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MYOSIN STRUCTURE. Proximity Measurements by Fluorescence Energy Transfer

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The results of energy transfer experiments on the proximity of six sites on the globular head region of myosin are discussed. A large hydrophobic crevice has been detected on each myosin head which is sufficiently large to accommodate six aromatic rings simultaneously. In the crevice is located a thiol residue not involved in activation of myosin Ca^{2+} ATPase and a lysine residue which is specifically trinitrophenylated with 2, 4, 6-trinitrobenzenesulfonic acid. A second sulfhydryl whose modification activates the Ca^{2+} ATPase is located near the hydrophobic thiol site. The tryptophan whose fluorescence is enhanced by ATP binding is sufficiently close to the thiols and lysine residue to quantitatively transfer its energy to probes at these sites. The site of myosin ATPase has been tentatively located as being near the other five sites by energy transfer to or from synthetic chromophoric substrates. Implications of these results on the possibility of determining the location of the myosin light chain and actin binding sites are discussed.

INTRODUCTION

The distance dependence of the efficiency of fluorescence energy transfer has been confirmed to be a "spectroscopic ruler" for determing the proximity of spectrally overlapping probes (1–3). If these probes are specifically attached to different sites on a protein or on two interacting proteins, an estimate of the distance separating the probes can be obtained from Förster's theory on interacting dipoles (4). Due to the filamentous nature of their aggregates, structural determination of the muscle contractile proteins by X-ray crystallography appears distant, and fluorescence energy transfer may be the only feasible method for determining the structure and association of muscle proteins in the range of 10–75 Å. By comparison, the efficiency of fluorescence energy transfer is sufficient to span the diameter of the actin monomer and the width, but not the length, of the myosin head (5).

Extensive research has been done on the chemical modification of myosin by a variety of reagents (for review see reference 6). Proximity of the reaction sites of many of the reagents to the ATPase site has frequently been inferred from alteration of the various metal ATPase activities of myosin. Since the ATPase activity is localized in the region forming the crossbridge to actin, the sites of reaction have been assumed to be on the head of myosin. This has been conclusively demonstrated for some reagents by specific papain cleavage of the head portion of myosin (subfragment 1) from the highly helical tail (5, 7–9).

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In this paper, the close spatial proximity of six sites on the myosin head is demonstrated by flurorescence energy transfer and the existence of a large hydrophobic crevice is shown. A more detailed examination of the evidence for energy transfer between the sites will be presented elsewhere (10).

MATERIALS AND METHODS

Experimental details will be published elsewhere as will synthesis of the new probes MIANS,[1] IAANS, IANBD, 5-IAS, DNAETP, and ϵ-AMPPNP (10). The synthesis of 1, 5-IAEAmNS (11), IA TEMPO (12), and TNPATP (13) has been described previously. Fluorescence measurements were made with a Perkin Elmer MPF 4 fluorometer at 10°. Measurements were made in the ratio mode which corrects the spectrum for wavelength variation of the lamp intensity but not for photomultiplier response. Flurorescence measurements were generally performed at an optical density less than 0.02 cm^2/mmole at the excitation wavelength to avoid "inner filter" artifacts. Where this was not entirely possible, as in the binding of 1, 8-ANS and the ATP analogs, a correction was made for the transmittance at the center of the cuvet. In most cases the transfer efficiency was determined by the quenching of the donor fluorescence in the presence of the acceptor. In a few spectrally favorable cases, an increase in excitation intensity of the acceptor at wavelengths where the donor was the primary absorbing species was observed, but use of this to determine the transfer efficiency was generally impractical. The myosin used was rabbit skeletal myosin and all measurements were made in 0.6 M KCl buffered with 50 mM Tris or TES buffers.

RESULTS

The existence of at least two (14, 15), probably three (16), and possibly four (17) exceptionally reactive cysteine residues in each myosin head has been proposed on the basis of chemical modification studies and partial peptide mapping. The result of this multiplicity of thiols is extreme confusion in interpretations and nomenclature. No longer can we speak of the "fast thiol" of myosin as a single entity. Its "fastness" depends on the structure and reactivity of the reagent used for its modification and, in the absence of detailed peptide analysis, its precise location must remain uncertain. Recently, the spatial proximity and ATPase effect of two of these thiols has been elegantly demonstrated by covalent cross-linking with p-phenylenedimaleimide (15).

Three covalent hydrophobic probes (IAANS, MIANS, and IANBD in Fig. 4) have been prepared combining the environment-sensitive fluorescence properties of the anilinonaphthalenesulfonate (18) and 7-nitro-4-aminobenz-2-oxa-1, 3-diazole (19) chromophores with the reactive iodoacetyl and maleimidyl functions (10, 20). To develop appreciable fluorescence, these probes require both a cavity in which the chromophore is protected from aqueous deactivation of the excited state and a reactive amino acid side chain capable of forming a covalent conjugate. Unless both conditions are met, little

[1] The structure of all compounds abbreviated is given in Fig. 4. R_0, the distance in the Förster equation (4) at which the calculated energy transfer efficiency is 50%.

fluorescence develops. That myosin has such a hydrophobic pocket is seen in Figs. 1 and 2. When myosin is incubated with increasing mole ratios of the three probes until essentially all of the probe has reacted, the primary sites of reaction yield strongly fluorescent conjugates while the fluorescence of the probe in the secondary sites is considerably weaker and, in the case of IANBD, almost nonexistent. The stoichiometry of labeling indicates two highly fluorescent sites per mole of myosin (one per head). The linearity of the curve between 0 and 2 moles incubated per mole of myosin indicates that the quantum yield and microenvironment of the two sites are identical.

With the hydrophobic thiol residue as a reference point, the proximity of the hydrophobic crevice to other landmarks on myosin was determined by fluorescence energy transfer. A schematic of the conclusions is given in Fig. 3. The structures of the probes used to establish these results are given in Fig. 4 and the energy transfer couples observed listed in Table I.

From analysis of the effect on myosin Ca^{2+} ATPase, it is possible to distinguish between the two thiols in sites B and C (Fig. 3). Modification of site B with IAA, 1, 5-IAEAmNS or IA TEMPO significantly enhances the Ca^{2+} ATPase while the three covalent hydrophobic probes have little effect on the Ca^{2+} ATPase. Prior modification of site B with IAA decreases the rate, but not the extent, of reactions with MIANS. This indicates that the thiols are unique. The spin label, IA TEMPO, which has been used extensively to investigate myosin (21, 22), also does not block reaction with MIANS, and further, no coupling of the magnetic dipole of IA TEMPO with the electric dipole of MIANS with resulting fluorescence quenching was observed, indicating that the probes are somewhat separated (23). That sites B and C do overlap when large probes are used was shown when 1, 5-IAEAmNS, which strongly activates myosin Ca^{2+}ATPase, was found to prevent reaction of site C with MIANS.

Reaction with covalent hydrophobic probes at site C does not block the noncovalent interaction of myosin at site D with either 1, 8-ANS or 2, 6-ANS (24). Energy transfer between the sites was shown in both directions. Bound 1, 8-ANS is an efficient energy transfer acceptor from MIANS at site B and, as an energy transfer donor, had its fluorescence strongly quenched by IANBD at the same site.

From its effect on myosin ATPase, a lysine residue that is particularly reactive to trinitrophenylation with TNBS has been identified as being near the active site (25). This work has recently been repeated and the two sites of reaction found to be identically situated with one mole on each head of myosin (9). Although the resulting ϵ-trinitrophenyl is not fluorescent, its broad absorption spectrum makes it a useful energy acceptor from fluorescent probes at sites B–D. Prior reaction of the thiols with fluorescent probes did not prevent reaction with TNBS and prior reaction with TNBS did not prevent reaction with the fluorescent probes, which demonstrates that the sites are not spatially overlapping.

Use was made of the enhancement of the intrinsic fluorescence of the protein on binding MgATP to demonstrate that the tryptophan whose fluorescence is enhanced is close to sites B, C., and E. In Fig. 2, the apparent loss in ability of MgATP to induce fluorescence enhancement with an increasing extent of IANBD modification at site C is shown. The probable cause is the quantitative energy transfer from the fluorescence-enhanced tryptophan to the probe. A similar loss in fluorescence enhancement was ob-

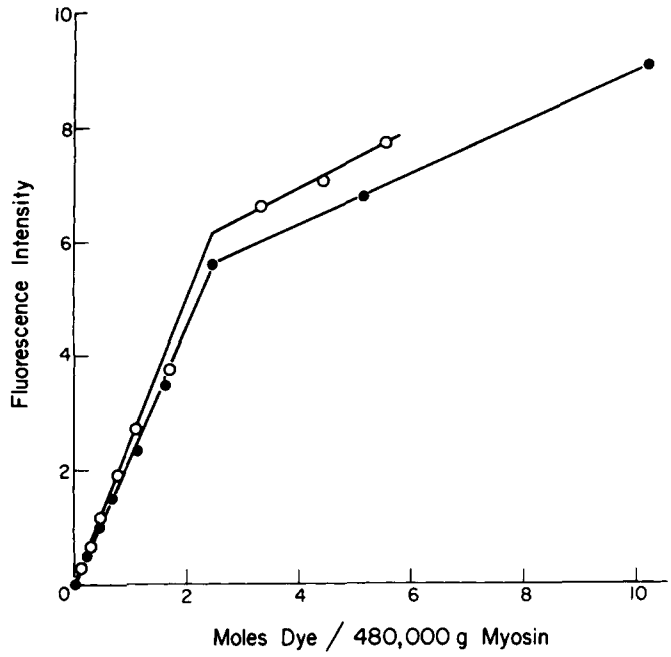

Fig. 1. Reaction of myosin with increasing mole ratios of MIANS (○) and IAANS (●). After reaction of 40 μM myosin in 0.6 M KCl, 50 mM TES, pH 7, at 0°C for 20 hr with the indicated ratios of dye the solutions were diluted 100-fold with the same buffer and the fluorescence intensity was measured at 420 nm for MIANS and 450 nm for IAANS with excitation at 330 nm for both.

served when site E was modified with TNBS or site B was modified with 1, 5-IAEAmNS.

Although there is a great amount of circumstantial evidence from kinetic effects, increase in affinity of TNBS myosin for substrates (9, 25), and implied interaction of the thiols with metal ions (15), direct evidence for proximity of the myosin ATPase site to these other sites is lacking. While conclusive on the proximity of sites B–F, energy transfer to or from synthetic substrates at the ATPase site has been inconclusive. Although energy transfer that is reversed by adding ATP has been observed between the couples in Table I (lines 17–21), the efficiency has been lower than expected for close proximity of the ATPase site to the other sites. The difficulty appears to be in the weak binding of the synthetic ATP analogs to the modified enzyme. The high concentration of analog needed to saturate the site is precluded by the high fluorescence blank of unbound ϵ-AMPPNP and the high absorbance of DNAETP and TNPATP leading to an "inner filter" artifact. The presence of the ATPase site in this scheme must remain tentative until stronger evidence of its proximity is found.

DISCUSSION

The proximity of five landmarks and existence of a large hydrophobic crevice on the myosin head has been clearly demonstrated by fluorescence energy transfer (10). While strongly implicated by kinetic (14, 15) and chromatographic (9) evidence, the fact

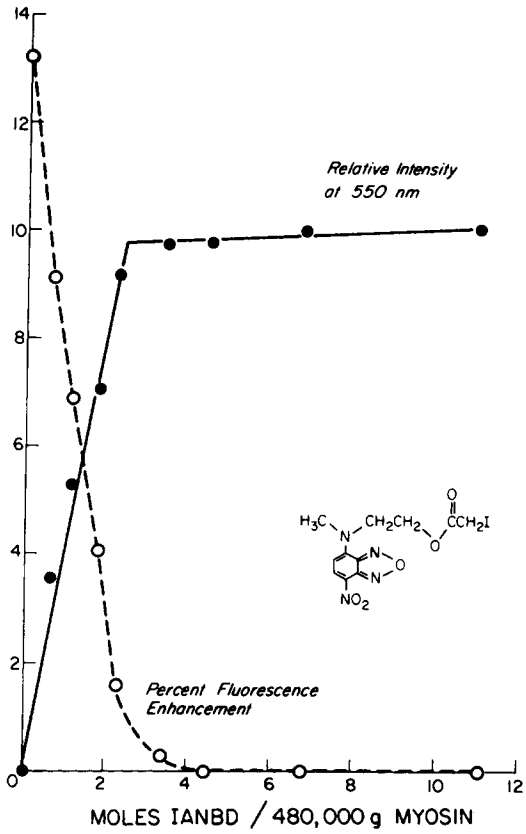

Fig. 2. Reaction of myosin with increasing mole ratios of IANBD under the same conditions as described in Fig. 1. The emission was measured at 550 nm with excitation at 480 nm. (•–•) emission of IANBD; (o–o) loss of fluorescence enhancement at 328 nm in the presence of 0.5 mM MgATP with excitation at 300 nm in the presence of the energy acceptor.

that this is the region of the active site has been difficult to demonstrate unambiguously, probably due to the weak binding and high absorbance of the chromophoric ATP analogs.

Initial observations indicate that it will not be possible to calculate anything other than a maximum distance between the sites in Fig. 3. In almost every case the transfer efficiency approached 100%, indicating that the probes are located in a compact region separated by distances much less than the calculated R_0 value for the donor-acceptor pairs (4). Only in the case of IANBD at site C to TNBS at site D, in which the spectral overlap is low, does the transfer efficiency appear to be less than 90%. The maximum distance of separation at which the transfer efficiency exceeds 90% is 22 Å if R_0 = 30 Å, and 36Å if R_0 = 50Å. Except under quite favorable spectral circumstances, it is difficult for R_0 to greatly exceed 50Å in transfer from a fluorescent donor to a single acceptor molecule. Given the uncertainties in measuring the absolute quantum yields, extinction coefficients, and especially the dipole orientation factors of the specifically bound dyes, estimation of the characteristic R_0 value for the donor-acceptor pairs is difficult; how-

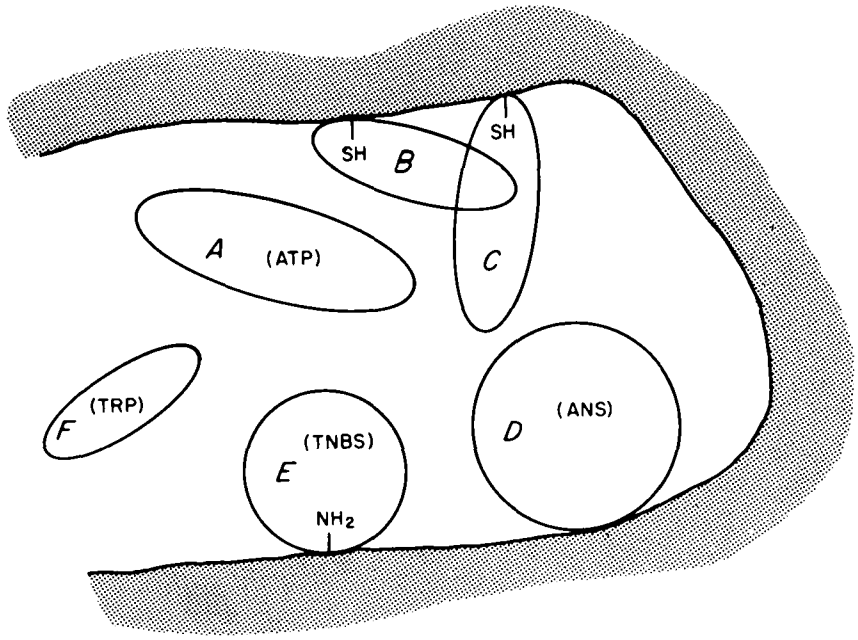

Fig. 3. Scheme of a structure for the myosin crevice based on the proximity measurements from excited state energy transfer. The two thiol sites B and C are spatially overlapping. The noncovalent hydrophobic pocket is site D (24) and the reactive lysine residue (9, 25) is site E. The tryptophan whose fluorescence is enhanced on ATP binding is part of the protein at site F. The site of myosin ATPase has tentatively been placed in the scheme at site A.

ever, unless the orientation factor is fortuitously favorable, the maximum distance between any two sites in Fig. 3 appears to be less than about 35 Å, which is a very small distance compared with the approximate 1,500 Å length of the myosin molecule and even the approximate 150 Å length of the myosin head (5). In its minimum dimensions, the hydrophobic cavity must be sufficiently large to accommodate the six aromatic rings of MIANS and 1, 8-ANS while leaving the lysine at site D and presumably ATP sites available for reaction (Fig. 5). To fit this number of probes into a region simultaneously would require that the cavity be quite large.

A second difficulty in determining the distance between sites in a large protein such as myosin is in assuming that all protein molecules are quantitatively labeled with the donor-acceptor pair. Fortunately, the kinetic selectivity of modification with the probes is sufficiently favorable in myosin to label single sites with a high degree of specificity up to about 90% of reaction. The solvation factors promoting the exceptional reactivity of the thiols at sites B and C with various alkylating reagents and the lysine at site D with TNBS are likely to be the same factors involved in the fluorescence enhancement of the environment-sensitive probes. Decreased aqueous solvation around the thiol and ammonium groups probably increases the acid strength of the group, making its dissociation to the more reactive and nucleophilic thiolate anion or free amine favored at the neutral pH used for labeling (16).

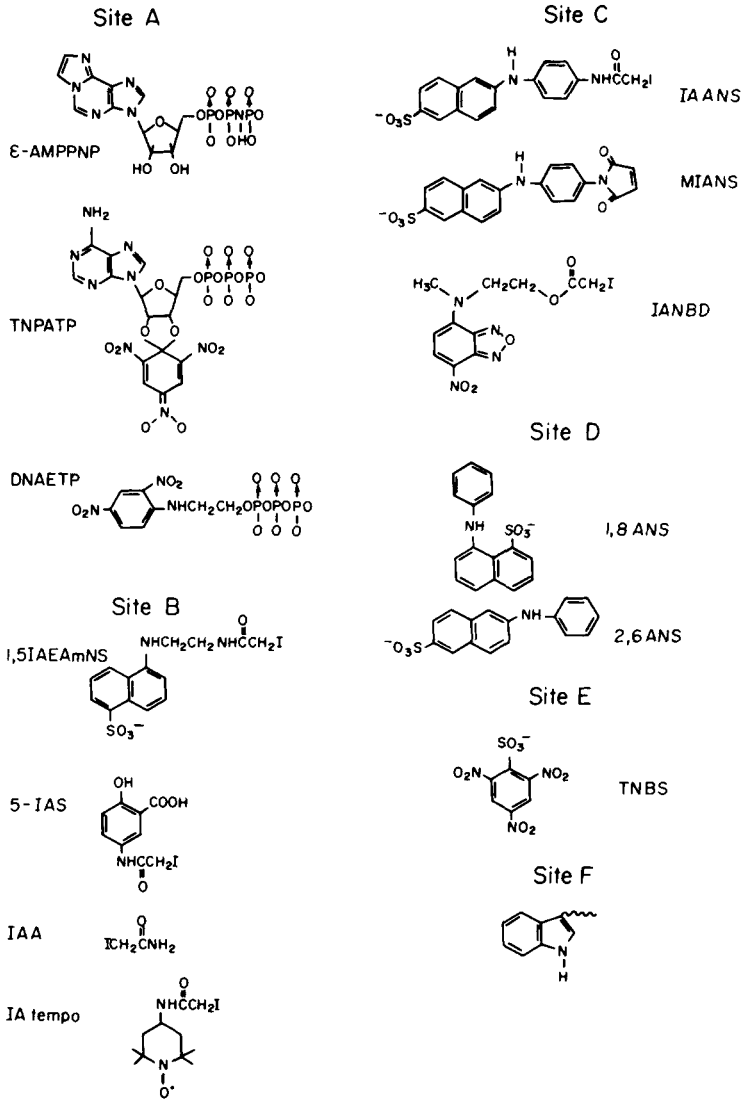

Fig. 4. Structures of the probes incorporated into the sites in Fig. 3.

Having these reference points on myosin, the question is what further information on the structure and interactions of myosin can be gained? Two extensions of mechanistic importance are obvious. First, are the low molecular weight "light chains" of myosin located near this region where they may interact with the active site? Second, is this region located very near the actin binding site with direct coupling of the ATPase energy with motion on actin, or is it near the "hinge" region of myosin (26) where the contractile energy coupling is transmitted through motion of the entire myosin head? Fluorescence energy transfer may be useful in resolving both problems. Although reconstitution of func-

TABLE I. Energy Transfer Couples Observed

	SITE				
	A	B	C	D	E	F
1			MIANS ⟶	1,8–ANS		
2			IANBD ⟵	1,8–ANS		
3			IANBD ⟵	2,6–ANS		
4			IANBD ⟵			Trp
5		1,5–IAEAmNS ⟵				Trp
6					TNBS ⟵	Trp
7				1,8–ANS ⟶	TNBS	
8				2,6–ANS ⟶	TNBS	
9			IANBD ⟶		TNBS	
10			MIANS ⟶		TNBS	
11			IAANS ⟶		TNBS	
12		1,5–IAEAmNS ⟶			TNBS	
13		5–IAS ⟶			TNBS	
14		IAA	MIANS			
15		IA TEMPO	MIANS			
16		1, 5–IAEAmNS	no reaction			
17	ϵ–AMPPNP ⟶				TNBS	
18	DNAETP ⟵	1,5–IAEAmNS				
19	DNAETP ⟵		MIANS			
20	DNAETP ⟵		IAANS			
21	TNPATP ⟵		IAANS			

tionally active myosin from mixtures of its subunits has not been particularly successful for the rabbit muscle enzyme (27), more success has been reported for some other species (28) and it is a simple matter to label the isolated light chains with a fluorescent donor or acceptor before reconstitution and to determine the proximity of the light chain to the reference points on the heavy chain by energy transfer. The second more fundamental problem of the proximity of the ATPase site to the actin binding site is amenable to

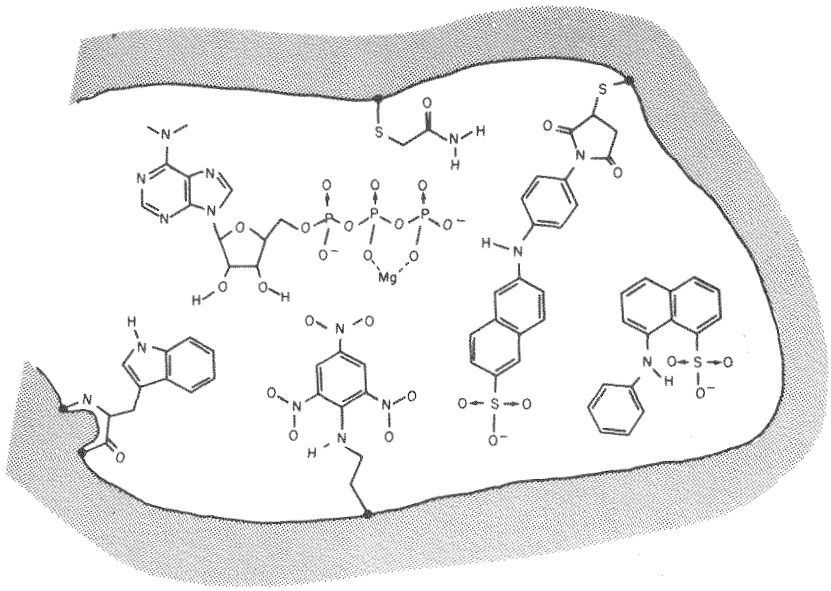

Fig. 5. A chemical structure of the myosin cavity. MgATP is bound to site A, iodoacetamide to site B sulfhydryl, MIANS on the site C sulfhydryl,1, 8-ANS bound noncovalently in a hydrophobic region, the lysine in site E trinitrophenylated with TNBS, and the tryptophan located in site F.

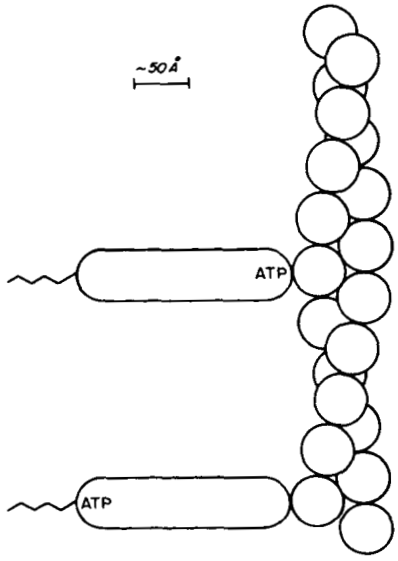

Fig. 6. A scheme of the interaction of the myosin head with the actin helix using the dimensions for myosin given in reference 5. Two possible locations of the ATP site are indicated. If the situation is as in the top of the figure, energy transfer is possible between probes on myosin and actin, but if the ATP site is nearer the hinge, energy transfer is precluded by the approximate 50 Å limitation on the interaction.

solution by energy transfer only if the ATPase site is within about 60 Å of actin, which is the approximate limit of interaction of a fluorescent donor on myosin and acceptor on actin or vice versa. The problem is illustrated schematically in Fig. 6. Since the myosin head has been pictured as an ellipse with a major axis of about 160 Å (5) and the actin binding site is almost certainly toward the end of the ellipse opposite the helical tail (although not necessarily at the tip as drawn in Fig. 6), energy transfer from an ATPase site located near the hinge to a probe on actin would be improbable due to the great distance. Thus, a more complete notion of the three-dimensional assembly and motion of the contractile proteins can be gained from the method of fluorescence energy transfer.

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REFERENCES

1. Latt, S. A., Cheung, H. T., and Blout, E. R., J. Am. Chem. Soc. 87:995 (1965).
2. Stryer, L., and Haugland, R. P., Proc. Nat. Acad. Sci. 58:719 (1967).
3. Haugland, R. P., Yguerabide, J., and Stryer, L., Proc. Nat. Acad. Sci. 63:23 (1969).
4. Förster, T., Ann. Physik. 2:55 (1948). Technical Translation TT564, Nat. Res. Council of Canada.
5. Mendelson, R. A., Morales, M. F., and Botts, J., Biochemistry 12:2250 (1973).
6. Tonomura, Y., "Muscle Proteins, Muscle Contraction and Cation Transport," pp. 115–139, University Park Press (1973).
7. Ohe, M., Seon, B. K., Titani, K., and Tonomura, Y., J. Biochem. 67:513 (1970).
8. Seidel, J. S., Chopek, M., and Gergely, J., Arch. Biochem. Biophys. 142:223 (1971).
9. Mühlrad, A., Lamed, R., and Oplatka, A., J. Biol. Chem. 250:175 (1975).
10. Haugland, R. P., to be published.
11. Hudson, E. N., and Weber, G., Biochemistry 12:4154 (1973).
12. Rosantzev, E. G., and Krinitzkaya, L. A., Tetrahedron 21:491 (1965).
13. Hiratsuka, T., and Uchida, K., Biochim. Biophys. Acta 320:635 (1973).
14. Reisler, E., Burke, M., and Harrington, W. F., Biochemistry 13:2014 (1974).
15. Reisler, E., Burke, M., Himmelfarb, S., and Harrington, W. F., Biochemistry 13:3837 (1974).
16. Kabasawa, I., Murayama, K., Kimura, M., and Sekine, T., J. Biochem. 75:1275 (1974).
17. Yount, R. G., Frye, J. S., and O'Keefe, K. R., Cold Spring Harbor Symp. Quant. Biol. 37:113 (1972).
18. Stryer, L., Science 162:526 (1968).
19. Ghosh, P. B., and Whitehouse, M. W., Biochem. J. 108:155 (1968).
20. Haugland, R. P., J. Supramol. Struct. 3:192 (1975).
21. Seidel, J. C., and Gergely, J., Cold Spring Harbor Symp. Quant. Biol. 37:187 (1972).
22. Stone, D. B., and Prevost, S. C., Biochemistry 12:4206 (1973).
23. Koblin, D. D., Kaufmann, S. A., and Wang, H. H., Biochem. Biophys. Res. Commun. 53:1077 (1973).
24. Cheung, H. C., and Morales, M. F., Biochemistry 8:2177 (1969).
25. Kubo, S., Tokura, S., and Tonomura, Y., J. Biol. Chem. 235:2835 (1960).
26. Burke, M., Himmelfarb, S., and Harrington, W. F., Biochemistry 12:701 (1973).
27. Dreizen, P., and Gershman, L. C., Biochemistry 9:1677 (1970).
28. Szent-Györgyi, A. G., Szentkiralyi, E. M., and Kendrick-Jones, J., J. Mol. Biol. 74:179 (1973).