Studies of Myosin Conformation by Fluorescent Techniques*

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ABSTRACT: The fluorescense of myosin A was quenched by its interaction with 8-anilino-1-naphthalenesulfonate.

The quenching is shown to result from a transfer of the excitation energy from myosin chromophores to the adsorbed dye, which fluoresces maximally at 468 nm. This is demonstrated by the fluorescence excitation spectrum of bound 8-anilino-1-naphthalenesulfonate in which the fluorescence intensity with excitation in the 280-nm region is several times larger than that expected from the absorption spectrum of the dye. Native myosin showed a larger transfer than myosin which had been modified by *p*-mercuribenzoate, EDTA, Cd^{2+}, and Zn^{2+}. The decrease in transfer is attributed to changes in myosin conformation resulting from the modifications.

The possibility of myosin deformation in the regions known to contain active centers is important because such deformation may be related to the origin of mechanical thrust in muscle contraction. In this paper we examine for such deformation by a new method.

The ATPase activity of myosin A can be modified by various types of agents, often of different chemical reactivity and structure. Small amounts of *p*-mercuribenzoate, Cd^{2+}, and Zn^{2+} activate the enzyme, while larger amounts of the same agents are inhibitors. Evidence obtained by Levy *et al.* (1962) on the temperature dependence of the ATPase activity of both native and *p*-mercuribenzoate-modified myosin suggests a different conformation for the modified enzyme. The possibility of a different conformation is also demonstrated by the pH dependence of myosin NTPase activity, and has been discussed by Rainford and coworkers (1964). The attempt to show a conformational difference by optical rotatory dispersion has not been too fruitful (Tonomura *et al.*, 1963), presumably due to the enormous size of the myosin molecule as compared with the size of the active-site region. This is not surprising since the optical rotatory dispersion of proteins is often not a very sensitive measure of small conformational changes (Kagi, 1964; Fasella and Hammes, 1964).

In recent years several workers have shown that certain fluorescent substances are sensitive probes for studying changes of macromolecular conformation. The dye, 8-anilino-1-naphthalenesulfonic acid, is almost nonfluorescent in water, but becomes highly fluorescent when adsorbed to certain proteins (Weber and Laurence, 1954). It is this spectral property that makes 8-anilino-1-naphthalenesulfonic acid useful for probing the structure of hydrophobic regions in macromolecules. Duke and coworkers (1966) have studied the interaction of myosin A with 8-anilino-1-naphthalenesulfonic acid. They found a very small enhancement of the 8-anilino-1-napthalenesulfonic acid fluorescence using native myosin, and a very large enhancement using the enzyme modified by *p*-mercuribenzoate. Lim and Botts (1967) have examined the aging and temperature effects on the fluorescence of the myosin–8-anilino-1-naphthalenesulfonic acid complex. Our present work is concerned with additional aspects of this interaction and with other ways in which 8-anilino-1-naphthalenesulfonic acid can be used to reveal localized deformation on myosin.

Materials and Methods

Myosin A was prepared from rabbit skeletal muscle by a modification of the Szent-Gyorgi method (Tonomura *et al.*, 1966), and the completed preparation was dialyzed against either 0.6 or 0.3 M KCl overnight. Protein concentration was determined by a modified Lowry procedure (Gellert *et al.*, 1959). Molar concentrations were based on a molecular weight of 4.8×10^5. Tris was from Sigma and *p*-mercuribenzoate from Calbiochem. All common chemicals were of reagent grade. The potassium salt of 8-anilino-1-naphthalenesulfonic acid was obtained by first converting the sodium salt (Eastman Chemical) into the magnesium salt, which was then recrystallized three times in hot water after filtering through Norit and dried at 110° for several hours in a vacuum oven. Its extinction coefficient was 5800 cm^2/mmole at 350 nm, in agreement with that recently reported by Laurence (1966). A solution of the purified magnesium salt was exchanged for potassium on a column of Dowex 50 resin before use. Thin-layer chromatography with Eastman Chromagram K301R (Eastman Chemical) showed both the magnesium and potassium salts to be reasonably homogeneous. Magnesium assay with a Perkin-Elmer atomic absorption spectrophotometer 303 showed the potassium salt to

* From the Cardiovascular Research Institute, University of California, San Francisco Medical Center, San Francisco, California 94122. *Received December 26, 1968.* This work was supported by research grants from the Muscular Dystrophy Association, U. S. Public Health Service (GM 14076), National Science Foundation (GB 4754), and American Heart Association (60 CI 8).

† Senior Postdoctoral Trainee, National Institutes of Health training grant (HE 5251) from the National Heart Institute.

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contain negligible Mg^{2+} (1×10^{-6} M, near the detectability of the instrument) and no detectable Na^{+}.

Fluorescence measurements were performed on a ratio spectrofluorometer, consisting of two Jarrell-Ash 0.25-m grating monochromators and a quantum counter consisting of a 5-mm layer of 5×10^{-3} M rhodamine G in ethylene glycol. Fluorescence intensity and emission spectra were recorded on a Beckman stripchart recorder from the front surface of a 1-cm cuvet with a slit width of 2 mm, which yielded a half-band width of 10 nm. The angle between the excitation beam and the direction of observation is 37°. A fraction of the excitating radiation from a 150-W xenon lamp (Hanovia) emerging from the excitation monochromator is reflected off a quartz plate situated at 45° to the exciting beam, and falls on the quantum counter. An RCA 1P21 photomultiplier was used to detect the quantum counter signal. The remaining portion of the exciting beam is focused on the front surface of the cuvet. The image of the slit of the excitation monochromator is focused on the entrance slit of the emission monochromator. The sample fluorescence was detected by an RCA 1P28 photomultiplier. The ratio of the fluorescence signal from the emission monochromator to the signal from the quantum counter was recorded by using a ratio detector. Ratio recording through the use of a quantum counter corrects for changes in the incident intensity as a function of wavelength (Parker, 1958), and also eliminates the need for an external standard to correct for intensity fluctuations of the lamp. The emission spectra shown here are direct tracings which have not been corrected for variations of the detector system with wavelength.

Corrected excitation spectra were obtained directly through the ratio detector. A Corning 3-73 filter was used to isolate the desired fluorescence when the emission was observed at right angles to the excitation. An RCA 1P28 photomultiplier was used to detect sample fluorescence. The spectra were also corrected for absorbance of the samples. Temperature control was achieved by circulating water through the cuvet holder.

Fluorescence quantum yield was determined by the comparative method of Parker and Reese (1960), with quinine hydrochloride as a reference. 8-Anilino-1-naphthalenesulfonic acid bound to bovine serum albumin was also used as a reference since its quantum yield has been measured to be 0.75 (Weber and Young, 1964). Measurements on the reference materials gave results within 10% of each other.

Fluorescence excited-state lifetime was measured with a nanosecond fluorimeter (Hundley *et al.*, 1967) in the laboratory of Professor Lubert Stryer at Stanford University.

The fluorescence intensity developed rapidly upon mixing myosin and 8-anilino-1-naphthalenesulfonic acid and it began to slow down after 10 min (Duke *et al.*, 1966). The intensity, however, never quite reached a saturation value, but kept increasing slowly in time. Because of this time dependence, all measurements were started exactly 15 min after the addition of the dye to the enzyme. Unless stated otherwise, all fluorescence measurements were made in 0.3 M KCl–0.05 M Tris buffer (pH 8). Absorbance of bound 8-anilino-1-naphthalenesulfonic acid was obtained as a difference spectrum.

Modification of myosin by *p*-mercuribenzoate was carried out on ice using incubations of at least 24 hr. The incubation with other modifiers (EDTA, Cd^{2+}, and Zn^{2+}) was 60 sec at room temperature before 8-anilino-1-naphthalenesulfonic acid was added.

Results

Binding of 8-Anilino-1-naphthalenesulfonic Acid. The intensity of light fluoresced from a system in which 8-anilino-1-naphthalenesulfonic acid has been equilibrated with myosin is maximal when the exciting radiation is of wavelength 380 nm, and the maximum of the fluorescence itself occurs at 468 nm. Since the quantum yield of 8-anilino-1-naphthalenesulfonic acid in water is only 0.004 (Stryer, 1965), the observed fluorescence is almost entirely from myosin–8-anilino-1-naphthalenesulfonic acid complexes.

If a protein, P, has n sites at which it can bind a dye, D, and if a property such as the fluorescence, F, arises only from complexes of P and D, then empirically at a fixed total concentration of P, *viz.*, P_0, one can always evaluate the ratio between the fluorescence arising when the total concentration of dye is arbitrary, *viz.*, D_0, and the maximum possible fluorescence, F_∞, arising when D_0 is made indefinitely large. If, in order to rationalize this relation, one assumes that (1) each site binds dye independently of the occupancy of other sites, with intrinsic dissociation constant, K, and (2) the fluorescence per site, $\hat{f}$, is the same for every site, then

$$\frac{F}{F_\infty} = \frac{\hat{f}(D_0 - D)}{\hat{f}nP_0} = \frac{D}{D + K} \text{ or}$$

$$\frac{1}{F} = \frac{1}{F_\infty} + \left(\frac{1}{F_\infty K}\right)\frac{1}{D} \quad (1)$$

Equation 1 suggests that $1/K$ can be readily evaluated from a table of values of F and D_0 (assuming that in excess dye, $D \cong D_0$). Professor G. Weber (private communication) has commented usefully on the two assumptions underlying eq 1. Anderson and Weber (1965) have shown that in situations in which energy is transferred from a protein chromophore to ligand fluorophores the quantum yields of the latter must all be nearly the same if an "isoemissive" point exists, as it does in our data. Weber has shown that, yields aside, the other source of deviations from linearity in a plot of eq 1 is heterogeneity in microscopic dissociation constants (introduces a term in $1/D^2$ in eq 1). Since, in fact, the plot of our data is linear (Figure 1) we can be reasonably confident that we are indeed evaluating a satisfactory average intrinsic dissociation constant, K. At 20° K is 4.93×10^{-5} M and at 8.7° it is 5.76×10^{-5} M. From this temperature dependence we can roughly estimate $\Delta H \cong 2.28$ kcal mole^{-1}, and therefore $\Delta S \cong 26$ eu at 20°.

Equation 1 provides an estimate of $F_\infty = \hat{f}nP_0$; since we know P_0, we can obtain $\hat{f}n$, but not f *and* n. However, if D_0 is kept constant and P_0 is increased without limit,

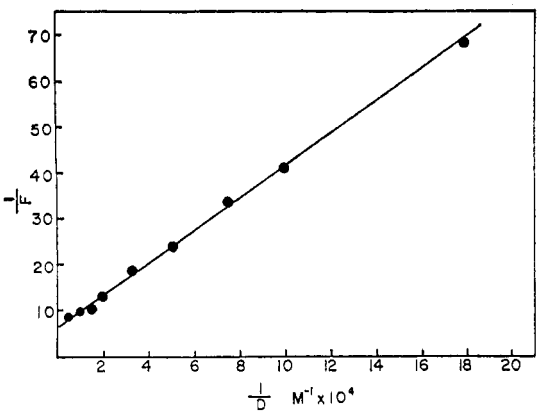

FIGURE 1: Binding of 8-anilino-1-naphthalenesulfonic acid to myosin A. Myosin concentration fixed at 1×10^{-5} M, total 8-anilino-1-naphthalenesulfonic acid concentration was varied between 5.5×10^{-6} and 2×10^{-4} M, 20.0°. Excitation was 380 nm and fluorescence was observed at 470 nm.

we see that in the region of great protein excess *protein* binding sites constitute the "ligand," and that if we think of the complex of such a site with a dye molecule as a substance PD, then the mass law can be written as

$$\frac{D_0}{PD} = 1 + \frac{K}{nP_0 - PD}$$

Reintroducing the relationship, $F = \hat{f}PD$, and noting that by hypothesis, $nP_0 \gg D_0 > PD$, we can write

$$\frac{D_0}{F} = \frac{1}{\hat{f}} + \frac{K}{n\hat{f}P_0} \quad (2)$$

Since K is known, a plot of eq 2 will allow an estimate of n. We find that, at the time of measurement, $n = 1.14$ for native myosin.

The weak binding makes it difficult to determine the quantum yield of the bound dye by the comparative method since it was not feasible to bind all of the 8-anilino-1-naphthalenesulfonic acid molecules to myosin and to have a reliable measurement of the absorbance of the bound dye. However, an estimate was made of the fraction of the total dye bound from the equilibrium dissociation constant for the system at 1×10^{-5} M total 8-anilino-1-naphthalenesulfonic acid and 3.15×10^{-5} M myosin. With this estimate the absorbance of the bound dye at 366 nm was calculated from the total absorbance of the sample (free dye and bound dye). When this was done, the quantum yield was found to be 0.48 with excitation at 366 nm.

Energy Transfer. Figure 2 shows the emission spectra of myosin and the myosin–8-anilino-1-naphthalenesulfonic acid complex excited at 295 nm, at which both myosin and 8-anilino-1-naphthalenesulfonic acid absorb. In the absence of 8-anilino-1-napthhalenesulfonic acid the fluorescence peak at 345 nm arises from excitation of the tryptophan residues. When 8-anilino-1-naphthalenesulfonic acid is present, the tryptophan fluorescence is quenched, and a second peak appears in the 475-nm region. The latter is the 8-anilino-1-naphthalenesulfonic acid fluorescence. With increasing 8-anilino-1-naphthalenesulfonic acid concentration, the tryptophan fluorescence is progressively quenched. Since at 295 nm the absorbance of 8-anilino-1-naphthalenesulfonic acid is very small, the quenching of the myosin fluorescence cannot be due entirely to attenuation of the exciting intensity by the dye. A more plausible explanation appears to be a transfer of excitation energy from myosin chromophores to the bound dye. Figure 2 also shows an isoemissive point at 416 nm. The energy transfer is also demonstrated from the excitation spectrum (Figure 3). The lower curve is the absorption spectrum of the bound dye, which has been normalized so that it coincides with the excitation spectrum in the longer wavelength region. For a given fluorescent species, its excitation spectrum is expected to be identical with its absorption spectrum. This is the case for the region >300 nm. Below 300 nm the observed fluorescence is several times larger than the absorption spectrum. The additional fluorescence results from a transfer of the excitation energy in the 280-nm region from the tryptophan residues to the bound 8-anilino-1-naphthalenesulfonic acid. The amount transferred is at a maximum near 280 nm since myosin itself absorbs maximally in this vicinity. The efficiency of transfer, E, is given by the following relationship

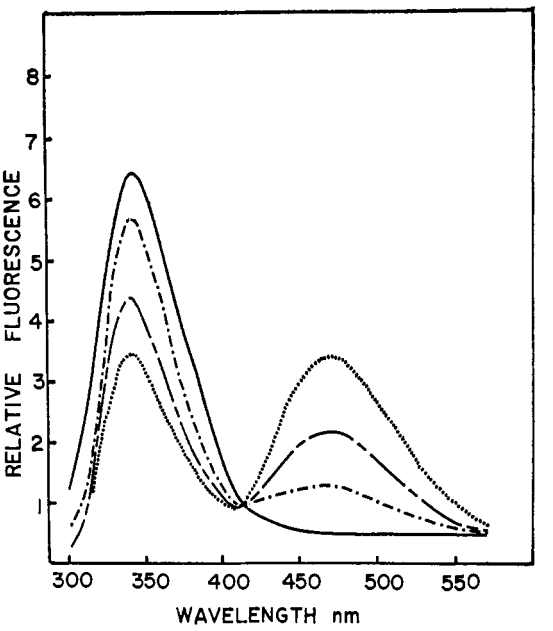

FIGURE 2: Fluorescence emission spectra of myosin–8-anilino-1-naphthalenesulfonic acid. 2.1×10^{-5} M myosin, excitation was 295 nm, (- - - -) myosin alone, (·—·—·) myosin plus 1×10^{-5} M 8-anilino-1-naphthalenesulfonic acid, (———) myosin plus 3×10^{-5} M 8-anilino-1-naphthalenesulfonic acid, and (·····) myosin plus 6×10^{-5} M 8-anilino-1-naphthalenesulfonic acid.

$$\frac{F_{280}}{F_{380}} = \left(\frac{A_{380}}{A_{280}}\right)\left(\frac{1 - 10^{-A_{280}}}{1 - 10^{-A_{380}}}\right)\left(\frac{A_{280}^{d} + EA_{280}^{m}}{A_{380}^{d}}\right) \quad (3)$$

where F_{280} and F_{380} are the fluorescence observed at 280 and 380 nm, A_{280} and A_{380} are the total absorbance at

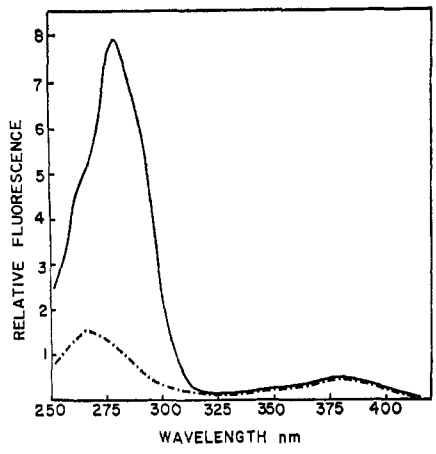

FIGURE 3: Excitation spectrum of native myosin–8-anilino-1-naphthalenesulfonic acid. 5×10^{-7} M myosin, 2×10^{-5} M 8-anilino-1-naphthalenesulfonic acid, 20°. Upper curve: excitation spectrum of 8-anilino-1-naphthalenesulfonic acid in myosin–8-anilino-1-naphthalenesulfonic acid complex. Lower curve: absorption curve of bound 8-anilino-1-naphthalenesulfonic acid in this system; the region of 380 nm has been normalized to coincide with the excitation spectrum in this region.

280 and 380 nm, and the superscripts d and m refer to the dye and myosin. If the linear approximation for 10^{-A} is valid, eq 3 reduces to

$$\frac{F_{280}}{F_{380}} = \frac{A_{280}^{d} + EA_{280}^{m}}{A_{380}^{d}}$$

Equation 3 provides an estimate of the efficiency of transfer. For native myosin, E is 13.5%.

Modification by p-Mercuribenzoate. Duke *et al.* (1966) observed that titration of SH in myosin with *p*-mercuribenzoate resulted in a small increase in the fluorescence up to about 80% of the titration. Further titration led to an abrupt increase to a much higher value when the enzyme becomes inhibited. Excitation spectra of myosin–8-anilino-1-naphthalenesulfonic acid were obtained with myosin samples which had been modified by *p*-mercuribenzoate to various extents. Figure 4 shows the excitation spectrum of a sample in which 40 SH groups had been titrated and the enzyme was completely deactivated. A comparison of Figure 4 with Figure 3 immediately reveals that the transfer of excitation energy in the modified enzyme is less than that in the native enzyme. In Table I are listed the values of transfer efficiency for native and *p*-mercuribenzoate-modified myosin, and for myosin modified by other agents.

The emission maximum undergoes a small blue shift when SH groups are titrated. With 16 moles of *p*-mercuribenzoate/mole of myosin, the λ_{em} is 465 nm, and with 40 moles of *p*-mercuribenzoate, λ_{em} is 463 nm.

Modifications by Other Agents. The other modifiers studied were EDTA, Cd^{2+}, and Zn^{2+}. At levels of these agents which are known definitely to affect the enzymatic activity of myosin, the fluorescence when excited at 380 nm was enhanced. The extent of enhancement was small when Zn^{2+} and EDTA were the modifiers. These results are shown in Figure 5. Transfer of excitation energy was also measured in the presence of these modifiers; the efficiency in each case is included in Table I.

TABLE I: Efficiency of Excitation Energy Transfer from Myosin Chromophores to Adsorbed 8-Anilino-1-naphthalenesulfonate.[a]

Modifier	% Transfer
None (native)	13.5
2×10^{-4} M EDTA	11.5
2×10^{-4} M Zn^{2+}	9.1
2×10^{-4} M Cd^{2+}	5.3
16 moles of *p*-mercuribenzoate	9.5
25 moles of *p*-mercuribenzoate	9.2
40 moles of *p*-mercuribenzoate	3.2

[a] The amounts of *p*-mercuribenzoate refer to the amounts per mole of myosin.

Discussion

Bound 8-anilino-1-naphthalenesulfonic acid differs from free 8-anilino-1-naphthalenesulfonic acid in water in two aspects. One is the enormous enhancement of the quantum yield, q, in the bound dye, and the other is the blue shift of the emission maximum, λ_{em}, from 514 nm in water to 468 when bound to myosin. Similar changes have been observed when 8-anilino-1-naphthalenesulfonic acid is bound to bovine serum albumin (Daniel and Weber, 1966; Weber and Young, 1964): λ_{em} = 469 nm, q = 0.75; apomyoglobin (Stryer, 1965): λ_{em} = 454 nm, q = 0.98; and apohemoglobin (Stryer, 1965): λ_{em} = 457 nm, q = 0.92. Spectral changes in the same direction have also been demonstrated when free 8-anilino-1-naphthalenesulfonic acid is transferred from water to less polar media (Stryer, 1965): ethylene glycol (λ_{em} = 484 nm, q = 0.15), ethanol (λ_{em} = 468 nm, q = 0.37), and *n*-octyl alcohol (λ_{em} = 464 nm, q = 0.63). The dependence of the emission maximum upon the polarity of environment is a consequence of the dipolar interaction of the excited state of the fluorescent species with solvent molecules. The excited-state energy is lowered as a result of the dipolar interaction with polar solvent molecules. Thus the emission in polar solvents is expected to be of higher wavelength than in nonpolar media. The quantum yield in general increases with decreasing polarity of the environment, although other deactivating processes may also be important in determining the over-all yield. The quantum yield of 0.48 and the λ_{em} at 468 nm indicate that the 8-anilino-1-naphthalenesulfonic acid binding site on myosin is quite nonpolar, and therefore that 8-anilino-1-naphthalenesulfonic acid probes the hydrophobic regions of the enzyme. The estimated values of $\Delta H°$ (+2.28 kcal mole^{-1})

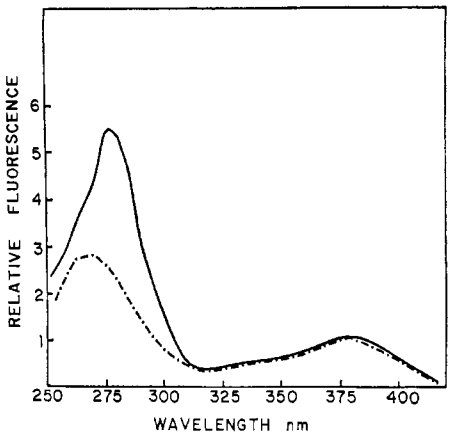

FIGURE 4: Excitation spectrum of 8-anilino-1-naphthalenesulfonic acid bound to *p*-mercuribenzoate-modified myosin. 5×10^{-7} M myosin, 2×10^{-5} M 8-anilino-1-naphthalenesulfonic acid, 20°. *p*-Mercuribenzoate concentration was 40 moles/mole of myosin. Upper curve: excitation spectrum. Lower curve: absorption spectrum of bound 8-anilino-1-naphthalenesulfonic acid in this system.

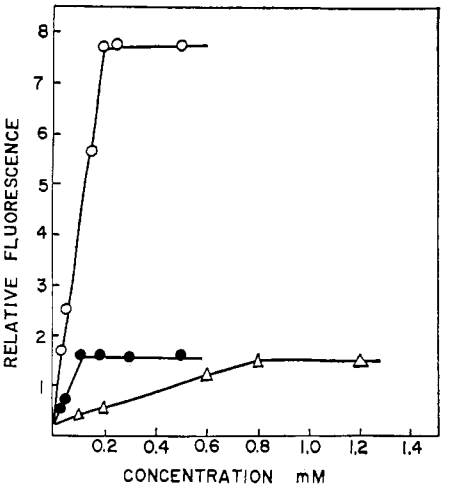

FIGURE 5: Fluorescence of 8-anilino-1-naphthalenesulfonic acid bound to myosin in the presence of Cd^{2+}, Zn^{2+}, and EDTA. 1×10^{-5} M myosin, 2×10^{-5} M 8-anilino-1-naphthalenesulfonic acid, 20°. Excitation wavelength was 380 nm, and emission was observed at 470 nm. (○) Cd^{2+}, (●) Zn^{2+}, and (△) EDTA.

and $\Delta S°$ (+26 eu) are subject to some uncertainty since the values of K are obtained when small changes are still occurring in the fluorescence efficiency of the solutions. Nevertheless, they are consistent with what is expected for hydrophobic interaction.

We have found that 8-anilino-1-naphthalenesulfonic acid does not appear to bind to light meromyosin, whereas it does bind to heavy meromyosin with an enhancement between 30 and 50% of that of myosin. From these observations the binding site on myosin appears to be located on the head of the molecule. A comparison of the measured excited-state lifetime and the lifetime calculated from the quantum yield affords an indirect test on the validity of the assumptions of eq 1. The quantum yield, 0.48, was determined through the use of the dissociation constant from eq 1 in order to obtain an estimate of the actual absorbance of the bound dye which was responsible for the fluorescence emission. The quantum yield q is related to the intrinsic lifetime τ_0 of 8-anilino-1-naphthalenesulfonic acid and the actual lifetime τ of 8-anilino-1-naphthalenesulfonic acid bound to myosin by $\tau = q \times \tau_0$. The value of τ_0 is close to 22 nsec (Hundley *et al.*, 1967). From this τ is estimated to be 10.6 nsec. This value compares favorably with the value 10.1 nsec directly measured with a nanosecond apparatus. This agreement may be regarded as an indirect confirmation of the validity of the linear relationship of eq 1.

When two different chromophores are in close proximity, the electronic excitation energy of one may be transferred to the other if certain basic spectral requirements are met. The requirements are: (1) the upper electronic energy levels of the donor must be higher than those of the acceptor, and (2) the two sets of upper energy levels must be partially overlapped. Such spectral properties are present in the myosin–8-anilino-1-naphthalenesulfonic acid complex, with the myosin molecule as a potential donor and the bound 8-anilino-1-naphthalenesulfonic acid as the acceptor. Such a transfer as demonstrated in Figures 2 and 3 suggests that the 8-anilino-1-naphthalenesulfonic acid site is very close to some tryptophan residues. If the transfer efficiency obeys Förster's theory (Förster, 1959), it is proportional to an inverse sixth power of the separation between the donor and acceptor, and dependent upon the mutual orientation of the two chromophores involved. The tryptophan residues involved probably are on the head of the myosin molecule, where the 8-anilino-1-naphthalenesulfonic acid site is located, since the distance over which transfer can take place cannot be much larger than 50 Å. The low transfer efficiency of 13.5% may seem somewhat surprising since there are some 20 tryptophan residues/myosin molecule. Based on the fact that 57% of the myosin molecule is the heavy meromyosin portion (Komiz *et al.*, 1962) and the assumption that only the tryptophan residues on the head are within transfer distance, the observed efficiency could be adjusted to 24%. It is possible to estimate the distance, R_0, at which the efficiency is 50%. However, such a value of R_0 would not provide any useful information on the distance between the donor and the acceptor because there are over ten tryptophan residues in the region of the 8-anilino-1-naphthalenesulfonic acid site. Since the excited-state lifetime of tryptophan in myosin is not available, the estimate of 24% as a lower bound for the transfer efficiency remains uncertain.

The transfer efficiency is sensitive to small perturbations of the enzyme conformation. This is so because of the inverse sixth power relationship and the orientation dependence. Each of the modified myosin samples shows a smaller transfer efficiency than the native enzyme. Under modifier conditions which are known to be activating, such as 2×10^{-4} M Zn^{2+}, 2×10^{-4} M Cd^{2+}, 2×10^{-4} M EDTA, 16 moles of *p*-mercuriben-

zoate, and 25 moles of *p*-mercuribenzoate/mole of myosin, the decrease is not very large. But for an inhibited enzyme (40 moles of *p*-mercuribenzoate), the decrease is large, down to only 3.2%. Regardless of whether the enxyme is activated or inhibited, the conformational changes as seen by 8-anilino-1-naphthalenesulfonic acid are always in the direction of decreasing transfer efficiency. While there is no observable change in the emission maximum when myosin is modified by EDTA, Zn^{2+}, or Cd^{2+}, there is a small blue shift when SH groups are titrated. This shift suggests that the new environment of 8-anilino-1-naphthalenesulfonic acid is less polar than in the native enzyme. Each of these results shows that enzymatic modification of myosin is accompanied by a deformation. The changes observed here do not necessarily involve the active site but concomitant with them are changes in catalytic efficiency. They do not appear to involve a gross change in structure since optical rotatory dispersion measurements do not show a significant change (Tonomura *et al.*, 1963). If the perturbations reported here could be regarded as a reflection of changes in the region of active centers, they would suggest that small changes in conformation result in activation and additional changes bring about deactivation.

Acknowledgments

We are especially grateful to Professor G. Weber for much valuable discussion, and for several constructive criticisms of this paper. We are equally indebted to Professor L. Stryer, for invaluable advice on the construction of our apparatus and for assisting us with the measurement of excited-state lifetime.

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