Haberkorn, R. A., Griffin, R. G., Meadows, M. D., & Oldfield, E. (1977) *J. Am. Chem. Soc. 99*, 7353.
Hinz, H. J., & Sturtevant, J. M. (1972) *J. Biol. Chem. 247*, 3697.
Hitchcock, P. B., Mason, R., Thomas, K. M., & Shipley, G. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3036.
Jacobs, R., & Oldfield, E. (1979) *Biochemistry 18*, 3280.
Ladbrooke, B. D., Williams, R. M., & Chapman, D. (1968) *Biochim. Biophys. Acta 150*, 333.
Lee, A. G. (1975) *Biochim. Biophys. Acta 413*, 11.
Lee, A. G. (1978) *Biochim. Biophys. Acta 507*, 433.
Mabrey, S., & Sturtevant, J. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3862.
Mabrey, S., Mateo, P. L., & Sturtevant, J. M. (1978) *Biochemistry 17*, 2464.
Owicki, J. C., & McConnell, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4750.
Phillips, M. C., & Finer, E. G. (1974) *Biochim. Biophys. Acta 356*, 199.
Privalov, P., Plotnikov, V. V., & Filimonov, V. V. (1975) *J. Chem. Thermodyn. 7*, 41.
Rouser, G., Nelson, G. J., Fleischer, S., & Simon, G. (1968) in *Biological Membranes* (Chapman, D., Ed.) pp 5–69, Academic Press, London.
Rubinstein, J. L. R., Smith, B. A., & McConnell, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 15.
Shimshick, E. J., & McConnell, H. M. (1973a) *Biochem. Biophys. Res. Commun. 53*, 446.
Shimshick, E. J., & McConnell, H. M. (1973b) *Biochemistry 12*, 2351.
Tsong, T. Y. (1975) *Biochemistry 14*, 5415.
van Dijck, P. W. M. (1979) *Biochim. Biophys. Acta 555*, 89.
van Dijck, P. W. M., de Kruijff, B., van Deenen, L. L. M., de Gier, J., & Demel, R. A. (1976a) *Biochim. Biophys. Acta 455*, 576.
van Dijck, P. W. M., van Zoelen, E. J. J., Seldenrijk, R., van Deenen, L. L. M., & de Gier, J. (1976b) *Chem. Phys. Lipids 17*, 336.
Verkleij, A. J., Ververgaert, P. H., de Kruijff, B., & van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 373*, 495.
von Dreele, P. H. (1978) *Biochemistry 17*, 3939.

Binding of Heavy Meromyosin and Subfragment-1 to Thin Filaments in Myofibrils and Single Muscle Fibers†

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ABSTRACT: The binding of fluorescently labeled heavy meromyosin (HMM) and heavy meromyosin subfragment-1 (S-1) to thin filaments of myofibrils and of rabbit psoas muscle fibers was measured under conditions of rigor and contraction. The fragments diffused rapidly into the myofibrillar space and bound specifically to the thin filaments. The fragments bound strongest and in a uniform fashion to myofibrils in which the competition from indigenous myosin was abolished by removing it with Hasselbach–Schneider solution. Under these conditions, the rigor K_a values for HMM and S-1 were 1.5×10^6 M^{-1} and 4.8×10^4 M^{-1}, respectively. The stoichiometry of binding was measured by independently estimating the concentration of actin sites. S-1 was found to be capable of saturating all available actin sites in a myofibril or a fiber, but HMM could only occupy 50% of the sites.

The study of the binding of myosin fragments to actin has attracted attention for two important reasons. First, association of the myosin–product complex with actin constitutes one of the intermediate steps in the actomyosin ATPase cycle. In addition, binding studied yield information about the interaction between the two heads of the myosin molecule and about the role such interactions (if any) play in the contractile process (Taylor, 1977; Tonomura & Inoue, 1977). To date, the results of these investigations have suggested the values for the rigor binding to actin of the single-headed fragment of myosin (S-1)[1] (Margossian & Lowey, 1975, 1976; Marston & Weber, 1975; Highsmith et al., 1976) and of the double-headed fragment HMM (Takeuchi & Tonomura, 1971; Eisenberg et al., 1972; Margossian & Lowey, 1973; Highsmith, 1978) but were unable to determine unambiguously whether the interactions between the heads occur (Highsmith, 1978; Greene & Eisenberg, 1980). All these studies have been carried out in solution, taking advantage of the fact that myosin fragments are soluble at low ionic strength. In muscle, however, actin filaments are arranged in a well-defined spatial configuration with fixed interfilament distances, where steric effects may be important in the binding of myosin heads. Further, in vitro experiments uniformly utilized purified actin preparations, and it is not at all clear what effect the presence of the regulatory proteins tropomyosin and troponin might have on the affinity of a cross bridge to actin. Tropomyosin makes actomyosin interactions cooperative (Bremel et al., 1972) presumably through propagated conformational change in the actin molecules (Weber & Murray, 1973), and it is conceivable

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[1] Abbreviations used: HMM, heavy meromyosin; S-1, heavy meromyosin subfragment-1; EGTA, 2,2′-ethylenedioxybis(ethyliminodiacetic acid); IAF, 5-iodoacetamidofluorescein; 1,5-IAEDANS, *N*-(iodoacetyl)-*N*′-(1-sulfo-5-naphthyl)ethylenediamine; $NaDodSO_4$, sodium dodecyl sulfate; H-S, Hasselbach–Schneider solution; P_i, inorganic phosphate.

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that such changes, in turn, affect myosin binding. For these reasons we have attempted to investigate the binding of myosin fragments to actin under conditions resembling as closely as possible those of the in vivo binding of myosin cross bridges.

The preparations used in these experiments were isolated myofibrils from rabbit psoas muscle. Myofibrils are well suited to this kind of experiment because they are readily and rapidly penetrated by molecules as large as myosin. To estimate binding during contraction, we have used single glycerol-extracted rabbit psoas muscle fibers from which the sarcolemma has been removed by mechanical skinning. This is because fibers can be held isometrically throughout the contraction. Because actin is immobilized in the myofibrillar matrix and is at the same time easily accessible to solutes such as myosin fragment, the binding can be conveniently assessed by the method of equilibrium dialysis (Marston & Tregear, 1972; Marston, 1973). In the present work, the equilibrium binding of HMM and S-1 to the myofibrils and fibers under conditions of rigor and contraction has been investigated by recording the differences in the fluorescence intensity between the preparation loaded with the fluorescently labeled fragments and the free fragment in the surrounding.

Experimental Procedures

Myofibrils and Muscle Fibers. Thin strips of rabbit psoas muscle have been glycerinated for a period of between 2 weeks and 3 months in a solution containing 80 mM KCl, 2 mM EGTA, 2.5 mM ATP, 5 mM $MgCl_2$, 5 mM sodium phosphate buffer, pH 7.0, and 50% glycerol. To prepare myofibrils, we first equilibrated a strip of muscle fibers for 0.5 h at 0 °C with the above solution but lacking ATP and glycerol (i.e., rigor solution). Rigor muscle (2–4 g) was then homogenized in a Sorvall Omni-Mixer homogenizer with 10 mL of rigor solution at the No. 10 setting for 60 s in ice. The myofibrils were filtered through gauze, resuspended in rigor solution, and used no later than 2 days after preparation. To prepare single fibers, we first equilibrated glycerinated muscle with the extracting solution but containing 15% rather than 50% glycerol. Single fiber was dissected and the sarcolemma was removed as previously described (Borejdo & Oplatka, 1976). The fiber was laid on a microscope slide at rest length, and the two ends were stuck to the glass with a drop of a Super Glue 3 (Woodhill Permatex, Cleveland, OH). The central segment of the fiber (not including the glued ends) was covered by a cut strip of a cover glass. Myofibrils and isolated fibers were perfused by supplying a drop of an appropriate solution at one edge of the cover slip and sucking it with the piece of a blotting paper at the other end.

Solutions. The solutions contained the following: rigor, 80 mM KCl, 2 mM EGTA, 5 mM $MgCl_2$, and 5 mM sodium phosphate buffer, pH 7.0; relaxing, as above plus 2.5 mM ATP; contracting, the same as relaxing except that EGTA was replaced by 0.1 mM $CaCl_2$. Myosin was extracted from myofibrils and fibers by the application of the modified Hasselbach–Schneider (H–S) solution: 0.5 M KCl, 0.1 M phosphate buffer, pH 6.5, 10 mM pyrophosphate, and 2 mM $MgCl_2$. H–S solution was prepared freshly before each experiment. Protein irrigating solutions had rigor or relaxing composition with varying HMM or S-1 concentrations added as required.

Proteins. The procedures described by Lowey & Cohen (1962) and by Lowey et al. (1969) were followed for the preparation of HMM and S-1, respectively. They were further purified and concentrated by ammonium sulfate fractional precipitation. The purity was checked by sodium dodecyl sulfate slab gel electrophoresis, wherein myosin impurity in all preparations was found to be less than 1%. The fragments were labeled with 5-iodoacetamidofluorescein (IAF) (Molecular Probes, Plano, TX) which is particularly well suited for this kind of study because it combines the intense fluorescence of the fluorescein chromophore (quantum yield $Q = 0.8$; extinction coefficient $\epsilon = 2.9 \times 10^{-16}$ cm^2) and a sulfhydryl selectivity of the iodoacetyl functional group. Labeling was carried out by adding a 2 molar excess of dye to HMM and 1 mol of dye/mol of S-1; the reaction proceeded for 24 h at 0 °C in the dark and was followed by an extensive dialysis against rigor solution. Ca^{2+}-activated ATPase activity of the labeled HMM was 0.85 μmol of P_i (mg·min)$^{-1}$. A 5 molar excess of actin over HMM activated Mg^{2+}-ATPase of native and labeled HMM to 0.08 and 0.06 μmol of P_i (mg min)$^{-1}$, respectively.

Protein solutions were stored at concentrations larger than 20 μM and were diluted immediately before experiments. ATP was added to relaxing or contracting solutions containing HMM or S-1 before use. All measurements were made at room temperature (20–23 °C).

Fluorescence measurements were performed under the Zeiss Axiomat microscope equipped with a 100-W mercury superpressure lamp source under incident light illumination. An earlier part of this work was done under a Zeiss Universal microscope. Zeiss exciter filters BC 38 and BG 12 were used in combination with a dichroic mirror selected to reflect all wavelengths below 510 nm. Zeiss barrier filter 44 and 50 further blocked light at the excitation wavelength. Planapochromat 100 × 1.3 NA objective collected fluorescent light and projected it into the circular measuring diaphragm which had a diameter equivalent to 6.4 μm in the object plane. The light which passed through the diaphragm was incident on to the photocathode of an RCA 931A photomultiplier operating at −1200 V. The anode current was passed through a 1-kΩ resistor, and voltage across it was converted into a 16-bit binary number and fed into an Apple II microcomputer. Although capable of collecting 5×10^4 samples/s, the computer read the fluorescence only 10 times/s. The voltage could also be read directly across the anode resistor with the voltmeter.

The binding of labeled myosin fragments was measured after a 4-min incubation of the myofibrils or the fiber in 0.2 mL of an appropriate solution containing the fragment. In the case of myofibrils the rate of the fluorescence increase after the addition of labeled fragments was immeasurably fast, while in the case of the fiber it was such that after 60 s no further increase could be observed. The measurement of the myofibrillar or fiber fluorescence was followed by the measurement of the fluorescence of the surrounding medium in the vicinity of the sample. Figure 1 shows myofibrils of representative size at sarcomere lengths of 2.6–2.8 μm after irrigation with HMM and illustrates the relative sizes of the myofibril and the measuring diaphragm. The results are, of course, independent of the size of the diaphragm because the same diaphragm is used for measuring the fluorescence of the sample and of the surroundings. The results are also independent of the thickness of the irrigating solution and of the muscle fiber because these are much greater than the depth of focus of the objective. With the Planopochromat objective (100×, NA = 1.3) and the wavelength of the fluorescent light $\lambda = 520$ nm, the depth of focus is 0.6 μm. This is of the order of the thickness of the myofibril, however, and so an effort was made to choose the myofibrils of the same diameter (and, therefore, presumably, thickness). The readings were taken within the shortest time possible (typically 2 s) to minimize the exposure of the sample to the exciting light. The shutter admitting the fluorescent

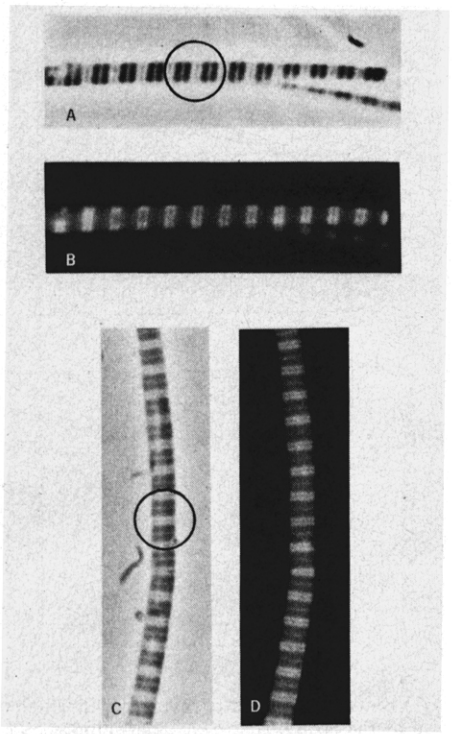

FIGURE 1: Photomicrographs of a myofibril loaded with the fluorescently labeled HMM (6.3 mg/mL) showing the relative size of the measuring diaphragm. In this case, the HMM in the surrounding medium has been washed out to enhance the contrast. (A and B) Normal myofibril; sarcomere length = 2.8 μM. (C and D) Myofibril showing HMM binding to the center of the sarcomere from which data was not taken; sarcomere length = 2.6 μm. (A and C) Phase contrast. (B and D) Fluorescence. Magnification: 1285×.

light was opened, and at the same time the computer began the data collection. Twenty readings were taken during the time the shutter was open. Invariably the first reading was the greatest, and the subsequent readings declined at the rate consistent with the quantum yield for the photobleaching of fluoresceine of 0.5×10^{-5} (Borejdo, 1979) (i.e., the half-time for the decay of fluorescence was ~30 s). The biggest reading was taken as that corresponding to sample fluorescence. The fluorescence of the surroundings showed slower bleaching, most likely because degraded fluorophores were rapidly exchanged through diffusion with the surrounding free molecules. The differences between the sample and medium fluorescence is referred to as the excess fluorescence and is proportional to the concentration of bound myosin fragment.

Data Analysis. The data for myosin fragment binding as a function of free fragment concentration were analyzed to give a statistical best fit to the simple binding curve. For S-1 this approach is obviously justified, but in the case of HMM which binds to two actin sites the situation is complicated by the fact that the number of adjacent actin sites available for binding decreases fast when the HMM/actin ratio approaches 1/2. Peller (1975) and Hill (1978) have dealt with this problem theoretically, and Greene & Eisenberg (1980) have applied Hill's formulas to their data. However, it can be shown [e.g., Greene & Eisenberg (1980)] that when the HMM/actin ratio is low, i.e., actin filaments are far from being saturated with HMM, the simple binding curve adequately describes the situation. In the present work the HMM/actin ratio was always low (see below), and consequently the data could be satisfactorily fit to the simple binding equation. We show, in addition, that in the HMM concentration range used a fit to a more complex isotherm gives essentially the same result (cf. Figures 4 and 5).

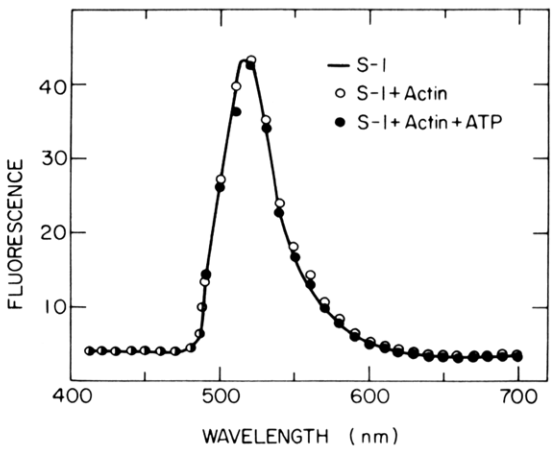

FIGURE 2: Comparison of emission spectra of IAF-labeled S-1 and of a labeled S-1–actin complex. (—) 6 μM S-1 in rigor solution; (O) 6 μM S-1 and 23 μM actin in rigor solution; (●) 6 μM S-1 and 23 μM actin in relaxing solution. The excitation wavelength was 490 nm. Vertical units are arbitrary.

Let n_f and n_s, respectively, denote the number of fluorescently labeled fragments in the illuminated volume of the sample and in the surroundings. If ϕ denotes the fraction of myosin fragments bound at any given concentration, then

$$\phi = (n_f - n_s)/n_{max} = K_a C/(1 + K_a C) \quad (1)$$

where n_{max} is the maximal number of bound molecules, K_a is the constant, and C is the concentration of free ligand (myosin fragment). Because the total protein concentration in the sample was low (see below), excluded volume effects were neglected in the analysis, i.e., the concentration of free HMM in the myofibrillar space and outside were assumed equal. If the total illuminating light flux Φ is not too great, the power of the fluorescent radiation is proportional to the number of molecules n of the single fluorescent solute in the illuminated volume. The photomultiplier current which is used to detect the fluorescence intensity is then

$$i = g\epsilon Q\Phi n \quad (2)$$

where g is the overall collection efficiency of the microscope–photomultiplier system, ϵ is the extinction coefficient of the fluorophore, and Q is its quantum yield. Because neither ϵ nor Q change upon the binding of labeled fragments to actin (see below), the observed photocurrent increment between the sample and the surroundings is

$$\Delta i = i_f - i_s = g\epsilon Q\Phi(n_f - n_s) = g\epsilon Q\Phi n_b \quad (3a)$$

where

$$n_b = n_f - n_s \quad (3b)$$

and therefore $n_b = \Delta i/(g\epsilon Q\Phi)$, $n_{max} = i_{max}/(g\epsilon Q\Phi)$, and

$$\phi = n_b/n_{max} = \Delta i/i_{max} \quad (4)$$

$$\Delta i = i_{max} K_a C/(1 + K_a C) \quad (5)$$

Similarly

$$\Delta i/C = K_a i_{max} - K_a \Delta i \quad (6)$$

in the Scatchard form.

That the quantum yield and the extinction coefficient of the bound fragment do not differ from those of the free fragment was checked by comparing the emission spectra of free and bound S-1. Figure 2 shows that the spectra are identical and that the addition of Mg–ATP also has no effect on the spectrum.

The data were fit to eq 5 by the two-parameter fit in which all possible combinations of K_a and i_{max} were examined by the

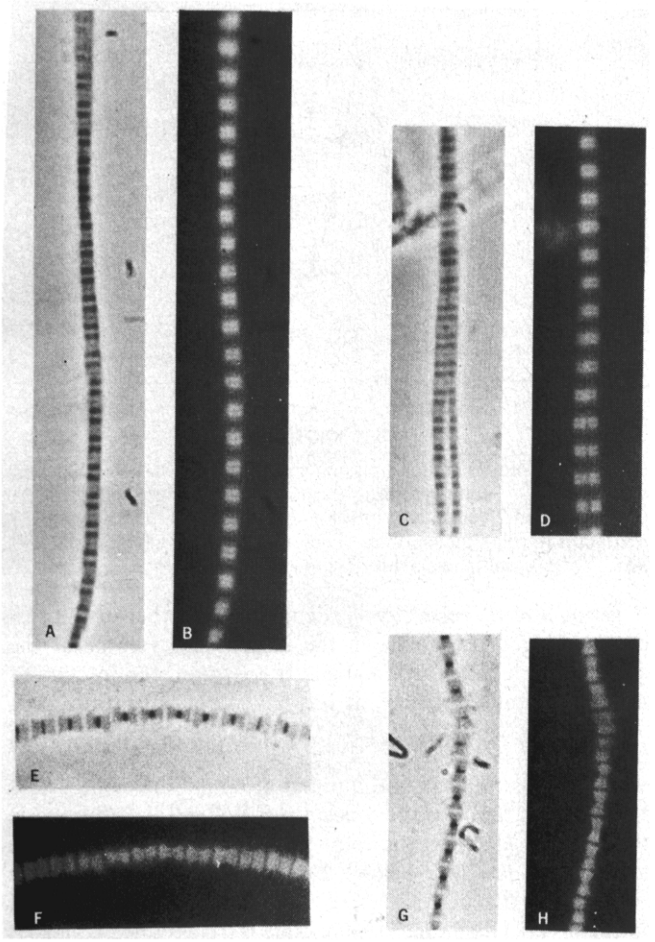

FIGURE 3: Photomicrographs at myofibrils irrigated with fluorescent HMM. (A–D) Native myofibril under phase contrast (A and C) and fluorescent (B and D) illumination. (E–H) Myofibril from which myosin has been extracted by a 2-min treatment with the Hasselbach–Schneider solution. (E–G) Phase contrast. (F–H) Fluorescence. Magnification: 1375×.

computer, the one giving the smallest root mean square (rms) deviation being chosen. The data were analyzed by the Weizmann Institute's IBM 370/165 computer.

Results

Binding of Heavy Meromyosin to Myofibrils. Myofibrils offer little resistance to the diffusional access of the solutes of the incubating medium. Within a few seconds after the application of fluorescent HMM or S-1, little further increase in the myofibril fluorescent could be observed.

Figure 3A–D shows examples of the phase contrast and fluorescence images of intact myofibrils irrigated with HMM. In Figure 3B,D it can be seen that HMM binds preferentially to the I bands and in particular that the A bands remain almost completely dark. It therefore appears that HMM cannot bind to the overlap zone, being prevented by the occupancy of the actin sites by the indigenous myosin. [Another possibility is that it successfully competes with myosin and does bind in the overlap zone but myosin somehow quenches the fluorescence. HMM certainly binds in the overlap zone at high ionic strength because we have observed that irrigation of the myofibril with HMM at 0.6 M KCl results in the formation of "ghosts" containing no A band. This effect was first observed by Szentkiralyi (1961).] It should be pointed out that we have also seen myofibrils in which HMM is bound to the center of the sarcomere. One such example is illustrated in Figure 1D. It is not clear why such binding should occur in myofibrils

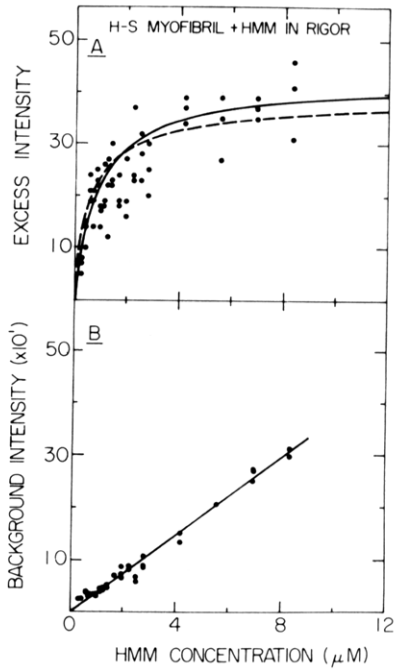

FIGURE 4: (A) Binding of HMM to myofibrils in which myosin has been removed by the Hasselbach–Schneider solution. The solid line shows the least-squares fit to the simple binding curve (eq 5). The broken line is the fit of the data to the equation derived by Peller (1975) and Hill (1978) for the case when HMM binds with both heads only. K_a and i_{max} derived from this kind of fit were not significantly different from those reported in Table I. (B) The relationship between HMM concentration and fluorescence intensity.

which had a H zone, i.e., the ends of actin filaments did not meet in the center. We have been careful to study binding only in myofibrils which did not show any such fluorescence in the center of the sarcomere.

In view of the above, it appears that the simplest way to circumvent difficulties associated with competition from myosin and nonuniform binding is to remove myosin by treating the myofibrils with the Hasselbach–Schneider solution. The myofibrils were irrigated with the H–S solution for a few minutes and then washed thoroughly with the rigor solution. Figure 3E–H shows the phase contrast and fluorescence images of myofibrils from which myosin has been removed and which have been irrigated with HMM. It is clear that HMM binds over the whole length of the fibril with the exception of the narrow zone in the center of the sarcomere corresponding to the original H zone and with the exception of the Z lines.

The binding of HMM to such preparations was measured at fragment concentration from 0.27 to 11 μM. Above 11 μM HMM the accuracy of the measurement (~10%) was not sufficient to resolve the increase in fluorescence above the background (free HMM) fluorescence. Indeed, the fluorescence of the surroundings is related to the ligand concentration C by $i_s = \alpha C + D$ (cf. Figure 4B). (D may not be zero due to the blank fluorescence of the glass in the experimental cell and the fluorescence of the microscope lenses.) If the accuracy of the photocurrent measurement is A, then the maximal free ligand concentration L_{max} at which the increase can still be resolved over the background fluorescence can be shown to be

$$2L_{max} = \frac{i_{max}}{A\alpha} - \frac{1}{K_a} - \frac{D}{\alpha} - \frac{1}{K_a\alpha}[(\alpha + K_aD - i_{max}K_a/A)^2 - 4K_a\alpha D]^{1/2} \quad (7)$$

For example, for HMM $\alpha = 36 \times 10^6\ \mu A/M$, $D = 0\ \mu A$ (cf.

Table I: Binding of HMM to Myofibrils and Fibers[a]

state	condition[b]	$K_a \times 10^{-5}$ (M^{-1})	C_{max}[c] (μM)	S (mol of HMM/mol of actin)	N (no. of assays)
rigor	H-S myofibrils	14.9 ± 5.2[d]	16.2 ± 3.3	0.22	6
rigor	native myofibrils	7.9 ± 3.5	42.6 ± 17.6[e]	0.18	4
rigor	H-S fibers	3.8 ± 0.2	28.8 ± 12.0	0.25	8
rigor	native fibers	2.2 ± 0.5	46.8 ± 14.4[e]	0.09	5
contract	H-S fibers	0.16 ± 0.16			6

[a] At 20-23 °C and 80 mM KCl; for composition of rigor, relaxing, and contracting solutions see Experimental Procedures. [b] Native refers to intact myofibrils and to single-skinned glycerinated muscle fiber of rabbit psoas. H-S denotes the same preparations but after extraction of myosin by the modified Hasselbach-Schneider solution. [c] Calculated by using eq 8. [d] Mean ± SD of N assays (see column 6). [e] Taking into account that only half of the myofibril is fluorescent.

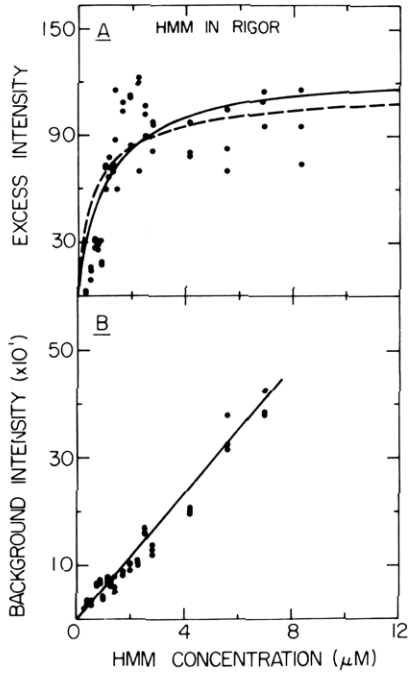

FIGURE 5: (A) Binding of HMM to native myofibrils. The solid line shows the least-squares fit of the data to eq 5. The dashed line shows an alternative fit to the binding data as described in the legend to Figure 4. (B) The relationship between free HMM concentration and fluorescence intensity.

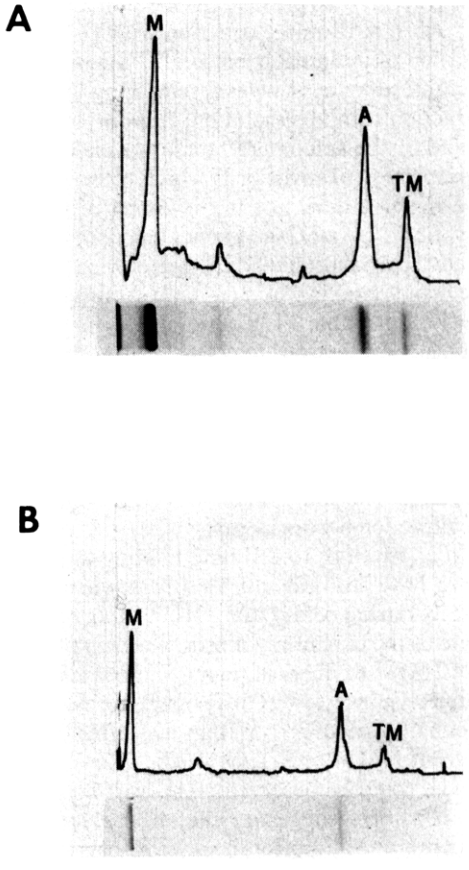

FIGURE 6: Gel pattern and densitometric scan of $NaDodSO_4$-solubilized preparations, 10% polyacrylamide, 0.1% $NaDodSO_4$, and 0.2 M Tris-glycine buffer, pH 8.5. (A) Native preparations, 20 μg of applied protein. (B) H-S-extracted preparations, 30 μg of applied protein.

Figure 4B), $i_{max} = 44\ \mu A$, and $A \approx 10\%$, then $L_{max} = 23\ \mu M$. In practice, maximum concentration turned out to be always a little lower.

HMM binding to H-S-extracted myofibrils increased with the concentration of the fragment in an approximately hyperbolic manner (Figure 4A). The mean dissociation constant K_d of the bound HMM was 0.67 μM (Table I). The maximal amount bound C_{max} was calculated as

$$C_{max} = (\eta i_{max} - D)/\alpha \quad (8)$$

where η^{-1} is the (measured) fraction of the volume viewed by the microscope objective occupied by the myofibril. This expression is accurate as long as the geometrical collection efficiency of the objective does not vary over the field of view and the quantum yield and the extinction coefficient of the dye attached to HMM do not change upon ligand binding. Because the field diaphragm opening was small and because IAF fluorophore was used, (Figure 2) these conditions are met. The data obtained from several myofibrillar preparations are summarized in Table I.

Figure 5 shows the binding of HMM to native myofibrils. The fit to the hyperbolic curve is considerably poorer, the association constant is smaller, and the maximal occupancy of the actin sites is lower (Table I). In computing C_{max} for intact myofibrils, an account was taken of the fact that HMM was limited to the I bands (Figure 3A), i.e., that the fraction of the volume viewed by the microscope objective which was fluorescent was lower.

To compute the fraction of actin sites occupied by HMM, it is necessary to estimate the concentration of C_{actin} in the myofibrils. This was done by dissolving myofibrils of known concentration in 20% $NaDodSO_4$ and performing $NaDodSO_4$ slab gel electrophoresis of the solution. The gels were then scanned, and the actin (as well as myosin and tropomyosin) concentration in the sample was determined by comparing the size of the appropriate peak with known standards which had been electrophoresed at increasing concentrations. The ratio of the actin concentration in the sample to the total protein concentration gives the actin concentration in myofibrils. The

Table II: Binding of S-1 to Myofibrils and Muscle Fibers[a]

state	condition	$K_a \times 10^{-4}$ (M^{-1})	C_{max} (μM)	S (mol of S-1/mol of actin)	N (no. of assays)
rigor	H-S myofibrils	4.8 ± 0.8	65 ± 15	0.91	4
rigor	native myofibrils	4.0 ± 5.4	47 ± 11	0.20	5
rigor	H-S fibers	2.9 ± 1.2	116 ± 67	1.01	9
rigor	native fibers	3.0 ± 0.8	117	0.22	2

[a] For conditions see Table I.

same procedure was applied to determine the actin concentration in muscle fibers. Figure 6 shows the electrophoretic pattern of native and H–S-extracted fibers. In the native myofibrils, actin was present at the concentration of 9.8 mg/mL. After a 10-min extraction with H–S solution, the concentration of actin dropped to 2.97 mg/mL. There was a considerable amount of myosin remaining in the myofibrils after extraction with H–S solution. This myosin is located in the M band of the sarcomere: under phase contrast we see a large proportion of myofibrils which after extraction with H–S give rise to a dark line in the center of the sarcomere. These myofibrils are lacking H zones before myosin extraction and are obviously shortened during glycerination procedure. We have avoided taking binding data from such myofibrils.

The ratio S in Table I is a ratio of C_{max} to C_{actin} as determined above. The reasons why this ratio is so small are discussed later.

Binding of Heavy Meromyosin to Fibers. It has proven impossible to measure binding of HMM to myofibrils during relaxation or contraction. When myofibril "ghosts" are irrigated with HMM and relaxing or contracting solutions are added, contraction occurs as reported earlier (Oplatka et al., 1974). In an attempt to estimate binding under these conditions, we have utilized muscle fibers which can be held isometrically during contraction. To be able to meaningfully extrapolate to the case of myofibrils, we have first determined binding of HMM to fibers in rigor. Skinned single fibers also offer little resistance to the diffusional access of the solutes of the incubating medium. Within 60 s after the application of fluorescent HMM or S-1, no further increase in the fiber fluorescence could be observed. This was not true of intact (not skinned) fibers, suggesting that after glycerination alone the cell membrane still offers a physical barrier for the penetration of myosin fragments.

HMM binding to H–S-extracted fibers increased with the concentration of fragment in an approximately hyperbolic manner (Figure 7A). The data from several preparations are summarized in Table I. The quality of the data obtained from these studies allowed for the representation of the data in the Scatchard form (Figure 7B). Table I also summarizes the results of experiments on the binding of HMM to native fibers.

The stoichiometry of binding was estimated for fibers in the same manner as for myofibrils. In the native fibers, actin was present at the concentration of 22.5 mg/mL, while myosin and tropomysin were present at concentrations of 51 and 12.5 mg/mL, respectively. After a 10-min extraction with the H–S solution, the concentration of actin dropped to 4.8 mg/mL (myosin and tropomyosin to 9.2 and 1.6 mg/mL, respectively). Table I lists $S = C_{max}/C_{actin}$ under a variety of conditions.

It was possible to investigate binding of HMM to H–S-extracted fibers during contraction; as shown in Table I, K_a was decreased 23-fold. Under contracting conditions, the scatter of the experimental points was too large for the meaningful determination of C_{max}, while K_a could only be estimated with 100% accuracy. These effects were most likely

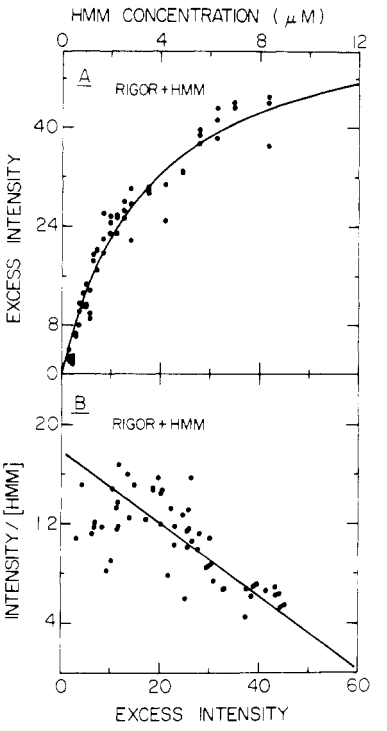

FIGURE 7: (A) Binding of HMM to muscle fiber in which myosin has been removed by the Hasselbach–Schneider solution. The line shows the least-squares fit to the simple binding curve (eq 5). (B) Scatchard plot of the HMM binding curve shown in (A). The data point corresponding to 0.4 μM HMM in (A) is not included in (B).

associated with the fact that the fibers irrigated with sufficiently concentrated HMM contracted even though myosin was absent (Borejdo & Oplatka, 1976). The contraction results in the collapse of the organized structure of the sarcomeres and hence in reduced permeability of fibers to solutes.

The results summarized in Table I suggest that HMM binds ~4 times weaker to extracted fibers than to extracted myofibrils in rigor. This may be due to the inability of the HMM to penetrate into the center of the fiber, and therefore the binding to myofibrils is taken as the true indicator of the in vivo binding. If this factor of 4 holds for contracted preparations as well, it would suggest the value of 0.64×10^5 M^{-1} for the in vivo binding constant of myosin heads to actin filaments during contraction.

Binding of S-1 to Myofibrils and Fibers. The practical concentration range from S-1, estimated on the basis of eq 7 and confirmed experimentally, was up to ~50 μM. Figure 8A shows the binding curve of S-1 to H–S-treated myofibrils. The curve was fitted to eq 5 in a manner described earlier. Table II summarizes several experiments done on both myofibrils and fibers. For myofibrils, K_a (Table II) is 20–30-fold smaller than that of HMM. The maximum amount of bound C_{max} was calculated by using eq 8 and estimating α and D from the relationship between free S-1 fluorescence and S-1 concentration such as that shown in Figure 8B. It is clear that

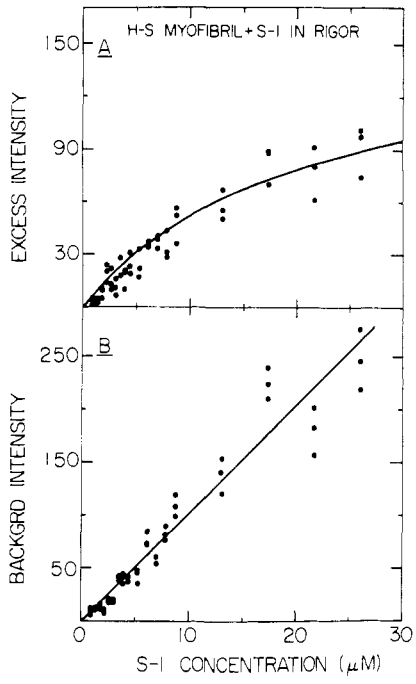

FIGURE 8: (A) Binding of S-1 myofibrils from which myosin has been removed. The line is the least-squares fit to the single binding curve. (B) The relationship between free S-1 concentration and fluorescence intensity.

in each case significantly more S-1 than HMM bound to the myofibrils.

Discussion

The principal advantage of our method of assessing binding of myosin fragments to actin is that the assay conditions closely resemble a situation prevailing in vivo. Actin is present in its native form at a concentration greater than practically possible in in vitro experiments, and it is arranged in a regular array which may, in itself, affect the binding characteristics. There is little doubt that the binding of myosin fragments is a specific one, i.e., represents actin–HMM or actin–S-1 formation. This has been shown earlier [e.g., Szentkiralyi (1961), Garamovölgyi et al. (1975), and Sanger (1975)] and is supported by our microscopic observation of myofibrils irrigated with the myosin fragments (cf. Figure 3).

The use of extrinsic protein fluorescence is an accurate and rapid method of assessing binding (Pesce et al., 1971). At the same time, the technique necessitates some precautions which must be brought into focus here. First, the insoluble myofibrillar network constitutes a barrier for the penetration of fragments. This necessitates the use of the myofibrils. Even the skinned muscle fibers which equilibrate rapidly and uniformly with the bathing medium give rise to the binding constant 4 times lower than that of myofibrils, suggesting that the diffusional barriers are not entirely removed by glycerination and mechanical skinning. Second, photodegradation of fluorescein is rapid and further enhanced by the fact that bound myosin fragments are immobilized in the myofibrillar space and the bleached products cannot be easily exchanged with the fresh fluorophores outside. (This is not true of the free myosin fragments outside of the myofibril which can rapidly exchange with unbleached fragments from outside of the experimental volume.) Thus, a given area of the preparation must be exposed to the illuminating light for the shortest possible time ($\leq$2 s), and the readings must be taken rapidly. The use of the on-line microcomputer is a convenient way of gathering this kind of rapidly decaying data. Finally, care must be taken that the labeling of the myosin fragment does not affect the binding itself. In fact, there are indications that the specific labeling of SH_1 groups of myosin or HMM results in the modification of the Michaelis–Menten V_{max} and K_d in the ATP-hydrolyzing system, most likely through the reduction in the rate-limiting transition from the refractory state to the nonrefractory state (Mulhern & Eisenberg, 1976, 1978). However, it is likely that labeling with IAF has little effect on rigor binding; modification of SH_1 by another iodoacetyl derivative *N*-(iodoacetyl)-*N'*-(1-sulfo-5-naphthyl)ethylenediamine (1,5-IAEDANS) was shown not to translate into a detectable difference between affinities of labeled and unlabeled S-1 (Highsmith et al., 1976). Similarly, blocking $-SH_1$ with iodoacetamide reduced actin–HMM and actin–S-1 association constants by only 25–45% (Greene & Eisenberg, 1980) and, further, maximum rigor tension developed by the fiber labeled with 1,5-IAEDANS and IAF was equal to that of the control native fibers (Borejdo & Putnam, 1977).

The results of the present work provide values for the affinity and stoichiometry of binding of HMM and S-1 to actin under conditions resembling those prevailing in vivo. At 24 °C and 80 mM KCl, pH 7.0, the association constant for HMM was 1.5×10^6 M^{-1}, while under the same conditions S-1 bound 30 times weaker. Further, while S-1 was able to saturate all actin binding sites in myofibrils, HMM could occupy less than 50% of the sites.

The affinity of HMM for actin in myofibrils measured here is a little lower than the values so far reported for in vitro experiments. Sedimentation methods yielded values of K_a varying between 4×10^7 M^{-1} [7 °C, 0.1 M KCl, pH 7.0; Margossian & Lowey (1973)] and 2×10^6 M^{-1} [4 °C, 20 mM KCl, pH 7.0; Eisenberg et al. (1972)], and recently as high as 3×10^9 M^{-1} [22 °C, 0.22 M KCl; Greene & Eisenberg (1980)]. Takeuchi & Tonomura (1971), using light-scattering methods, found $K_a = 4 \times 10^6$ M^{-1} (4 °C, 0.1 M KCl, pH 8.8), while Highsmith (1978), using time-resolved fluorescence depolarization methods, measured $K_a = 1.6 \times 10^7$ M^{-1} (4 °C, 0.15 M KCl, pH 7.0). It is impossible that the residual myosin remaining in the myofibrils after the application of the H–S solution decreases the HMM affinity by competing for actin sites, because our measurements were done on myofibrils in which no myosin was left. Further, we have discussed reasons for believing that the binding is little affected by the presence of the fluorophore. It thus appears that in vivo binding constant is slightly smaller than the in vitro constant. One reason for the observed difference may be the organization of actin into I bands in the sarcomere. This organization may lead to an enhancement of electrostatic or long-range interactions which normally average out in solution. Another possible reason may be the presence on actin filaments of the regulatory protein tropomyosin. Tropomyosin was shown to be present in myofibrils also after myosin extraction (Figure 6). Tropomyosin is known to impart the potentiating effect on actin filaments at high S-1 concentrations (Bremel et al., 1972; Weber & Murray, 1973). Even though Bremel et al. (1972) found no differences in the cofactor activity of regulated and unregulated actin, when actin was present in large excess over S-1, it is possible that equilibrium binding of S-1 at low S-1/actin ratios is affected by the presence of tropomyosin and/or Ca^{2+}.

The affinity constant during contraction is reduced about 20-fold in comparison with rigor. It is interesting to point out that the K_a for contracting muscle is close to the apparent binding constant for the formation of the ternary complex between actin, 5'-adenylyl imidodiphosphate [AMP-P(NH)P],

and S-1 measured at 22 °C, 0.2 M KCl, and pH 7.0, since $1.0 \times 10^4\ M^{-1} < K_a < 1.5 \times 10^4\ M^{-1}$ (Greene & Eisenberg, 1978) and to the apparent association constant for the binding of HMM to actin in the presence of ATP in 0.1 M KCl at 23 °C (Borejdo, 1979).

Under rigor conditions, S-1 bound to extracted myofibrils with $K_a = 4.8 \times 10^4\ M^{-1}$. The ratio of the binding constant of HMM and S-1 to actin is ~30. This number falls in the range between the values reported by Margassian & Lowey (1976) and Highsmith (1978) on the one hand and Greene & Eisenberg (1980) on the other. The ratio of affinities of HMM to S-1 suggests several possible models of binding of the two-headed myosin moiety (Highsmith, 1978). Reduced mobility of a free head of HMM when one is bound and a strained state for both heads when both are attached are two likely possibilities.

Table II shows that the stoichiometric molar binding ratio of S-1 is unity, indicating that S-1 is capable of forming a 1:1 complex with G-actin. Thus, the steric arrangement of actin in a three-dimensional lattice does not prevent full saturation of the actin binding sites. In vitro determination of binding stoichiometry also gave a 1:1 binding ratio of S-1 to actin (Young, 1967; Moore et al., 1970; Takeuchi & Tonomura, 1971; Margossian & Lowey, 1973). In contrast, it appears that in myofibrils or in the muscle fibers added HMM cannot saturate all available actin sites: we find that at most 50% of actin could be filled with HMM. In vitro estimations, on the other hand, suggested the values of the stoichiometric ratio of 1 (Young, 1967; Tawada, 1969) and 2 (Gergely & Kohler, 1957; Tonomura et al., 1962; Takeuchi & Tonomura, 1971; Eisenberg et al., 1972; Margossian & Lowey, 1973; Greene & Eisenberg, 1980).

In the case of HMM it is possible that the stoichiometric ratio is decreased through the binding of a ligand to nonconsecutive free lattice residues. To estimate these effects, we have calculated (by Monte Carlo simulations) the fraction of unoccupied actin binding sites when HMM binds to the two actin sites separated by one or two actin monomers; HMM is large enough (Mendelson & Kretzschmar, 1979) to span the required distance. Our calculations show that at ligand concentrations such as those used in the present experiments (i.e., small ratios of HMM to actin when the lattice succeeds in "resisting" saturation) 40% of the actin sites remain unoccupied when actin binding sites are separated by one monomer and 60% remain unoccupied when separated by a dimer.

Another reason for the observed low S value for HMM may lie in the fact that the binding data was interpreted by the classical Scatchard independent binding site treatment. The interaction of multiple binding site ligand with the one-dimensional lattice systems may require a different type of theoretical analysis [cf. McGhee & von Hippel (1974)]. When a ligand molecule binds to n consecutive lattice residues (actin monomers) and when partial binding ("dangling") is not allowed, the experimental determination of the saturated ligand concentrations becomes difficult. McGhee & Von Hippel (1974) have shown that the extrapolation of actual data from the usually attained middle portion of the saturation range may underestimate the stoichiometry of binding. In the present experiments, this underestimation may be particularly severe because the ligand concentration was low (cf. eq 7) and the actin concentration was high, allowing for experimental exploration of only the low end of the saturation range. In other words, the low S value may be an artifact associated with the data extrapolation. To settle the question of the HMM binding stoichiometry in myofibrils, it will be necessary to increase HMM–actin ratios which could be done if fluorescence signals could be measured with better accuracy.

Added in Proof

Since this article was submitted for publication, a relevant article by Greene & Eisenberg (1980a) appeared which showed that the equilibrium binding of S-1 to regulated actin filaments at low S-1/actin ratios is weak in the absence of Ca^{2+}.

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References

Borejdo, J. (1979) *Biopolymers 18*, 2807.
Borejdo, J., & Oplatka, A. (1976) *Biochim. Biophys. Acta 440*, 241.
Borejdo, J., & Putnam, S. (1977) *Biochim. Biophys. Acta 459*, 578.
Bremel, R. D., Murray, J. M., & Weber, A. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 267.
Eisenberg, E., Dobkin, L., & Kielley, W. W. (1972) *Biochemistry 11*, 4657.
Garamvolgyi, N., Vaczy, K., & Biro, E. N. A. (1975) *Acta Biochem. Biophys. Acad. Sci. Hung. 10*, 267.
Gergely, J., & Kohler, H. C. (1957) *Conf. Chem. Muscular Contract.* [*Proc.*], 14.
Greene, L. E., & Eisenberg, E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 54.
Greene, L. E., & Eisenberg, E. (1980) *J. Biol. Chem. 255*, 549.
Greene, L. E., & Eisenberg, E. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2616.
Highsmith, S. (1978) *Biochemistry 17*, 22.
Highsmith, S., Mendelson, R., & Morales, M. F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 133.
Hill, T. L. (1978) *Nature* (*London*) *274*, 825.
Lowey, S., & Cohen, C. (1962) *J. Mol. Biol. 4*, 293.
Lowey, S., Slayter, H. S., Weeds, A. G., & Baker, H. (1969) *J. Mol. Biol. 42*, 1.
Margossian, S. S., & Lowey, S. (1973) *J. Mol. Biol. 74*, 313.
Margossian, S. S., & Lowey, S. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 671.
Margossian, S. S., & Lowey, S. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 1580.
Marston, S. (1973) *Biochim. Biophys. Acta 305*, 397.
Marston, S., & Tregear, R. (1972) *Nature* (*London*), *New Biol. 235*, 279.
Marston, S., & Weber, A. (1975) *Biochemistry 14*, 3868.
McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469.
Mendelson, R. A., & Kretzschmar, K. M. (1979) *Biophys. J. 25*, 20a.
Mulhern, S. A., & Eisenberg, E. (1976) *Biochemistry 15*, 5723.
Mulhern, S. A., & Eisenberg, E. (1978) *Biochemistry 17*, 4419.
Oplatka, A., Borejdo, J., & Gadasi, H. (1974) *FEBS Lett. 45*, 55.
Peller, L. (1975) *J. Supramol. Struct. 3*, 169.
Pesce, A. J., Rosen, C. G., & Pasby, T. C. (1971) *Fluoresc. Spectrosc.*, 203–240.

Sanger, J. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1913.
Szentkiralyi, E. M. (1961) *Exp. Cell Res. 22*, 18.
Takeuchi, K., & Tonomura, Y. (1971) *J. Biochem. (Tokyo) 70*, 1011.
Tawada, K. (1969) *Biophys. Biochim. Acta 172*, 311.
Taylor, E. W. (1977) *Trends Biochem. Sci. (Pers. Ed.) 2*, N32.
Tonomura, Y., & Inoue, A. (1977) *Trends Biochem. Sci. (Pers. Ed.) 2*, N32.
Tonomura, Y., Tokura, S., & Sekiya, K. (1962) *J. Biol. Chem. 237*, 1074.
Weber, A., & Murray, J. M. (1973) *Physiol. Rev. 53*, 612.
Young, M. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 2393.

Tubulin–Myosin Interaction. Some Properties of Binding between Tubulin and Myosin†

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ABSTRACT: This report presents evidence suggesting the direct binding between tubulin and myosin: (1) coprecipitation of tubulin with myosin occurred at a low ionic strength at which no precipitation of tubulin by itself occurred; (2) the amount of tubulin coprecipitated was unchanged when the coprecipitate was washed thoroughly; (3) about 2 mol of tubulin dimer could bind per mol of myosin at the maximum under our experimental conditions. The binding of about 1 mol of tubulin dimer was influenced by the presence of F-actin, but that of the other 1 mol of tubulin dimer was uninfluenced. In the former binding, tubulin or actin which bound first to myosin was suggested to have a priority. With regard to the priority of the binding, a similar result was obtained from the experiments of tubulin interference in actin activation of myosin Mg^{2+}-ATPase. The tubulin–myosin binding occurred moderately even at 0 °C and was not affected by Ca^{2+} (2 mM), colchicine (200 μM), or Mg-ATP (4 mM), reflecting that the ability of tubulin to bind to myosin was different from the ability of tubulin to form microtubules and that the nature of tubulin–myosin binding was different from that of F-actin–myosin binding. Besides tubulin–myosin interaction, a possible interaction between microtubule-associated proteins (MAPs) and actomyosin was suggested from the data that MAPs activated actomyosin Mg^{2+}-ATPase activity while purified tubulin inhibited the activity.

Attention has been increasingly directed to the roles of the actomyosin system and microtubule (tubulin–dynein) system in cell motility [for review, see Goldman et al. (1976)]. Furthermore, the idea that the two systems may function coincidentally in a certain cellular motile phenomenon has been widely entertained; for example, axoplasmic transport (Hoffmann & Lasek, 1975), release of neurotransmitters at synaptic endings (Thoa et al., 1972), movement of pigment granules in pigment cells (Malawista, 1975), chromosome movement in mitosis (Sanger, 1975), and modulation of cell surface proteins (Nicolson, 1976). It is possible to infer that both systems function not only independently but also cooperatively through the interactions between the components contained in both systems.

As one of such interactions, a tubulin–myosin interaction has so far been studied. Mohri & Shimomura (1973) observed a superprecipitation-like phenomenon in a tubulin–myosin mixture. Alicea & Renaud (1975) showed tubulin–myosin interaction by measuring myosin ATPase activity which was markedly stimulated by tubulin. However, this result was not reproducible as shown by Castle et al. (1976). On the other hand, Gozes et al. (1975) reported that tubulin and actin synthesized in vitro from brain mRNA failed to separate from each other even if they were treated by the purification procedures specific for the respective proteins. The result strongly suggested the occurrence of actin–tubulin or tubulin–myosin interaction. This urged us to investigate the tubulin–myosin interaction in detail. The present paper describes the evidence suggesting that about 2 mol of tubulin dimer can bind per mol of myosin and that the binding attributes of the 2 mol are likely to be different from each other.

Materials and Methods

Preparation of Proteins. Microtubule protein was prepared from porcine brain by the procedure of Shelanski et al. (1973) except that the reassembly buffer of Weisenberg (1972) was replaced by that of Kuriyama (1975). As "microtubule protein", the fraction obtained after two cycles of polymerization and depolymerization was used. For the preparation of purified tubulin and microtubule-associated proteins (MAPs), the microtubule protein was applied to a phosphocellulose column preequilibrated with 5 mM imidazole hydrochloride buffer (pH 6.6) containing 50 mM KCl (buffer A) and eluted with buffer A. The protein fraction eluted in the void volume was used as purified tubulin (PC-tubulin). The MAPs fraction was then eluted with 5 mM imidazole hydrochloride buffer (pH 6.6) containing 0.8 M KCl and desalted by passage through a column of Sephadex G-25 preequilibrated with buffer A.

Myosin was extracted from rabbit skeletal muscle as described by Perry (1955) and purified by ammonium sulfate precipitation. Purified actin was prepared from rabbit skeletal muscle by the method of Hirabayashi & Hayashi (1970).

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. This was performed by using 7.5% polyacrylamide gel by the method of Weber & Osborn (1969) unless otherwise specified. Staining of gels was done with 0.25% Coomassie brilliant blue solution containing 45.4% methanol and 9.2% acetic acid for more than 6 h, and free dye was rinsed out with 20% ethanol containing 10% acetic acid for 2–3 days.

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