 1)$ and $R_0^6 = (8.79 \cdot 10^{-5}) n^{-4} Q \kappa^2 J$, where n is the refractive index (taken as 1.4), κ^2 is the orientation factor, and J is the overlap integral. J was determined as described previously [3] and found to be the same ($1.62 \cdot 10^{15}$ $M^{-1} \cdot cm^{-1} \cdot nm^4$) in the presence and absence of MgADP. $R(2/3)$ was calculated from various observed parameters and by assuming $\kappa^2 = 2/3$. $R(\min)$ and $R(\max)$ were calculated from the minimum (0.049) and maximum (2.94) values of κ^2 derived from measured depolarization factors of attached donor and acceptor as described previously [3]. All measurements were carried out in 60 mM KCl/30 mM Tes (pH 7.5) at 10°C and either without or with added MgADP (2 mM).

	No MgADP	Plus MgADP
τ_0 (ns)	20.4 ± 0.2	20.5 ± 0.2
τ (ns)	5.1 ± 0.2	5.6 ± 0.4
E	0.75	0.73
Q	0.52	0.47
$R(\min)$ (Å)	26.0	26.7
$R(2/3)$ (Å)	40.2	40.5
$R(\max)$ (Å)	51.5	51.8

Energy transfer in S-1(AEDANS, DDPM)

When S-1 in which SH_1 had been modified by AEDANS was reacted with DDPM in the presence of MgADP, the Ca^{2+}-ATPase activity declined rapidly. In a typical experiment with a 2-fold molar excess of DDPM, the activity was reduced to less than 5% of the activity of unmodified S-1 in 5 min (data not shown). Absorbance measurements showed that the extent of DDPM incorporation was in excess of 0.9. These results are indicative of SH_2 modification of DDPM and in agreement with Dalbey et al. [4], who investigated the labeling of SH_2 by DDPM in detail.

Shown in Fig. 2 is the biexponential decay pattern of AEDANS in S-1(AEDANS, DDPM). The long component (τ_1) was 19.9 ± 0.8 ns ($\alpha_1 = 0.69$) and the short component (τ_2) was 8.9 ± 0.4 ns ($\alpha_2 = 0.31$). The long component was identical with the single lifetime observed with S-1 singly modified with AEDANS. The short lifetime also originated from donor emission because DDPM is not fluorescent. Their fractional amplitudes were in agreement with the proportions of the donor and acceptor probes incorporated into the S-1 sample. The results from two preparations yielded an average transfer efficiency of 58%, corresponding to $R(2/3) = 27.4$ Å. The results obtained with a 490 nm 3-cavity filter were indistinguishable from those with a 470 nm filter. When a 5-fold molar excess of (Mg^{2+} + ADP) was added, the decay pattern remained biexponential. The long lifetime was unchanged (20.1 ns), but the short component decreased to 3.7 ns with $\chi^2_R = 1.2$. This decrease in τ_2 corresponded to an increase in transfer efficiency to 0.81 and a decrease in $R(2/3)$ to 22.6 Å assuming no change in the orientation factor. The donor acceptor pair AEDANS/DDPM attached to SH_1 and SH_2, respectively, was sensitive to added nucleotide in agreement with the previous report [4]. With both labeled preparations, the decay pattern was biexponential both in the absence of added MgADP and in the presence of a 5-fold molar excess of the nucleotide. We were unable to resolve the decay into three components when MgADP was present in large excess. However, when the concentration of MgADP was in the range 1–3 molar excess of S-1, a third decay component was detected which was in the range 1.4–2.0 ns with a relatively large uncertainty (ca. ± 1.5 ns). We were confident of the presence of a third lifetime under these conditions, since the chi-square ratio was in the range 1.1 and 1.6 and the autocorrelation function of the weighted residuals between experimental data and the chosen decay function was reasonably random. The purpose of this set of experiments was to establish whether in our hands and with the pulse nanosecond technique we were able to observe the nucleotide effect that was previously reported. The present results confirm the previous observations [4]. The agreement indicates that the observed differences between the two pairs of probes AEDANS/IAF and AEDANS/DDPM are not due to different experimental techniques. We did not analyze the decay data further as a function of MgADP concentration because this was done in detail by the other workers.

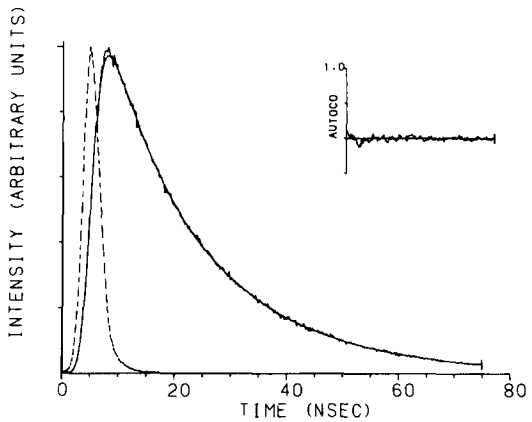

Fig. 2. Emission decay of AEDANS attached to the SH_1 of myosin subfragment-1 in S-1(AEDANS,DDPM), 10°C. Subfragment-1 was 5.3 μM in which AEDANS was 4.4 μM and DDPM was 1.4 μM. Solid line is the best fit of the observed decay data to a biexponential function: $\tau_1 = 19.9 \pm 0.7$ ns, $\tau_2 = 8.9 \pm 0.6$ ns, $\alpha_1 = 0.69$, $\alpha_2 = 0.31$ and $\chi^2_R = 1.18$. The dashed curve is lamp profile and inset is the autocorrelation function of the weighted residuals between the chosen decay function and observed data. Emission was isolated with a 490 nm interference filter.

Distance between SH_1 and ATPase site

The fluorescence polarization spectrum of TNP-ADP is shown in Fig. 3A. This spectrum is essentially the same as that of TNP-ADP determined in the presence of a large excess of S-1 in

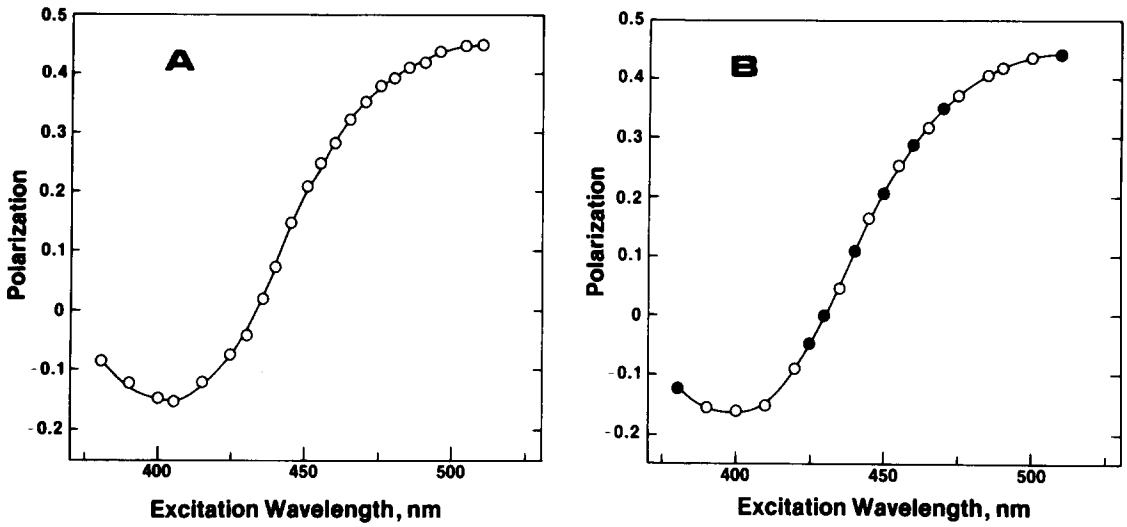

Fig. 3. Polarization spectra of TNP-ADP. (A) TNP-ADP (11.5 μM) in glycerol, 3.5°C. Emission was monitored at 560 nm. (B) TNP-ADP (9.6 μM) in the presence of subfragment-1 (40.5 μM) in which SH_1 was modified by iodoacetamide/60 mM KCl/30 mM Tes (pH 7.5). Open circles, 20°C; closed circles, 6°C. The polarization data determined in the presence of native, unmodified subfragment-1 are indistinguishable from the data shown in Fig. 3B.

which SH_1 was moified by IAA (Fig. 3B). The polarization profile of TNP-ADP has been previously investigated and shown to be independent of medium viscosity [16]. The polarization shown in Fig. 3A can be taken as the fundamental polarization of the nucleotide [16]. At 510 nm, this value is 0.351 (fundamental polarization 0.448). The limiting anisotropy of TNP-ADP bound to S-1-(IAA, SH_2) was 0.349 (polarization 0.446). The same result was obtained from either isothermal

TABLE II

ENERGY TRANSFER PARAMETERS BETWEEN SH_1 AND ATPase SITE OF MYOSIN SUBFRAGMENT-1

The donor probe AEDANS was attached to SH_1 and the acceptor was TNP-ADP. Subfragment-1 concentration was in the range 5–15 μM, of which 80–90% was modified at SH_1 by AEDANS. The TNP-ADP concentration was maintained at 40–50% of that of subfragment-1. The lifetime values are averages from three different preparations of S-1-(AEDANS,SH_2) and duplicate measurements on each preparation. The chi-square ratio of the lifetime fits was in the range 1.18–1.51. The quantum yield, Q, the overlap integral, J, and the depolarization factor of donor, $\langle d_D^x \rangle$, were determined with S-1(AEDANS,SH_2) in the presence of 2 mM MgADP, and in 60 mM KCl/30 mM Tes (pH 7.5) and at 10°C. The depolarization factor of acceptor ($\langle d_A^x \rangle$) was determined in the same medium with MgADP omitted.

τ_0 (ns)	20.3 ± 0.3
τ (ns)	4.4 ± 0.3
Q	0.47
E	0.78
J ($M^{-1} \cdot cm^{-1} \cdot nm^4$)	$6.24 \cdot 10^{14}$
$\langle d_D^x \rangle$	0.979
$\langle d_A^x \rangle$	0.997
R(min) (Å)	15.2
$R(2/3)$ (Å)	32.8
R(max) (Å)	44.1

TABLE III

EFFECT OF TRYPTIC DIGESTION ON DONOR-ACCEPTOR DISTANCES IN MYOSIN SUBFRAGMENT-1

For SH_1-SH_2 separation, SH_1 was modified with donor AEDANS and SH_2 with acceptor IAF. In the case of the SH_2-ATPase site, SH_1 was labeled with AEDANS as donor and TNP-ADP bound to the ATPase site was acceptor. Samples containing donor and acceptor were digested as described in text. After the digestion was stopped, the samples were stored at 0°C and used for lifetime measurements within 1–2 h. The lifetime measurements were made at 10°C under conditions similar to those described in Tables I and II.

	SH_1-SH_2 Separation		SH_1-ATPase site
	−MgADP	+MgADP	
τ_0 (ns)	20.8 ± 0.4	20.6 ± 0.3	20.2 ± 0.2
τ (ns)	6.3 ± 0.5	6.0 ± 0.2	3.3 ± 0.3
E	0.70	0.71	0.84
R(min) (Å)	27.2	27.1	14.4
$R(2/3)$ (Å)	42.1	41.8	30.9
R(max) (Å)	53.8	53.5	43.5

measurements or as a function of temperature. The bound TNP-ADP was highly immobilized, with a depolarization factor essentially unity.

The transfer efficiency from AEDANS attached to SH_1 to TNP-ADP that was bound to S-1-(AEDANS,SH_2) was determined at 10°C by measuring the lifetime of the attached donor. Listed in Table II are the results obtained from this set of experiments. The range in R is 15–55 Å, with $R(2/3)$ being 32 Å. The lower limit of R is a factor of two smaller than $R(2/3)$.

Effect of tryptic digestion on energy transfer in subfragment-1

When S-1 is tryptic digested, the heavy chain is cleaved into three fragments. It was of interest to examine whether nicking of the heavy chain could perturb the gross structural features of S-1. The results on energy transfer determined with tryptic digested samples are listed in Table III for the two donor-acceptor pairs, AEDANS/IAF and AEDANS/TNP-ADP. Aliquots of the digested samples were run on SDS polyacrylamide gels. In each case the heavy chains was found to have been cleaved into three segments with the anticipated molecular weights (results not shown). The tryptic action appears to increase the SH_1-SH_2 distance by about 1 Å. There was a small increase in the transfer efficiency between AEDANS at SH_1 and bound TNP-ADP at the active site, corresponding to a small decrease (approx. 5%) in the donor-acceptor separation. The changes are small, but reproducible from two different preparations.

Discussion

Determination of resonance energy transfer involving extrinsic probes by the steady state methods requires homogeneous labeling and an accurate knowledge of labeling stoichiometry. In our previous work, the SH_1-SH_2 distance was estimated from steady-state measurements. Since the previous steady-state results differ from those which were determined by phase nanosecond fluorimetry and with a different energy acceptor, it was necessary to reinvestigate the SH_1-SH_2 separation. To rule out possible heterogeneous labeling of light chains and to minimize uncertainties in the extent of labeling, we first repeated our previous study by using donor-acceptor (AEDANS/IAF) labeled subfragment-1 preparations that had been exchanged with isolated alkali light chains and by determining transfer efficiency with the pulse naosecond method. The agreement between the present and previous results strengthens the previous conclusion that the AEDANS/IAF donor-acceptor pair does not sense structural perturbation in S-1 that is induced by Mg-nucleotide binding at the ATPase site and transmitted to the SH_1-SH_2 region of the heavy chain. This observation is in contrast with the AEDANS/DDPM pair attached to the same sites. The lack of an apparent nucleotide effect is not due to the inability of the doubly modified S-1 to interact with nucleotide. Our previous study showed that the rigor complex formed between actin and such modified S-1 was dissociated by ATP as demonstrated from fluorescence polarization.

Since we were able to reproduce the main results of Dalbey et al. [4] with the donor-acceptor pair AEDANS/DDPM, including a pronounced MgADP effect on the transfer efficiency, it is clear that caution must be exercised in interpreting energy transfer results. It should not be surprising that the best estimate of molecular distances by this method is to some extent dependent upon the size and structure of the extrinsic probes, as has been discussed previously [1,3]. The fact that the measured distance $R(3/2)$ between SH_1 and SH_2 differs by some 12 Å as reported by two different acceptors is of little consequence, since $R(2/3)$ is not likely a realistic parameter to describe the separation between the two sites. This is due in part to the lack of a precise knowledge of the orientation factor κ^2. In addition, it is not known whether DDPM when attached to SH_2 is highly immobilized or has considerable motional freedom. The latter information is not available from fluorescence measurements because the probe is not fluorescent. Nevertheless, the lower and upper bounds of the AEDANS-DDPM separation in S-1 can be estimated, but with less certainty than with the AEDANS-IAF separation. The estimated range is 12–36 Å (based on estimated minimum $\kappa^2 = 0.007$ and maximum $\kappa^2 = 3.94$; and $J = 6.1 \cdot 10^{13}$ $M^{-1} \cdot cm^{-1} \cdot nm^4$ [4]). This range overlaps the lower end of the range estimated for the

AEDANS-IAF distance. In the absence of more accurate knowledge of κ^2 for either case and additional structural information, it cannot be unambiguously concluded that the two different acceptors reported different separations from the same donor.

A second concern of the present results is the apparent difference between the acceptors IAF and DDPM in sensing structural perturbation produced by MgADP. The critical transfer distance (R_0) is dependent upon the orientation factor κ^2. With S-1(AEDANS, IAF) addition of MgADP did not produce detectable changes in the rotational motion of both probes. Their depolarization factors were essentially unaltered. Since the bound nucleotide did not affect the observed transfer efficiency, the averaged relative spatial relationship of the two attached probes remained the same whether MgADP was present or absent. It is in this sense that the donor-acceptor separation in S-1(AEDANS,IAF) was not perturbed by bound nucleotide. If the true orientation factor was dependent upon the presence of bound nucleotide, the result would have indicated a change in R. This situation cannot be established.

The observed MgADP effect on S-1-(AEDANS,DDPM) is less straightforward to interpret. It is not known whether bound nucleotides affect the mobility of the attached DDPM, since this information cannot be obtained. A change in κ^2 from the estimated lower bound 0.007 to 2/3 would produce a 2-fold increase in R_0 and a change from 2/3 to 4.0 would produce a 34% increase. These changes in R_0 would be proportionally reflected in R if transfer efficiency and other parameters remained unchanged. Unless κ^2 (and hence R_0) could be shown to remain the same upon addition of MgADP, the resulting increased transfer efficiency would not necessarily reflect a decrease in R. If in the presence of nucleotides the average orientation of the two dipoles of donor and acceptor was perturbed such that the applicable κ^2 increased from 2/3 to 2.3, R_0 would increase from 29 to 36 Å. The observed increase in transfer efficiency would then be offset by this larger R_0 and the corresponding calculated donor-acceptor separation would remain at 27 Å. Similarly, a 3.5-fold increase in κ^2(min) would not cause any change in R(min). While the depolarization factor of AEDANS in S-1 was little affected by MgADP, the motional freedom of the attached DDPM might be sensitive to nucleotide binding. This possibility cannot be ruled out in spite of the fact that the absorption spectrum of the attached DDPM is unaffected by added nucleotides. The substituted phenyl ring of DDPM is relatively small compared to the bulky conjugated rings of fluorescein. The former probe in S-1 could be sufficiently mobile in the absence of bound nucleotide and this mobility could be appreciably modified due to structural perturbation induced by nucleotide binding. The large ring system of attached IAF might be constrained on the protein surface and remained relatively unperturbed by structural changes. In spite of these uncertainties, the observed increase in transfer efficiency does reflect some kind of structural perturbation occurring at the ATPase site that is transmitted to the SH_1-SH_2 region. The nature of this perturbation remains undefined. Because of the highly flexible nature of the polypeptide backbone in the SH_1-SH_2 region of the heavy chain [8], a relatively small perturbation in an adjacent domain could produce structural constraints on one of the -SH groups. The constraints may or may not be sensed by a given probe attached to the -SH group.

We have previously pointed out that the relatively large SH_1-SH_2 distance deduced from energy transfer is not incompatible with other lines of evidence that the segment of the heavy chain polypeptide between Cys-704 and Cys-694 is highly flexible [3]. The distance calculated from energy transfer efficiency is a time-averaged value which reflects a range of instantaneous distances arising from a time-dependent distribution of conformation within the heavy chain segment between the two cysteine residues. The separation between the two thiols may fluctuate over a wide range from 2–3 Å to a distance corresponding to a fully stretched polypeptide chain. In spite of these conformational dynamics, the time-averaged distance is still a useful parameter not only for characterizing proximal relationship but also for providing a measure of structural perturbation induced by ligand binding. While the present results pertain to a specific system, they point to the general necessity to use more than one donor-acceptor pair in assessing changes in proximity relationship and

structural perturbation. An additional uncertainty in the donor-acceptor separation is the finite sizes of the probes used which are expected to contribute to the measured distance [3,4]. This is particularly true for the AEDANS/IAF pair. One approach to minimize this problem is to use probes that are less bulky and less likely to impose structural distortion in the protein. It may be possible to exploit the symmetric luminescent property of certain cations in future studies so that the uncertainty in the orientation factor can be drastically reduced.

The Ca^{2+}-ATPase activity of myosin is severalfold enhanced when SH_1 is chemically modified and abolished if both SH_1 and SH_2 are blocked. These observations have raised the possibility of direct interaction between the ATPase site separation and one or both of the sulfhydryl groups. For direct interaction to be feasible, the SH_1-ATPase site must be reasonably small. The donor-acceptor separation between AEDANS at SH_1 and bound TNP-ADP at the ATPase site spans the range 15–44 Å. The present value of $R(2/3)$ determined at 10°C is in good agreement with that (32 Å) determined at 5°C by phase fluorimetry and previously reported by Weiel et al. [17], but smaller than the value 39 Å determined at 25°C [1]. As with the SH_1-SH_2 distance, it is not possible to assign a single value to the SH_1-active site separation. If the actual separation were at least $R(2/3)$, then the possibility of SH_1 being directly involved in either the binding or splitting of ATP by myosin would be unlikely. If the distance were closer to the estimated $R(\min)$, then direct involvement of SH_1 and SH_2 would become possible. Since the segment between SH_1 and SH_2 is highly flexible and at any instant their separation could be as small as 2–3 Å, it would seem that this distance fluctuation could also render the SH_1-active site separation transiently smaller than the average value of $R(\min)$. This transient fluctuation would make possible a direct interaction between the two sites. Although the available information is not sufficient to allow a definitive conclusion in this regard, the possibility of a direct involvement of SH_1 in the myosin active site activity remains an open question.

Controlled typtic digestion of the heavy chain of subfragment-1 produces three fragments. In the absence of denaturing agents, the cleaved S-1 appears to retain the original gross physical properties and Ca^{2+}-ATPase activity, but suffer a drastic reduction in the actin activated MgATPase activity [18]. These findings suggest that the cleaved S-1 may retain the overall tertiary structure but experience some subtle structural perturbation which inhibits actin activation. The energy transfer results obtained with tryptic digested S-1 are compatible with the early observations that the gross structural features of S-1 are preserved in the cleaved molecule. The separations between AEDANS and IAF located at SH_1 and SH_2, respectively, and AEDANS and SH_1 and TNP-ADP at the active site are not appreciably altered by heavy chain nicking. These results also confirm the recent report of Botts et al. [19], who found no change in either the lifetime of AEDANS attached to SH_1 or the rotational correlation time of the attached probe as a result of tryptic digestion. The small increase in the SH_1-SH_2 separation and the small decrease in the SH_1-active site distance are too small to warrant any conclusion at this time, although the same trends were observed with several different preparations. If these changes were significant, they might bear on the impaired actin binding that was observed with cleaved S-1 [19].

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