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Excitation Energy Transfer Studies on the Proximity between SH_1 and the Adenosinetriphosphatase Site in Myosin Subfragment 1†

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ABSTRACT: Excitation energy transfer studies were carried out to determine the distance between the adenosinetriphosphatase (ATPase) site and a unique "fast-reacting" sulfhydryl (referred to as SH_1) in myosin subfragment 1. The fluorescent moiety of the probe *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine was used as the donor attached at SH_1. The chromophoric nucleotide analogue 2'(3')-*O*-(2,4,6-trinitrophenyl)adenosine 5'-diphosphate was used as the acceptor noncovalently bound at the ATPase site. The energy transfer efficiency was found to be 56% by measuring the decrease in donor fluorescence lifetime. The critical transfer distance, $R_0(^2/_3)$, was determined to be 40.3 Å. Since both donor and acceptor are likely to be rigidly attached, a statistical interpretation of the data was applied [Hillel, Z., & Wu, C.-W. (1976) *Biochemistry 15*, 2105] to determine distances. The method yielded the following conclusions: most probable distance = 38.7 Å; maximum possible distance = 52 Å; 10% probability for the distance to be less than 20 Å; 3% probability to be less than 15 Å. It may be concluded that despite the great influence that the two sites exert on each other, it is not likely that SH_1 interacts directly with the ATPase site in myosin subfragment 1. This conclusion is in agreement with the findings of Wiedner et al. [Wiedner, H., Wetzel, R., & Eckstein, F. (1978) *J. Biol. Chem. 253*, 2763] and Botts et al. [Botts, J., Ue, K., Hozumi, T., & Samet, J. (1979) *Biochemistry 18*, 5157].

It is well-known that myosin subfragment 1 (S1)[1] (prepared from myosin by proteolytic digestion) retains its capacity to hydrolyze ATP and contains a pair of "fast-reacting" sulfhydryls (referred to as SH_1 and SH_2). Much current work is directed toward relating the conformational states of S1 with steps in the hydrolysis cycle. In this respect, it is of importance to find methods that are capable of defining the conformation of S1. One such method is based on singlet–singlet excitation energy transfer [see reviews by Fairclough & Cantor (1978) and Stryer (1978)], which has been used by a number of workers to measure distances between sites located either within or between contractile proteins (Marsh & Lowey, 1980; Takashi, 1979; Miki & Mihashi, 1978; Miki, 1979; Haugland, 1975). Of particular interest is the distance separating the ATPase site from SH_1 and SH_2, for it is well-known that modification of SH_1 and/or SH_2 affects the ATPase activities of myosin (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966). Conversely, the reactivity of SH_2 is affected by the presence of a bound nucleotide at the ATPase site (Yamaguchi & Sekine, 1966). Furthermore, recent work found that

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[1] Abbreviations used: S1, myosin subfragment 1; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; AEDANS-S1, S1 labeled with 1,5-IAEDANS; TNP-ATP, TNP-ADP, and TNP-AMP, 2'(3')-*O*-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate, -diphosphate, and -monophosphate, respectively; Hepes, *N*-2-(hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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cross-linking of SH_1 to SH_2 by various reagents caused the nucleotide to be trapped at the ATPase site (Wells & Yount, 1979, 1980; Wells et al., 1980).

At one time, it was proposed that the two sulfhydryls directly interact with the substrate MgATP (Burke et al., 1973; Reisler et al., 1974). More recently, however, it was reported that sulfhydryl modification with "small" ligands affects, but does not totally eliminate, the ATPase activity (Wiedner et al., 1978; Botts et al., 1979).

We have carried out energy transfer studies with the AEDANS moiety attached to SH_1 as the donor, and TNP-ADP noncovalently bound at the ATPase site as the acceptor. Fluorescence lifetime measurements yielded a transfer efficiency of 56%. When a statistical interpretation of the data (Hillel & Wu, 1976; Stryer, 1978) is used, the most probable distance was estimated to be 38.7 Å, with a 10% probability that the distance is less than 20 Å and a 3% probability that it is less than 15 Å. Thus, it appears unlikely that SH_1 is sufficiently close to the ATPase site to allow for direct interaction.

Materials and Methods

Materials. 1,5-IAEDANS was purchased from Aldrich. TNP-ATP, TNP-ADP, and TNP-AMP were purchased from Molecular Probes. ATP, ADP, and AMP and other commonly used reagents for buffers and routine analysis were from Sigma.

Protein Preparations. Myosin was prepared from rabbit back muscle (Balint et al., 1975). Chymotryptic S1 was prepared according to Weeds & Pope (1977). Labeling of S1 was carried out by using essentially the same methods described by Botts et al. (1979) and by Marsh & Lowey (1970): Freshly prepared S1 in 5 mM phosphate buffer and 40 mM KCl, pH 7.0, was incubated with an equimolar amount of 1,5-IAEDANS at 4 °C for 20 h in darkness. After the reaction was terminated with excess dithiothreitol, unreacted reagent was removed by dialysis against 10 mM Hepes buffer, 0.15 M NaCl, and 1 mM EDTA, pH 7.5. Prior to experimentation, the labeled protein was dialyzed overnight against 10 mM Hepes buffer, 0.1 M NaCl, and 5 mM $MgCl_2$, pH 7.5. All measurements were made in the above buffer at 25 °C.

Labeling stoichiometry was determined as follows: the concentration of the AEDANS moiety was determined from the absorbance at 337 nm using a molar extinction coefficient of 6100 M^{-1} cm^{-1} (Hudson & Weber, 1973). The concentration of S1 was determined from the absorbance at 280 nm using a specific absorbance of 0.77 $(mg/mL)^{-1}$ cm^{-1} (Young et al., 1965), and M_r = 115000 (Lowey et al., 1969). Since the AEDANS moiety also absorbs at 280 nm, it is necessary to substract its contribution from the total absorbance. This contribution was estimated to be $A_{280} = 0.174A_{337}$ by using published absorption data (Hudson & Weber, 1973). Typically, 0.9 mol of label per mol of S1 was incorporated. AEDANS-S1 was also prepared by chymotryptic cleavage of myosin that was labeled with 1,5-IAEDANS using the procedure described above.

Spectroscopy. Corrected steady-state fluorescence spectra were obtained on a Perkin-Elmer MPF-4A spectrofluorometer equipped with a DCSU-2 correction device. Absorption spectra were obtained on a Cary-15 spectrophotometer. Fluorescence lifetimes were obtained on a modified ORTEC 9200 nanosecond fluorometer, using procedures described by Tao & Cho (1979).

Miscellaneous Methods. Tryptic peptide analysis of AEDANS-S1 was carried out according to Balint et al. (1978).

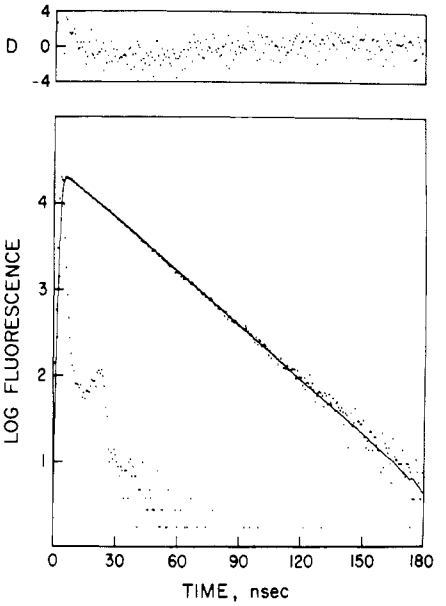

FIGURE 1: Fluorescence decay of AEDANS-S1 (5 μM). Dots are experimental points (F_e); solid line is calculated curve (F_c), using a single lifetime of τ = 20.6 ns obtained by the method of moments. Top panel is the deviation function $D = (F_c - F_e)/F_e^{1/2}$. Lower curve is the excitation.

Calcium ATPase activity was measured as described by Botts et al. (1979). The overlap integral was calculated numerically using Simpson's rule at 5-nm intervals on a PDP 11/03 computer.

Results and Discussion

The fluorescence decay of AEDANS-S1 is well described by a single exponential of lifteime $\tau = 20.6 \pm 0.2$ ns (Figure 1), in excellent agreement with the value reported by Mendelson et al. (1973) for myosin and S1 selectively labeled with 1,5-IAEDANS at SH_1 (Duke et al., 1976; Takashi et al., 1976). This lifetime varies by less than 1.0 ns between different preparations of AEDANS-S1 and AEDANS-S1 prepared from AEDANS–myosin. The calcium ATPase activity increased characteristically from 0.8 μmol mg^{-1} min^{-1} for unmodified S1 to 4.3 μmol mg^{-1} min^{-1} for AEDANS-S1. Tryptic digestion of AEDANS-S1 yielded only one fluorescent fragment identifiable by $NaDodSO_4$–polyacrylamide gel electrophoresis as the 20000-dalton fragment that contains SH_1 (Balint et al., 1978). These observations strongly indicate that under our labeling conditions, the AEDANS moiety is selectively attached at SH_1, in agreement with findings of Botts et al. (1979) and Marsh & Lowey (1980).

Since TNP-ATP is a substrate of myosin (Hiratsuka & Uchida, 1973), we can expect TNP-ADP to bind to S1 as an enzyme–product complex, and since the absorption spectrum of TNP-ADP overlaps substantially with the fluorescence spectrum of AEDANS-S1 (Figure 2), we can expect the bound TNP-ADP to quench the AEDANS-S1 fluorescence by energy transfer, provided that the two moieties are sufficiently close to each other. Titration of AEDANS-S1 with TNP-nucleotides showed that TNP-ATP and TNP-ADP quenched the fluorescence of AEDANS-S1 by ~45% (Figure 3). TNP-AMP quenched by only ~15%. When excess ATP or ADP was added to samples quenched by either TNP-ATP or TNP-ADP, the quenching was partially reversed. Excess AMP was not capable of reversing the quenching (not shown). Thus, only TNP-nucleotides that are specific for the ATPase site were capable of substantially quenching the fluorescence of AEDANS-S1. Conversely, only nucleotides that are specific

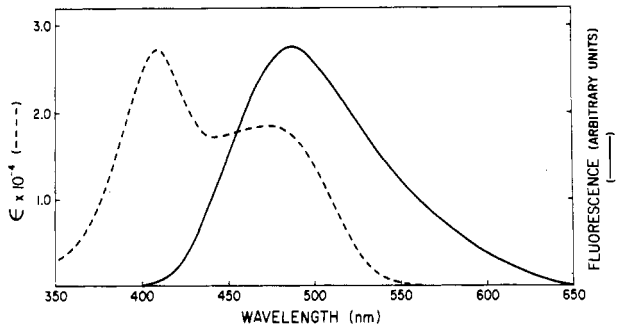

FIGURE 2: Solid curve: corrected fluorescence spectrum of AEDANS-S1 (2.15 μM). Dashed curve: absorption spectrum of TNP-ADP (23.7 μM) + S1 (83 μM).

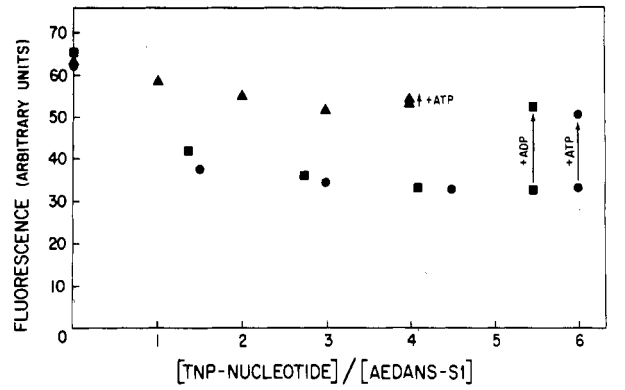

FIGURE 3: Fluorescence intensity of AEDANS-S1 (2.15 μM) at 475 nm against molar ratio of added TNP-nucleotide:AEDANS-S1. TNP-ATP (●), TNP-ADP (■), and TNP-AMP (▲). Trivial absorption was corrected with the equation $I_c = I_o \exp(-3.73 \times 10^4 c)$, where I_o is the observed intensity, I_c is the corrected intensity, and c is the molar concentration of TNP-nucleotide. This equation was obtained empirically by measuring under identical conditions the decrease in the fluorescence of 1,5-IAEDANS-labeled tropomyosin caused by the addition of TNP-nucleotides, which do not interact with tropomyosin.

for the ATPase site were capable of reversing the quenching, presumably by displacing the bound TNP-nucleotides. These observations indicate that TNP-ATP and TNP-ADP are bound, at least in part, at the ATPase site of S1.

The observations that TNP-AMP did quench the AEDANS-S1 fluorescence to a certain extent and that excess ADP or ATP did not completely reverse the quenching by either TNP-ADP or TNP-ATP lead us to believe that some quenching must occur by energy transfer to TNP-nucleotides bound at a site (or sites) other than the ATPase site. The ability of TNP-nucleotides to bind weakly at nonspecific sites in S1 was also noted by Moss & Trentham (1980) in a preliminary report. Before meaningful distance calculations can be carried out, it is clearly crucial to minimize, if not eliminate, the contribution of this nonspecifically bound TNP-nucleotide to the energy transfer process.

We have sought to minimize the extent of nonspecific binding by limiting the molar ratio, r, of TNP-ADP added to AEDANS-S1. Fluorescence lifetime measurements were carried out under four categories of conditions: (A) $r > 1.0$, no added ADP; (B) $r < 0.6$, no added ADP; (C) $r > 1.0$, excess ADP added; (D) $r < 0.6$, excess ADP added. By examining the number of decay components present in each decay curve, we could deduce whether nonspecific binding was present to a significant extent in each case. For case A, the decay curve was complex, requiring more than two exponentials to describe the data (not shown). That this behavior can be expected is illustrated in Figure 4, which shows that under these conditions we can expect at least three species to exist in solution, each

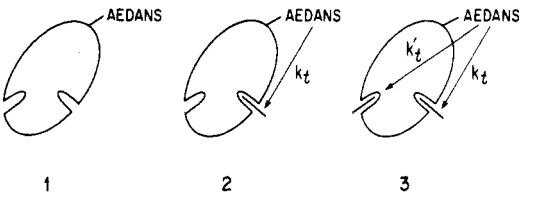

FIGURE 4: Schematic diagram illustrating the different species present when TNP-ADP was added to AEDANS-S1. AEDANS-S1 is assumed to possess two binding sites for TNP-nucleotides: the major one at the ATPase site that is specific for TNP-ATP and TNP-ADP, and a weak nonspecific site that binds all TNP derivatives. ATP and ADP are capable of displacing any TNP-nucleotides bound at the ATPase site, but not at the nonspecific site. At roughly stoichiometric amounts of TNP-ADP added to AEDANS-S1, three species can be expected to exist: (1) Species with neither site occupied, with a fluorescence decay time $\tau_0 = 1/k_0$, where k_0 is the decay rate of the AEDANS moiety in the absence of any acceptor. (2) Species with the ATPase site occupied by TNP-ADP, with $\tau_1 = 1/(k_0 + k_t)$, where k_t is the energy transfer rate from the AEDANS moiety to the TNP moiety ATPase site. (3) Species with both sites occupied, with $\tau_2 = 1/(k_0 + k_t + k_t')$, where k_t' is the energy transfer rate from the AEDANS moiety to TNP-ADP at the nonspecific site. Note that $\tau_0 > \tau_1 > \tau_2$.

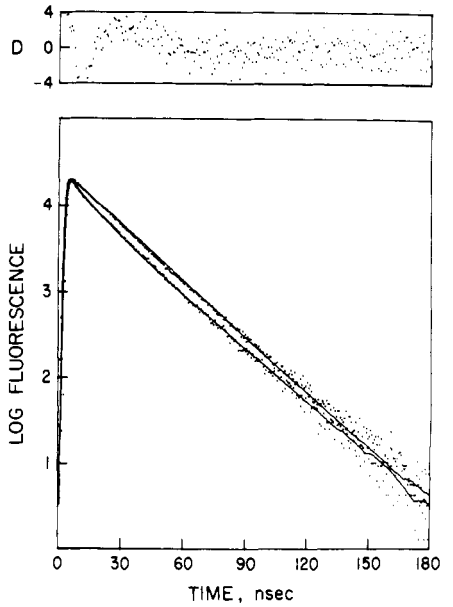

FIGURE 5: Upper curve: fluorescence decay of AEDANS-S1 (4.96 μM) with 2.82 μM added TNP-ADP and 1 mM added ADP. Lower curve: with 2.82 μM TNP-ADP, no added ADP. One lifetime of 19.4 ns was used to fit the upper curve. Two lifetimes of 8.6 and 20.8 ns were used to fit the lower curve. Top panel shows deviation function for lower curve.

with a distinct lifetime. The decay might be even more complex if there were more than one nonspecific site. Because of this complexity, and because of the inaccuracy inherent in the analyses of decay curves with more than two exponentials, no attempt was made to analyze such data. If, however, r was reduced to 0.6 or less (case B), the decay became well represented by two exponentials (Figure 5), presumably because a negligibly small amount of TNP-ADP was bound at the nonspecific site, such that only species 1 and 2 depicted in Figure 4 exist in solution. These observations were corroborated by the addition of excess ADP, which can be expected to displace TNP-ADP bound at the ATPase site. Thus, for case C, a two-component decay was observed, correponding to a species with neither site occupied by TNP-ADP and a species with only the nonspecific site occupied by TNP-ADP. When r was again reduced to 0.6 or below (case D), the decay became nearly a single exponential, corresponding to a single species with negligible TNP-ADP bound at the nonspecific

Table I: Fluorescence Decay Parameters of AEDANS-S1[a]

AEDANS-S1[b]	A_I	τ_I (ns)	A_{II}	τ_{II} (ns)
no additions				20.6
+1 mM ADP				19.6
+2.82 μM TNP-ADP + 1 mM ADP				19.4
+2.82 μM TNP-ADP	0.47	8.6	0.53	20.8

[a] Obtained by two exponential methods of moments. A_I and A_{II} are amplitudes; τ_I and τ_{II} are lifetimes. [b] AEDANS-S1 (4.96 μM) in 10 mM Hepes, 0.1 M NaCl, and 5 mM $MgCl_2$, pH 7.5, 25 °C.

site, and ADP replacing the TNP-ADP at the ATPase site. Based on these observations, we conclude that the extent of nonspecific binding is negligible as long as measurements are carried out at molar ratios of 0.6 or below.

Analysis of fluorescence decay curves obtained at $r = 0$ and at $r = 0.57$ yielded fluorescence lifetime data that can be used for quantitative analysis (Table I). Note that the presence of excess ADP causes the flurescence lifetime of AEDANS-S1 to slightly decrease from 20.6 to 19.6 ns, presumably via a conformational effect. The addition of TNP-ADP to AEDANS-S1 to a molar ratio of 0.57 in the *presence* of ADP causes a negligible change in the lifetime, indicating that even if a small amount of TNP-ADP was bound under these conditions, its effect is insignificant. When TNP-ADP was added to the same molar ratio of 0.57, but in the *absence* of excess ADP, two exponentials of roughly equal amplitude were obtained. As expected, the long lifetime of 20.8 ns agrees well with that for AEDANS-S1 in the absence of any added nucleotides. The short lifetime of 8.6 ns corresponds to $\tau_1 = 1/(k_0 + k_t)$ where k_t is evidently the desired energy transfer rate (see Figure 4 legend for definitions of k_0 and k_t), from which the separation distance can be calculated. Similar results were obtained both qualitatively and quantitatively for AEDANS-S1 prepared from AEDANS–myosin, for which $\tau_1 = 9.8$ ns was obtained.

The details of distance calculations based on energy transfer measurements have been amply described by recent reviewers (Fairclough & Cantor, 1978; Stryer, 1978) and will not be repeated here. Table II presents a summary of the results pertaining to the present study. As for most energy transfer studies, all the parameters can readily be determined, with the notable exception of the orientation factor κ^2. If the donor and/or acceptor can undergo rapid independent segmental motion, certain averaging methods can be used to limit the value of κ^2 (Dale & Eisinger, 1975). In this case, however, the acceptor, being noncovalently bound, is likely to be held fixed at several contact points. The donor is not likely to be flexible either, because Mendelson et al. (1973) reported that the polarization anisotropy of AEDANS-S1 decays with a single relaxation time that is consistent with the size of S1. We are left with little recourse but to employ a statistical interpretation of the results as described by Hillel & Wu (1976) and by Stryer (1978).

This approach assumes that the probability distribution function for the orientations of the donor and the acceptor is isotropic, i.e., the donor and the acceptor are just as likely to take on one orientation as another. Since each orientation gives rise to a value of κ^2, a probability distribution function of κ^2 can be obtained by either analytical methods (Jones, 1970) or Monte Carlo methods (Hille & Wu, 1976). The behavior of this function is such that certain values of κ^2 are statistically more likely to occur because a large number of orientations can give rise to these values. In contrast, the extreme values of κ^2 (0 and 4) are not as likely to occur because only a few

Table II: Summary of Energy Transfer Parameters

efficiency	$E = 0.56$[a]
overlap integral (cm^{-1} M^{-1} nm^4)	$J = 5.866 \times 10^{14}$ [b]
critical transfer distance (Å)	$R_0(^2/_3) = 40.3$[c]
most probably distance (Å)	$R(^2/_3) = 38.7$[d]
probability distribution functions (%)	P_R ($0 < R \leq 52$ Å) = 100[e] P_R ($0 < R \leq 20$ Å) = 10[e] P_R ($0 < R \leq 15$ Å) = 3[e]
cone semiangle (deg)	$\theta = 23$[f]
probability distribution functions (%)	$P_{R'}$ (24 Å $< R \leq 52$ Å) = 100[g] $P_{R'}$ (27 Å $< R \leq 44$ Å) = 80[g]

[a] $E = 1 - \tau_1/\tau_0$, where τ_1 and τ_0 are the lifetimes of the donor in the presence and absence of the acceptor, respectively. Here, $\tau_1 = 8.6$ ns; τ_0 was taken to be 19.4 ns in order to correct for the effect of nucleotide bound at the ATPase site, and the small amount of TNP-ADP bound at the nonspecific site. $E = 0.53$ for AEDANS-S1 prepared from AEDANS–myosin. [b] $J = \int F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda / \int F(\lambda) d\lambda$, where F is the corrected fluorescence spectrum of the donor and ϵ is the molar extinction coefficient of the acceptor. [c] $R_0(^2/_3) = [(8.79 \times 10^{-5})\kappa^2 n^{-4}\phi J]^{1/6}$, where ϕ is the donor quantum yield, taken to be 0.48 (Marsh & Lowey, 1980), n is the refractive index, taken to be 1.4 (Fairclough & Cantor, 1978), and κ^2 is the orientation factor, assumed to be $^2/_3$. [d] $R(^2/_3) = (E^{-1} - 1)^{1/6} R_0(^2/_3)$. Note that both $R(^2/_3)$ and $R_0(^2/_3)$ were obtained by assuming $\kappa^2 = ^2/_3$. [e] P_R ($R_1 < R \leq R_2$) is the probability that the distance R is between R_1 and R_2, with the assumption that both the donor and the acceptor are rigid. The values were obtained with the aid of Figure 4 in Hillel & Wu (1976). [f] θ is the semiangle of a cone within which the donor can undergo rapid reorientation. θ was calculated from the equation $(\cos\theta)(1 + \cos\theta)/2 = (A_1/A_0)^{1/2}$ (Kawato et al., 1977) where A_0 is the limiting anisotropy of the AEDANS moiety, taken to be 0.32 [Hudson & Weber (1973), as quoted by Wu et al. (1976)], and A_1 is the limiting anisotropy of AEDANS-S1, taken to be 0.25 (Mendelson et al., 1973). [g] $P_{R'}$ is equivalent to P_R, except that the donor is assumed to be flexible within a cone angle of 23°. The values were obtained with the aid of Figure 1 in Stryer (1978).

orientations give rise to these values. We have made use of this formalism to estimate the probability that the donor–acceptor separation is within certain ranges. Assuming that both the donor and the acceptor are rigid within the lifetime of the donor, we determined the range of R to be from 0 to 52 Å. According the Hillel & Wu (1976), the most probable distance is that given by $\kappa^2 = ^2/_3$; in this case, it is 38.7 Å. Using figures from Hillel & Wu (1976) or from Stryer (1978), we found that there is a 10% probability for R to be less than 20 Å, 3% for R to be less than 15 Å.

Actually, it is possible that the donor does possess some flexibility because the limiting anisotropy for AEDANS-S1 was reported to be 0.25 (Mendelson et al. 1973), whereas the limiting anisotropy for the AEDANS moiety itself is 0.32 [Hudson & Weber, 1973; as quoted by Wu et al. (1976)]. This decrease in limiting anisotropy can be attributed to rapid reorientation of the AEDANS moiety within a cone angle of 23° (Kawato et al., 1977). An averaging method can therefore be used to limit the range of R. Using Figure 1 in Stryer (1978), we estimated the range of R to be from 23.6 to 52 Å, with a 80% probability that R is between 26.7 and 44.4 Å.

It should be noted that there is an additional source of ambiguity in these determinations because the distances obtained here refer to the separation between the transition moments of the donor and the acceptor moieties, and not the separation between SH_1 and the dephosphorylation site. With the aid of molecular models, we estimated the distance from the center of the TNP moiety to the terminal phosphate group in TNP-ADP to be ~12 Å and the distance between the center of the dansyl group to the sulfhydryl attachment site in 1,5-IAEDANS to be ~10 Å. When the observed distance is taken to be 38.7 Å, then the distance between SH_1 and the dephosphorylation site can take on a range of values, with 38.7

Å as the most probable, and the extremes of 17 and 61 Å as the leeast probable.

In conclusion, it appears that SH_1 is not likely to be closer than 15 Å to the ATPase site. Thus, in spite of the striking manner in which the two sites affect each other, the interaction is probably not a direct one but one that is communicated via conformational distortion. In a preliminary report, Perkins et al. (1980) obtained similar energy transfer results using a different donor–accetor pair. The findings of Wiedner et al. (1978) and Botts et al. (1979) that modification of all the sulfhydryls in S1 by "small ligands" did not totally abolish the ATPase activity also argue against any direct interaction between SH_1 and the ATPase site. That cross-linking of SH_1 to SH_2 should diminish the rate at which adenine nucleotides dissociate from the ATPase site is also suggestive of an indirect effect rather than a direct one (Wells & Yount, 1979).

We recognize that although the prrobability is low it is still possible for the sites to be closer than 15 Å and that the observed energy transfer efficiency is lower than what it should be due to a fortuitously unfavorable donor–acceptor orientation. We are currently seeking additional donor–acceptor pairs that would allow further refinements of the measurement.

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