Interaction of Myosin Subfragments with F-Actin†

Sarkis S. Margossian‡ and Susan Lowey*

ABSTRACT: The effect of ionic strength, temperature, and divalent cations on the association of myosin with actin was determined in the ultracentrifuge using scanning absorption optics. The association constant (K_a) for the binding of heavy meromyosin (HMM) to F-actin was 1×10^7 M^{-1} at 20 °C, in 0.10 M KCl, 0.01 M imidazole (pH 7.0), 5 mM potassium phosphate, 1 mM $MgCl_2$, and 0.3 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid. K_a was the same for HMM prepared by trypsin or chymotrypsin. The affinity of subfragment 1 (S1) for actin under the same ionic conditions was 3×10^6 M^{-1}. Varying the preparative procedure for S1 had little effect on K_a. The small difference in binding energy between HMM and S1 suggests that either only one head can bind strongly to actin at a time or that free energy is lost during the sterically unfavorable attachment of the two heads to actin.

The generation of force between the thick and thin filaments of muscle is believed to be related to a change in conformation of the myosin "heads" (or cross-bridges) as they attach to actin (Huxley, 1969). X-ray diffraction and electron microscopy have, in fact, provided some evidence for a change in angle of the head relative to the rod as the muscle goes from a relaxed to a rigor state (Reedy et al., 1965; Moore et al., 1970). More recent structural studies using ATP analogues suggest that the myosin head may even exist in more than one conformational state while attached (Goody et al., 1975; Marston et al., 1976; Holmes et al., 1976). Models for muscle contraction, based on two or more attached states, have been proposed to account for the conversion of the free energy of ATP hydrolysis into the work done by a cross-bridge (Huxley and Simmons, 1971; Eisenberg and Hill, 1978). Here we have determined the energy to be obtained from the interaction of a single head (S1)[1] or a double-headed subfragment (HMM) with F-actin. We find that the second head contributes little to the free energy of binding, suggesting that the two heads cannot bind independently to F-actin.

Values for the binding of myosin and its subfragments to actin have been reported from many laboratories over the years (Young, 1967; Tawada, 1969; Takeuchi and Tonomura, 1971; Lowey, 1971; Eisenberg et al., 1972; Margossian and Lowey, 1973; Marston and Weber, 1975; Highsmith et al., 1976; Chantler and Gratzer, 1976), but agreement has been generally poor. Some of the ambiguities have been due to limitations in the available experimental techniques; others have resulted from differences in subfragment preparation; and, finally, some of the discrepancies have arisen from variations in the pH, temperature, and ionic strength under which the measurements were made. Two of the more recent determinations of the association constant for S1 and actin have given values of 10^7 M^{-1} at 25 °C, 0.1 M KCl (Marston and Weber, 1975) and 10^6 M^{-1} at 4 °C, 0.15 M KCl (Highsmith et al., 1976); the former value depends on the free concentration of iodo[^{14}C]acetamide-labeled S1 remaining in the supernatant after sedimentation of the acto-S1 complex, while the latter is based on a decrease in rotational mobility of fluorescent-labeled S1 on binding to actin. The difference between these values can be accounted for most simply by the difference in temperature between the two experiments: Chantler and Gratzer (1976) have shown that the acto-S1 interaction is endothermic and the binding constant increases by about an order of magnitude in this temperature range.

Although these values agree well with each other, they are at least ten times higher than the association constant published previously by Margossian and Lowey (1973). In order to reconcile these discrepancies, we have reexamined the binding of S1 to actin. The procedure followed, namely, that of sedimenting the complex in the analytical ultracentrifuge and measuring the absorbance of unbound S1 in the supernatant, has the advantage of simplicity and directness: the S1 need not be covalently modified by attached labels, and a minimum of assumptions are needed to interpret the data. Recent advances in the preparation of S1 have made it possible to minimize proteolytic degradation (Weeds and Taylor, 1975) and also to obtain subfragments which retain a full complement of light chains (Margossian et al., 1975). Furthermore, improvements in the scanning absorption optical system have made measurements at 230 nm routine, and it is now possible to determine protein concentrations of less than 30 μg/mL. When the effects of ionic strength, temperature, and divalent cations on the binding of S1 to actin are taken into account, we find an association constant (3×10^6 M^{-1} at 20 °C, 0.10 M KCl) which compares favorably with those of the recent literature (Marston and Weber, 1975; Highsmith et al., 1976).

Apart from the intrinsic interest in the association constant for the so-called rigor bond, a major purpose in reexamining S1 was to compare its binding to actin with that of HMM. Previous determinations for HMM have established a stoichiometry of 2 mol of F-actin monomer bound/mol of HMM, and an affinity constant of greater than 10^6 M^{-1} (Lowey et al., 1971; Eisenberg et al., 1972; Margossian and Lowey, 1973). Basic to any speculation of how the cross-bridges might bring about contraction is the question of whether the myosin heads bind to actin independently or whether some sort of cooperative

† From the Rosenstiel Basic Medical Sciences Research Center and the Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received February 21, 1978.* This work was supported by grants from the National Science Foundation (PCM 75-14790), the National Institute of Arthritis, Metabolism, and Digestive Diseases, United States Public Health Service (AM 17350), and the Muscular Dystrophy Association, Inc.

‡ Established Investigator of the American Heart Association.

[1] Abbreviations used are: S1, subfragment 1; HMM, heavy meromyosin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); AMP-PNP, adenylyl imidodiphosphate; DTT, dithiothreitol; EPR, electron paramagnetic resonance; BSA, bovine serum albumin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino]ethanesulfonic acid.

0006-2960/78/0417-5431$01.00/0

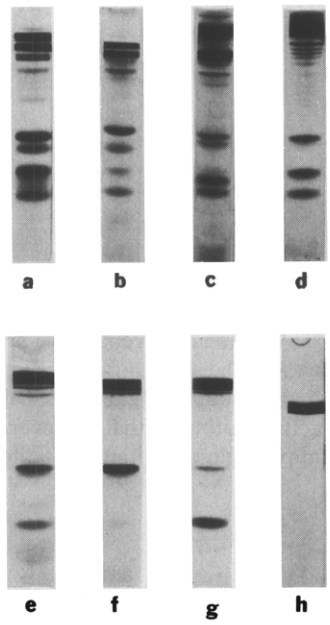

FIGURE 1: $NaDodSO_4$–gel electrophoresis of myosin subfragments and actin. Loads of 30 μg were run on 10% gels: (a) Mg·S1 and (b) EDTA·S1 prepared by papain digestion; (c) HMM prepared by tryptic and (d) α-chymotryptic digestion; (e) S1 prepared by α-chymotrypsin before chromatography; and (f) S1·A1 after fractionation on DE-52 as described under Materials and Methods; (g) S1·A2 isolated as in f; (h) actin.

interaction takes place between them. The low value for the binding of HMM to actin obtained in this study ($K_a = 1.0 \times 10^7\ M^{-1}$ at 20 °C, 0.10 M KCl) suggests that the two heads of myosin cannot bind independently and that steric hindrance may force them to attach to actin in nonequivalent positions. Preliminary reports of this work have appeared in abstract form (Margossian and Lowey, 1975, 1976).

Materials and Methods

Preparation of Proteins. Myosin was prepared from the muscles of the hind legs and back of New Zealand white rabbits as described by Holtzer and Lowey (1959). The stock myosin solution was stored at a concentration of about 5% in 0.5 M KCl, 0.05 M phosphate buffer (pH 6.5), with a crystal of thymol added to prevent bacterial growth.

Heavy meromyosin (HMM) was prepared by digesting a 2% myosin solution in stock buffer for 5 min at room temperature with either trypsin (Lowey and Cohen, 1962) or α-chymotrypsin (Weeds and Taylor, 1975) at a final concentration of 0.005%. Digestion with trypsin (Worthington) was terminated by adding soybean trypsin inhibitor to a concentration of 0.01%; α-chymotrypsin (Worthington) was inhibited with phenylmethanesulfonyl fluoride (Sigma) at a concentration of 0.1 mM. HMM was isolated in the supernatant by dialysis against 20 volumes of 0.01 M phosphate buffer (pH 6.5), 0.3 mM dithiothreitol (DTT) and further purified by fractionation between 43 and 57% saturated ammonium sulfate (ultrapure grade, Schwarz/Mann), 0 °C. In some instances, HMM was also chromatographed on DEAE-cellulose (Lowey et al., 1969).

Subfragment 1 (S1) was prepared according to Lowey et al. (1969) as modified by Margossian and Lowey (1973). A 2% myosin suspension in 0.2 M ammonium acetate (pH 7.1) with added 2 mM $MgCl_2$ or 2 mM EDTA was digested at room temperature for 7 min with 0.03 mg/mL papain (Worthington). The reaction was stopped with iodoacetic acid to a final concentration of 1 mM. Insoluble material was removed by centrifugation, and the supernatant was purified by chromatography on DEAE-cellulose (Whatman DE52) equilibrated in 0.05 M Tris-HCl (pH 7.9), 0.5 mM DTT containing either 2 mM $MgCl_2$ or 2 mM EDTA. The S1 was eluted with a linear gradient to 0.5 M KCl, and the protein in the front two-thirds of the peak was pooled and salted out between 47 and 58% saturated ammonium sulfate. Depending on the presence or absence of divalent cations, the preparation of S1 retains all its light chains (Mg·S1) or is missing the Nbs_2 light chain (EDTA·S1) (Margossian et al., 1975).

TABLE I: Extinction Coefficients of Myosin Subfragments (10^2 cm^2/g).

subfragment[a]	wavelength (nm) 230	236	280
HMM	52	21	6.0
EDTA·S1	60	25	8.1
Mg·S1	62	26	8.3
S1·A1	59	24	7.4
S1·A2	59	24	7.5

[a] Protein concentration was determined by micro-Kjeldahl; with the exception of S1·A1 and S1·A2, these values are an average of two to three determinations.

Subfragment 1 was also prepared with α-chymotrypsin as described by Weeds and Taylor (1975). If myosin is suspended in 0.12 M NaCl, 0.02 M sodium phosphate (pH 7.0), 1 mM EDTA and digested for 7 min with α-chymotrypsin as discussed above for HMM, a preparation of S1 lacking the Nbs_2 light chain is produced. The two components of S1, one containing the alkali 1 light chain (S1·A1) and the other composed of alkali 2 light chain (S1·A2), can be separated by ion-exchange chromatography under the same conditions as used for papain S1.

Figure 1 shows $NaDodSO_4$–polyacrylamide gels of representative samples of the subfragments discussed above.

F-actin was purified essentially by the method of Spudich and Watt (1971); details can be found in the legend to Table III (expts 10–12) of Margossian and Lowey (1973). This preparation gave a single band on $NaDodSO_4$–polyacrylamide gels (Figure 1h) and was usually greater than 90% polymerizable.

Sodium Dodecyl Sulfate–Gel Electrophoresis. Electrophoresis on polyacrylamide gels was carried out as described by Weber and Osborn (1969). Samples in 0.01 M sodium phosphate (pH 7) were boiled for 2 min in 1% $NaDodSO_4$ and 0.1% 2-mercaptoethanol before being applied to the gel. Electrophoresis was carried out for 5 h at 8 mA/tube (70 × 6 mm). The gels were then simultaneously fixed and stained by diffusion in 25% methanol and 10% acetic acid, containing 0.025% Coomassie brilliant blue stain; destaining was by electrophoresis in 10% methanol and 10% acetic acid.

Protein Concentrations. The extinction coefficients of dialyzed, clarified protein solutions were determined from absorbance measurements at 230, 236, and 280 nm, corrected for scattering by subtraction of the absorbance at 340 nm. Protein concentration was determined by the micro-Kjeldahl method, assuming 16% nitrogen. The values obtained for the different subfragments are summarized in Table I. The extinction coefficient for actin at 280 nm was taken as 11.0 (West et al., 1967).

Determination of ATPase Activity. Adenosine triphosphatase activity of myosin and its subfragments was assayed as described earlier (Margossian and Lowey, 1973). Calcium-activated ATPase was measured in 0.23 M KCl, 2.5 mM ATP, 2.5 mM $CaCl_2$, 0.05 M Tris-HCl (pH 7.9), and 0.1 mg of subfragment. The EDTA-activated ATPase contained 1

mM EDTA instead of calcium, and the KCl concentration was increased to 0.6 M. The actin-activated ATPase was measured in 0.01 M Tris-HCl (pH 7.9), 2.5 mM ATP, 2.5 mM $MgCl_2$, 0.1 mg of subfragment, and an increasing concentration of F-actin (0.2–5.0 mg/mL). All reactions were carried out at 25 °C in a volume of 2 mL; the reaction was initiated by the addition of ATP and terminated 5 min later with 1 mL of cold 15% trichloroacetic acid. Inorganic phosphate was measured by the method of Fiske and Subbarow (1925). Enzyme activities are reported in molar units [μmol of P_i (μmol of enzyme)$^{-1}$ s^{-1}] with molecular weights of 350 000, 130 000, 110 000, and 42 000 taken for HMM, Mg·Sl, EDTA·Sl (or chymotryptic S1) and actin, respectively.

Nucleotide Analysis. NaAMP-PNP was purchased from P-L Biochemicals. The purity of the reagent was analyzed by thin-layer chromatography (Bergmeyer, 1962) and judged to be greater than 90% pure.

Determination of Binding Constants in the Analytical Ultracentrifuge. The free concentration of subfragment remaining in the supernatant after sedimentation of the actin-bound subfragment was measured by absorption optics in the Model E analytical ultracentrifuge. The Multiplex attachment to the photoelectric scanning system allows five samples to be recorded in one run using the six-hole AN-G rotor with 12-mm double-sector cells and sapphire windows which were tested for their transmission of light at 230 nm. The performance of the entire optical system was tested with a series of solutions of known optical density at several wavelengths in a double-sector cell with distilled water as the reference liquid (Schachman and Edelstein, 1966). These data show a direct proportionality between the recorder deflections for solutions of maleate, tyrosine, and tryptophan and their optical density measured independently in a Zeiss spectrophotometer. The linearity of the recording system was also tested with known concentrations of HMM and BSA. Again, the recorder deflections were directly proportional to the spectrophotometric readings, multiplied by a factor of 1.12 to account for the optical path in the ultracentrifuge cell. Extinction measurements made at several wavelengths (280, 236, 230 nm) with the scanner agreed well with values determined in the Zeiss spectrophotometer. These experiments show that absolute concentrations can be determined reliably by recorder deflections, and effects of stray light are negligible.

In a typical experiment, actin at a concentration of 5 μM (0.2 mg/mL) was mixed with various concentrations of myosin subfragments (0.3–0.6 mg/mL) in 0.10 M KCl, 0.01 M imidazole (pH 7.0), 5 mM potassium phosphate, 1 mM $MgCl_2$, in a final volume of 2.0 mL. The imidazole was of a fluorimetric grade from Sigma. The samples were stirred slowly in the cold for 30 min and then centrifuged at 40 000 rpm to sediment the acto-subfragment complexes and any free F-actin. Within 10 to 20 min after reaching top speed, the supernatant could be scanned at 230, 236, or 280 nm, depending on the conditions of the experiment. The height of the plateau region in each scan was converted to protein concentration by means of a calibration plot of optical density vs. pen deflection. Unless otherwise specified, all runs were made at 20 °C. In the majority of the experiments, the "basic buffer" included either 0.3 mM EGTA or 0.1 mM $CaCl_2$.

The absorbance measured from the scans represents the sum of all proteins remaining in the supernatant after removal of the polymeric species by sedimentation. This value will, therefore, include not only the unbound myosin subfragment but also any unpolymerized actin and any denatured HMM or S1 incapable of binding to F-actin. Hence, it is essential to determine the amounts of such contaminants in the supernatant and to correct the optical density for their presence. The concentration of unpolymerized actin was determined in each experiment by running the amount of actin used in the reaction mixture in a separate cell along with the acto-enzyme samples. The correction for unpolymerized actin was usually no more than 10% of the total added actin. To test for inactive myosin subfragment, increasing concentrations of actin (5–30 μM) were added to a fixed amount (3 μM) of subfragment, and the supernatant was analyzed for unbound enzyme. It is apparent from the data in Figure 2 that less than 1% HMM remains in the supernatant at the highest excess of actin. Similar results were obtained with S1, and, therefore, no correction for inactive subfragment was applied to the data.

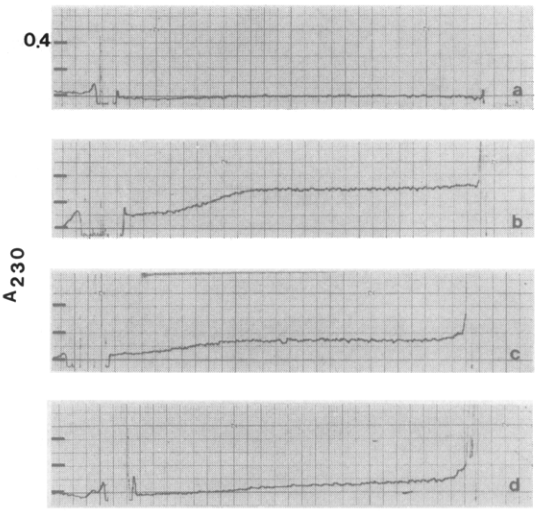

FIGURE 2: Test for unreactive HMM by "difference spectroscopy". To a fixed concentration of HMM (3 μM), increasing concentrations of F-actin were added: (b) 5, (c) 15, and (d) 30 μM. Actin at these same concentrations was included in the solvent sector of the ultracentrifuge cell. In a, 30 μM actin was included in both solvent and sample sectors to test the operation of the scanner. The solvent was 0.1 M KCl, 0.01 M imidazole (pH 7.0), 5 mM potassium phosphate, 1 mM $MgCl_2$, and 0.3 mM EGTA. Samples were run at 40 000 rpm and 20 °C and scanned at 230, 236, and 280 nm. Less than 1% free HMM was detected at the highest actin concentrations.

Since the absorbance of unpolymerized actin was large compared to the absorbance of unbound subfragment, particularly in the experiments described in Figure 2, it was advantageous to employ the technique of "difference spectroscopy" to reduce any error arising from the actin correction. In this procedure, the same amount of actin was added to the reference sector as was contained in the acto-HMM (or acto-S1) mixture in the sample sector of the centerpiece; thus, the recorded optical density presumably arises only from the subfragment. The absence of stray light and geometrical artifacts was demonstrated in a control experiment in which actin at 30 μM was placed in both sectors (Figure 2a); no optical density difference could be detected. In the majority of binding experiments, actin was run along with the test samples, and its absorbance was subtracted from the supernatant absorbance of the sample (see Figure 3). However, in the few experiments where the technique of difference spectroscopy was used, identical results were obtained.

The data were plotted according to the equation of Scatchard (1949):

$$\bar{\nu}/c = (\bar{\nu}_m - \bar{\nu})K_a$$

where $\bar{\nu}$ is the number of moles of subfragment bound per mole of F-actin monomer, c is the concentration of free subfragment obtained from the corrected scans, K_a is the association con-

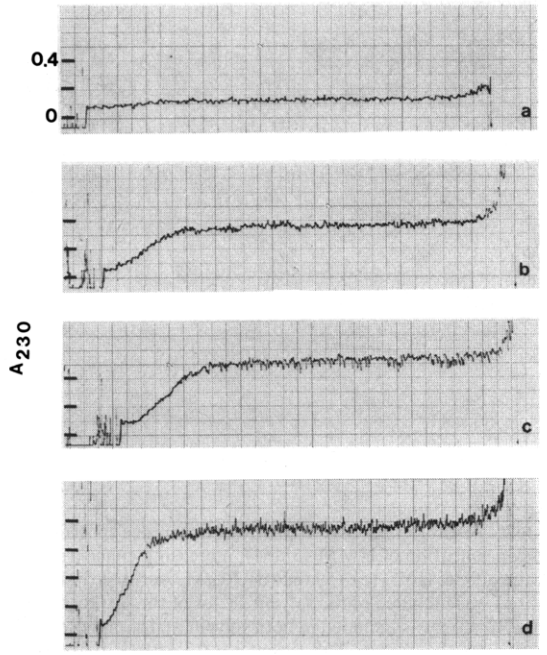

FIGURE 3: Scans of a typical binding experiment of Mg·S1 and F-actin. Conditions as in Figure 2, except that actin was run separately at 5.6 μM (a) and its absorbance subtracted from that of the samples. Thus, the solvent sector contained buffer as is customary in conventional centrifuge runs. The actin concentration was held constant in the samples at 5.6 μM, and Mg·S1 was added at increasing concentrations of 2.5, (b), 3.8 (c), and 5.0 μM (d).

stant for the reaction, and $\bar{\nu}_m$ is obtained by extrapolation of $\bar{\nu}/c$ to infinite subfragment concentration. The data were analyzed by linear regression, and K_a was obtained from the slope of the Scatchard plots.

Results

Characterization of Subfragments. Myosin and its subfragments were routinely assayed for calcium, EDTA-, and actin-activated ATPase to establish that the proteins were fully active. Table II summarizes the steady-state values for HMM and S1 prepared under a variety of digestion conditions. Although the Ca^{2+}-ATPases are somewhat erratic, the EDTA-activated ATPase is consistently lower, by at least one half, for S1 than for HMM or myosin (Eisenberg and Kielley, 1972; Margossian and Lowey, 1973). The actin-activated ATPase is the same, within experimental error, for the four different kinds of S1. Although the electrophoretic patterns of the subfragments vary (see Figure 1), depending on the digestion conditions and the type of proteolytic enzyme used, these extraneous cleavages do not seem to affect the enzymatic activity to any significant degree. Moreover, S1 is activated by actin to the same extent as a single head in HMM, confirming our previous conclusion that the two heads of myosin hydrolyze ATP independently (Margossian and Lowey, 1973). The apparent dissociation constant (K_m) for the interaction of enzyme and actin, obtained from double-reciprocal plots of activity vs. actin concentration (data not shown), indicates that the affinity of HMM for actin in the presence of ATP differs by no more than a factor of two from that of S1 for actin (Eisenberg and Kielley, 1972; Margossian and Lowey, 1973). Although the turnover rates suggests that the two heads of myosin can each bind to actin, the small difference in K_m values implies that they may have difficulty attaching simultaneously. A similar conclusion has been reached from the binding data in the absence of ATP to be described below.

TABLE II: Enzymatic Activity of Myosin and Its Subfragments.

subfragments[a]	ATPase activity (s^{-1}) Ca·ATP	EDTA·ATP	actin activated[c]
myosin	4.2	20.3	
HMM	4.6	20.6	36.3
EDTA·S1	2.4	11.2	17.0
Mg·S1	2.9	9.0	15.0
S1·A1[b]	5.0	7.3	14.7
S1·A2[b]	4.3	7.5	17.0

[a] Average of three to four determinations. [b] Average of two determinations from two different preparations. [c] Extrapolated value at infinite actin from Lineweaver–Burk plots of ATPase activity vs. actin concentration.

It has been reported that S1 preparations differing only in their alkali light-chain content differ also in their actin-activated ATPase activities (Weeds and Taylor, 1975; Wagner and Weeds, 1977). Our own measurements showed no significant difference between S1·A1 and S1·A2 in activity (see Table II), and the K_m for actin binding was only twofold smaller for S1·A1 (11 μM) than for S1·A2 (22 μM). However, since the results of Wagner and Weeds (1977) show considerable variation from one experiment to another, we feel that our own more limited data are well within the range of values reported by them.

In addition to finding that the enzymatic activity of S1 is not particularly sensitive to the choice of proteolytic enzyme used in its preparation, we have also found that the subfragments are remarkably homogeneous in size, as judged by sedimentation equilibrium (Yphantis, 1964). Both Mg·S1 and EDTA·S1 showed practically identical values of M_n, M_w and M_z over the entire range of concentration in the centrifuge cell (Margossian, Stafford, and Lowey, unpublished data). Moreover, the difference in molecular weight between Mg·S1 (130 000) and EDTA·S1 (110 000) can be accounted for by the Nbs_2 light chain, which is largely absent on $NaDodSO_4$ gels of EDTA·S1 (Figure 1b). Subfragment 1 prepared by chymotrypsin gave a molecular weight of 110 000 by sedimentation equilibrium. Although it showed fewer cleavages on $NaDodSO_4$ gels (Figure 1e–g), it was somewhat more polydisperse in molecular weight than the papain fragments.

The choice of protease in the preparation of S1 depends principally on the requirements of the experiment: if it is advantageous to have the Nbs_2 light chain intact, then papain is preferable; if, however, a minimum number of proteolytic cleavages in the heavy chain is desirable, chymotrypsin should be used. Since none of the S1 preparations can be considered "native" heads of myosin, it is recommended that several kinds of S1 be used, whenever possible, to minimize the artifacts introduced by proteolysis. The same argument, of course, applies to heavy meromyosin, which was prepared both by chymotrypsin and trypsin. Sedimentation equilibrium measurements on tryptic HMM indicated a highly homogeneous protein of molecular weight 350 000 (Margossian, Stafford, and Lowey, unpublished observations).

Binding of Subfragment 1 to F-Actin. The initial binding experiments were done without any special regard to the level of divalent cations either in the solvent used to digest the myosin or in the buffer used in the binding measurements. Binding constants obtained for S1 and actin under these conditions were in the range of 10^5 M^{-1} (Margossian and Lowey, 1973, 1975). Later it was discovered that the inclusion of $MgCl_2$ during the digestion of myosin helped to preserve the Nbs_2 light chain and

TABLE III: Association Constants for the Binding of S1 and HMM to Actin.[a]

subfragment	no. of determ	K_a (M)
Mg·S1	4	$(2.1 \pm 0.2) \times 10^6$
EDTA·S1	2	$(1.4 \pm 0.0) \times 10^6$
S1·A1	2	$(6.6 \pm 0.1) \times 10^6$
S1·A2	2	$(2.8 \pm 0.5) \times 10^6$
HMM-Trp[b]	10	$(1.0 \pm 0.2) \times 10^7$
HMM-Chym	3	$(1.0 \pm 0.2) \times 10^7$
HMM-Trp[c]	6	$(3.6 \pm 2.0) \times 10^6$
HMM-Trp[d]	2	$(3.0 \pm 0.6) \times 10^6$

[a] The solvent and temperature in all experiments, except where specified otherwise, were 0.1 M KCl, 0.01 M imidazole (pH 7.0), 5 mM potassium phosphate, 1 mM $MgCl_2$, 0.3 mM EGTA, at 20 °C. [b] The majority (7) of these experiments used 5 μM actin; in those experiments (3) where 2–3 μM actin was used, the results were the same. [c] These experiments contained 0.1 mM $CaCl_2$ in place of EGTA. [d] The temperature in these experiments was reduced to 5 °C, and the actin concentration was about 3 μM.

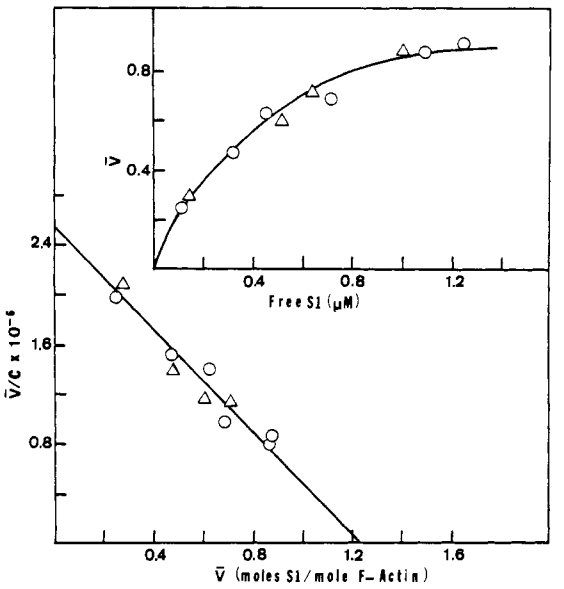

FIGURE 4: Binding of Mg·S1 to F-actin. Conditions as in Figure 3. When the samples could be scanned at more than one wavelength, results were averaged. Inset: Direct-binding isotherm of S1 to F-actin; two preparations. The value for K_a obtained from the Scatchard plot was 2.1×10^6 M^{-1}.

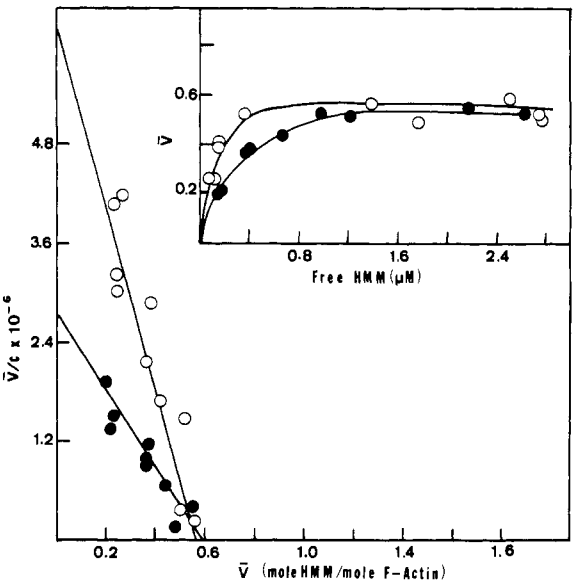

FIGURE 5: Binding of chymotryptic HMM to F-actin at 20 °C. Increasing concentrations of HMM were added to 5 μM F-actin in 0.1 M KCl, 0.01 M imidazole (pH 7.0), 5 mM potassium phosphate, 1 mM $MgCl_2$, and 0.3 mM EGTA (open circles). In a similar experiment, 0.1 mM $CaCl_2$ was substituted for EGTA (filled circles). Inset: Direct-binding isotherm of HMM to F-actin. The values of K_a from the Scatchard plots were 1.2×10^7 M^{-1} in EGTA, and 3.0×10^6 M^{-1} in calcium.

that subfragments rich in this light chain changed their affinity toward actin in the presence of calcium (Margossian et al., 1975). Calcium appears to reduce the affinity of Mg·S1 for actin, whereas EGTA increases the interaction. Subfragments devoid of Nbs_2 light chain, such as EDTA·S1 or chymotryptic S1, are insensitive to divalent cations (Margossian et al., 1975). Once the effect of calcium was realized, EGTA was routinely included in the basic buffer, and all subsequent binding experiments, except where specifically stated otherwise, were performed in 0.3 mM EGTA. Figure 3 shows a typical binding experiment in which increasing concentrations of S1 were mixed with a constant concentration of actin. The scans at 230 nm show the unbound S1 remaining in the supernatant after sedimentation of the polymeric species; the optical density in each trace has to be corrected for the absorbance of unpolymerized actin shown in the top scan of Figure 3a. Results calculated from such data are presented in Figure 4 in the form of a direct-binding isotherm (inset) and as a Scatchard plot from whose slope a K_a of 2.1×10^6 M^{-1} was determined. A limiting ratio of 1 mol of S1/mol of F-actin monomer was obtained from these plots. Deviations from 1.0 reflect the sizeable experimental error associated with this procedure. A summary of association constants determined for EDTA·S1 and Mg·S1 is given in Table III. As long as EGTA was included in the solvent, no significant difference was detected between different preparations. Binding experiments were also carried out on the fractionated S1·A1 and S1·A2 heads isolated by chymotryptic digestion of myosin (data not shown). For two different preparations of each isoenzyme, the association constant for S1·A1 was approximately twice that of S1·A2 (Table III). This difference is in the same direction as the values for K_m obtained from ATPase activity measurements for S1·A1 (11 μM) and S1·A2 (22 μM). Thus, despite the scatter in data, we believe the factor of two in K_a is probably real. The average value of K_a for the chymotryptic subfragments is only slightly larger (4.7×10^6 M^{-1}) than that obtained for the papain subfragments (1.7×10^6 M^{-1}), and not too much significance should be attached to this small difference.

Binding of Heavy Meromyosin to F-Actin. The binding of HMM to actin appeared to be the same whether the subfragment was prepared by tryptic or chymotryptic digestion of myosin; the association constant for a typical experiment represented in Figure 5 was 1.2×10^7 M^{-1}, and the extrapolated value for $\bar{\nu}$ was about 0.6. The deviation from the theoretical value of 0.5, expected for the binding of two actin molecules to a double-headed HMM, most likely reflects experimental error. The absorbance of free HMM in the region of the binding isotherm where half saturation of actin sites occurs is of a comparable magnitude to the optical density of unpolymerized actin, and, therefore, the error to be expected from such measurements is unavoidably large. Nevertheless, if a sufficiently large number of determinations are made (some by "difference spectroscopy"), the average value of 1.0×10^7 M^{-1} can be considered a reliable estimate of the affinity of HMM for actin (Table III).

Most of the unpolymerized actin is a "critical concentration"

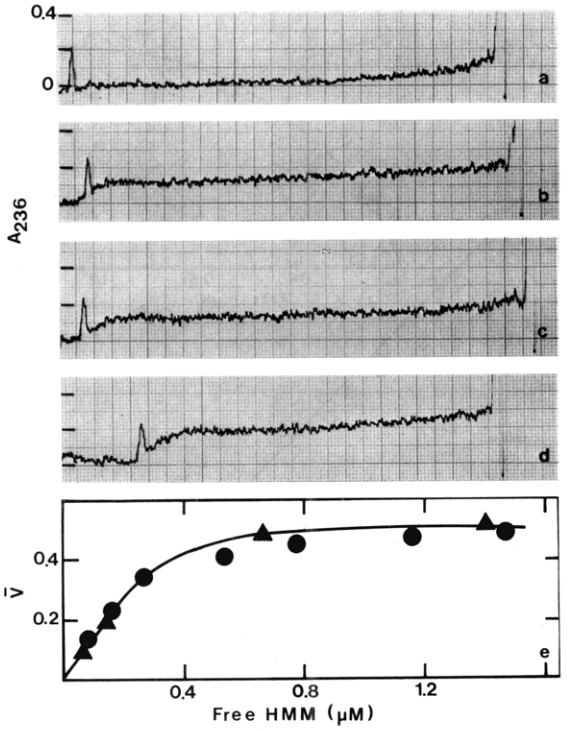

FIGURE 6: Binding of tryptic HMM to F-actin at 5 °C. The actin concentration was held constant at 2.7 μM (a), and HMM was added at increasing concentrations of 0.48 (b), 0.79 (c), and 1.19 μM (d). The samples were scanned at 230, 236 (data shown above) and 280 nm (the latter only at the higher concentrations of HMM). Absorbances at the different wavelengths were averaged to obtain the final values shown in the binding isotherm in e. The first three circles in e include the data in b–d.

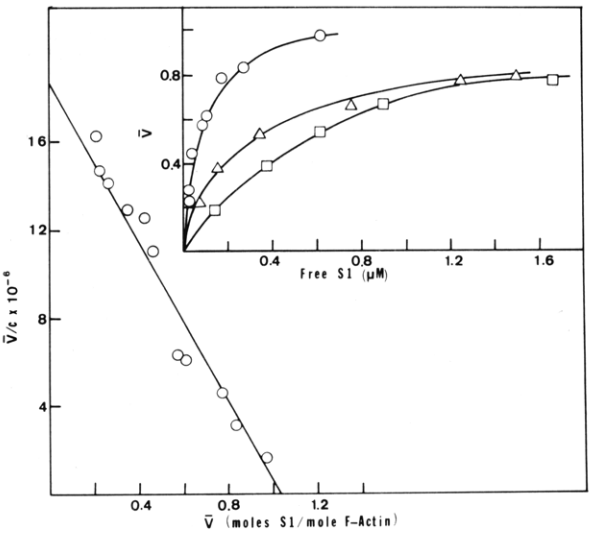

FIGURE 7: Binding of Mg·S1 to F-actin as a function of temperature. Conditions as in Figure 3, except that no KCl was included in the reaction mixture. Inset: Direct-binding isotherms at 5 (squares), 15 (triangles), and 25 °C (circles). The Scatchard plot is of an experiment at 25 °C. Values for K_a (μM) are as follows: 5 (1.4), 10 (1.8), 15 (3.4), 20 (5.1), and 25 °C (19).

of G-actin in equilibrium with F-actin (Oosawa and Kasai, 1962), but a small amount is probably denatured actin. The contribution of the latter can be reduced by working at lower actin concentrations. Although the majority of the experiments were performed at 5 μM actin, those using 2 to 3 μM actin gave similar results (Table III). Another means of increasing the amount of free HMM relative to the other components in the supernatant is to lower the temperature and thereby reduce the binding constant (see next section) (Figure 6 and Table III). The value so obtained at 5 °C, 3.0×10^6 M^{-1}, is consistent with that at 20 °C, assuming a standard enthalpy in the range of 10–20 kcal/mol (Chantler and Gratzer, 1976; Highsmith, 1978).

The effect of replacing EGTA in the standard buffer with 0.1 mM $CaCl_2$ was to reduce the association constant to 3.6×10^6 M^{-1} (average of six determinations) (see Figure 5 and Table III). This decrease in K_a for HMM parallels the loss in binding energy found for S1 in the presence of calcium (Margossian et al., 1975). It must be emphasized, however, that the effect of calcium on the interaction between myosin and actin has not been consistently observed, and we are unable, at present, to explain why some preparations of myosin (and its subfragments) do not show any sensitivity toward calcium. Possibly the state of phosphorylation of myosin is critical, but this question will require considerable further experimentation before it can be satisfactorily resolved.

Another additive which has the effect of reducing the association constant is the ATP analogue, adenylyl imidophosphate (AMP-PNP), which binds strongly to the nucleotide site of myosin without being hydrolyzed (Yount et al., 1971). Concentrations of 0.25 and 1.0 mM AMP-PNP in standard buffer (including 0.3 mM EGTA) caused the binding of HMM to actin to decrease to 2.1×10^6 M^{-1} (average K_a from two Scatchard plots). A stoichiometry of two actin monomers per mol of HMM was maintained. There was no significant difference between the results obtained at the two different concentrations of analogue and between these and a more limited number of determinations at 1.5 mM AMP-PNP, indicating that close to saturating levels of AMP-PNP were present. Thus, the behavior of AMP-PNP approximates that of ADP more closely than that of ATP, insofar as it has relatively little dissociating effect on the acto-myosin complex (Highsmith, 1976; Greene and Eisenberg, 1978).

Effect of Ionic Strength and Temperature on Binding. Values reported in the literature for the binding of myosin (and its subfragments) to F-actin have been obtained under a variety of ionic conditions and temperatures. To reconcile these diverse binding constants, we have investigated the effect of lowering the salt concentration and varying the temperature on the K_a for S1 and HMM. If 0.10 M KCl is omitted from the standard buffer, the binding constant for Mg·S1 at 20 °C becomes 5.1×10^6 M^{-1} (average of two determinations). The effect of temperature is considerably greater: In going from 5 to 25 °C (in the absence of added KCl), K_a increases from 1.4×10^6 to 1.9×10^7 M^{-1} or an order of magnitude (Figure 7). The binding constant of 1.9×10^7 M^{-1} at 25 °C [in 10 mM imidazole (pH 7.0), 5 mM potassium phosphate, 0.3 mM EGTA, 1 mM $MgCl_2$] was averaged from three separate determinations, a representative sample of which is given by the Scatchard plot in Figure 7. Although only single experiments were performed at 5, 10, and 15 °C, particular emphasis was placed on the 25 °C value in order that it might be compared to a published binding constant for S1 determined under similar conditions (Marston and Weber, 1975) (see Table IV). By plotting the association constants for S1 as a function of temperature according to the van't Hoff equation, a standard enthalpy of 21 kcal/mol was obtained. Experiments with HMM (average of two determinations) in the temperature range of 5 to 20 °C (in 0.1 M KCl) have led to an enthalpy of 17 kcal/mol, a value similar to that obtained for S1.

Discussion

We have shown that the absolute value of the association constant for the binding of a myosin head (S1) to F-actin is

TABLE IV: Comparison of Binding Constants for S1 and Actin with those in the Literature.

K_a (M)	conditions	method	reference
3×10^5	0.10 M KCl, 0.01 M Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 6-8 °C	sedimentation with UV absorption optics	Margossian & Lowey (1973)
3×10^6	0.10 M KCl, 0.01 M imidazole (pH 7.0), 1 mM $MgCl_2$, 5 mM KP_i, 0.3 mM EGTA, 20 °C	sedimentation with UV absorption optics	this work
6×10^7	0.01 M imidazole (pH 7.0), 1 mM $MgCl_2$, 10 mM KCl, 0.2 mM DTT, 0.1 mM $CaCl_2$, 25 °C	sedimentation with labeled S1	Marston & Weber (1975)
2×10^6	0.15 M KCl, 0.01 M Tes (pH 7.1), 4 °C	fluoresc depolarization with labeled S1	Highsmith et al. (1976)
2×10^7	0.10 M KCl, 0.10 M Tris-HCl (pH 8), 20 °C	ratio of rate constants	White & Taylor (1976)

highly dependent on the experimental conditions. Elevated temperatures enhance the binding, whereas additives such as calcium, salt, or nucleotides diminish the binding. If these variables are taken into account, however, there is remarkably good agreement among the results published by a number of laboratories (Table IV). This agreement is all the more impressive considering the wide variety of techniques used to measure this parameter, as well as the variation in the digestion conditions used to produce the subfragments. Our K_a of 3×10^6 M^{-1} given in Table IV is an average of all the S1 binding constants listed in Table III. The reason for this value being so much higher than our previously published constant of 3×10^5 M^{-1} can be ascribed, in part, to the presence of calcium in the medium, which we have now shown can reduce the interaction between S1 and actin (Margossian et al., 1975). Moreover, the earlier measurements were performed at lower temperatures (6-8 °C), where the interaction would be expected to be weaker. Also, some of the discrepancies no doubt arise from the limitations of the UV scanning optical system, which in the early 1970s was even less sensitive in recording low absorbances than it is today.

The advantage of measuring the free concentration of subfragment directly by a spectrophotometric method is that no chemical modification of the protein is necessary. Labeling a macromolecule with a fluorescent or radioactive probe invariably raises the question of whether the interaction will be affected by the label. This problem was approached experimentally, and the investigators satisfied themselves that no detectable difference in binding occurred between labeled and unlabeled S1; nevertheless, it introduces one more uncertainty into an experiment which already suffers from the deficiencies inherent in proteolytic fragments. Another advantage in the spectrophotometric procedure is the simplicity of the experiments, and the relatively small number of assumptions needed to interpret the data. In this respect, the "kinetic" constant derived from rate measurements (White and Taylor, 1976) is probably the least direct but still well within the range of reported values. The technique of fluorescence depolarization appears to be the most sensitive procedure for detecting micromolar concentrations of protein with high precision, and we note that the binding constant for S1 reported by Highsmith (1977) at 20 °C, $\sim 5 \times 10^6$ M^{-1}, is in good agreement with ours.

Having established a value for S1 in which one can have a measure of confidence, we have proceeded to redetermine the binding of HMM to actin. This was first attempted as early as 1971 (Lowey, 1971; Lowey et al., 1971), but the difficulties in scanning optical density at 230 nm permitted one only to *estimate* K_a as being greater than 10^6 M^{-1} (Margossian and Lowey, 1973). Although it is now possible to scan routinely at 230 nm, we are still limited to determining association constants no larger than about 10^7 M^{-1}; any stronger binding will reduce the absorbance of free subfragment to a level where it can no longer be measured with any precision. This problem has been largely circumvented by choosing conditions of temperature and ionic strength which favor weaker binding. At 0.1 M KCl and 20 °C, the average value of K_a for HMM prepared by both chymotryptic and tryptic digestion of myosin is close to 1×10^7 M^{-1} (Table III). This value again agrees well with that recently reported by Highsmith (1978; $K_a = 3.9 \times 10^7$ M^{-1} at 25 °C in 0.15 M KCl) using fluorescence depolarization. Upon reducing the temperature to 5 °C, as much as 20% of the HMM becomes dissociated from actin (Figure 6). This HMM is highly homogeneous in size, as determined by sedimentation equilibrium analysis, and contains no S1 or single-headed HMM, small amounts of which frequently contaminate HMM preparations. Essentially all of the HMM is sedimentable in the presence of a large excess of F-actin (Figure 2). Thus, we believe that the free HMM measured in the supernatant is in true equilibrium with F-actin and does not represent a denatured or nonbinding species of protein.

Energetics of the Interaction. More significant than the absolute value of K_a, in which some variation is unavoidable, is the finding that the two heads of HMM bind to actin only about ten times more strongly than the single head of S1. If both heads were to bind to actin quite independently, one might expect the K_a for HMM to be approximately the square of the equilibrium constant for S1, or K_{S1}^2. While recognizing that the assumption of adding the free energies of binding for S1 ($\Delta F°_{HMM} = 2\Delta F°_{S1} = -2RT \ln K_{S1}$) is without a firm thermodynamic basis (Jencks, 1975), it is, nonetheless, clear that the binding energy found for HMM is considerably less than what might be expected if no constraints were involved.

A good example of the energetic enhancement to be gained from the binding of a bivalent molecule to a substrate with multiple determinants is to be found in the literature of antibody-antigen interactions: Antibody prepared against the 2,4-dinitrophenyl (DNP) group was reacted with bacteriophage $\phi\overline{X}$ 174 multiply conjugated with the DNP group (Hornick and Karush, 1972). The intrinsic affinity of this antibody for the monovalent hapten, ϵ-*N*-DNP-lysine, was shown by equilibrium dialysis to be about 10^7 M^{-1}, whereas the association constant of anti-DNP immunoglobulin for the multideterminant phage particle was about 10^{10} M^{-1}. The difference of $\sim 10^3$ in affinity was attributed to the ability of antibody to combine with two determinants on the antigen (Hornick and Karush, 1972). A statistical mechanical treatment of this phenomenon has been developed by Crothers and Metzger (1972), which can account at least qualitatively for the experimental data described above. If K_1 is the equilibrium constant for the association of the first site of a bivalent molecule with a determinant and K_2 defines the interaction of the second site with a second determinant linked to the first, then

$K_2/K_1 = 3V/(2\pi<r>^3N)$, where N is the number of particles per volume V in the standard state used to define K_1. If this expression is applied to HMM, with r (the maximum separation of the two combining sites) taken as 100 Å, and K_1 is $\sim 10^6$ M^{-1} (the intrinsic constant for S1), then K_2 should be about 800 or $K_{obsd} \simeq 2K_1K_2 \simeq 10^3K_{S1}$. This formalism, similarly to the thermodynamic argument, again predicts a binding constant for HMM which is several orders of magnitude greater than what is actually observed.[2] One can only conclude that the second head of HMM is not binding, or binding weakly, or that the heads when linked together in HMM cannot attach to actin as strongly as when separated. It is difficult to distinguish between these alternatives: The similarity in enthalpies between HMM and S1 could be used in support of only one head binding, but the error in ΔH from a van't Hoff plot is too large to draw any firm conclusions. Considering the apparent immobility of both heads of HMM from fluorescence depolarization (Mendelson et al., 1973), saturation transfer EPR (Thomas et al., 1975), and conventional spin-labeling EPR (Seidel and Gergely, 1972), it seems more likely that both heads of HMM are binding to actin. It has been suggested that the heads may be binding to two different actin filaments (Offer and Elliot, 1978), and, indeed, this possibility cannot be excluded in muscle. However, solutions of acto-HMM and acto-S1 are similar in turbidity (White and Taylor, 1976), which suggests an absence of extensive cross-linking of actin filaments by HMM in vitro. We favor a picture of the rigor bond in which both myosin heads are attached to an actin filament. The use of binding energy to deform the molecules sufficiently to allow them to interact with actin can account for the drop in free energy which is observed experimentally.

Relation to Structural Models for the Cross-Bridge Cycle. The combined approaches of electron microscopy (Reedy et al., 1965; Moore et al., 1970), X-ray diffraction (Huxley and Brown, 1967; Miller and Tregear, 1972; Goody et al., 1975), and kinetic studies (Lymn and Taylor, 1971; Bagshaw et al., 1974) have led to a working model for the contractile cycle: The most elementary steps consist of dissociation of actomyosin by ATP, hydrolysis of ATP to a myosin-products intermediate, followed by reassociation of this intermediate complex with actin to complete the cycle. One of the conformational states of myosin revealed by the structural studies is a "relaxed state" in which the cross-bridges form a helical array about the thick-filament surface and the myosin heads lie more or less at right angles to the filament axis. Independent of whether myosin has bound ATP or ADP·P_i, as long as it is dissociated from actin, it gives an X-ray diffraction pattern characteristic of the relaxed state (Holmes et al., 1976). If the muscle is allowed to go into rigor (absence of nucleotide), the cross-bridges attach firmly to actin and make an angle of about 45° to the filament axis (Reedy et al., 1965). The extent of attachment is mainly determined by the repeat of the actin helix, and myosin heads can only attach to actin in certain favorable "target" areas (Reedy, 1968; Wray et al., 1978). Although the "rigor state" is nonphysiological, it is generally regarded as one of the transient states in contracting muscle and may resemble myosin at the end of the "power stroke".

A third conformational state of myosin has more recently been suggested by X-ray diffraction studies of muscles irrigated by the ATP analogue AMP-PNP (Barrington-Leigh et al., 1972; Lymn and Huxley, 1972; Goody et al., 1975). The diffraction pattern obtained with low concentrations of this analogue at 4 °C appears to be "intermediate" between that of a rigor and a relaxed state. This pattern could be interpreted as arising from a mixture of attached and free cross-bridges, but a strong argument against this explanation comes from the high degree of mechanical stiffness displayed by fibers in the presence of AMP-PNP (Goody et al., 1975), indicating no change in the number of attached cross-bridges from the rigor state.

While fully recognizing that conclusions from solution studies cannot be readily applied to an intact muscle where the proteins are present in high concentration and are associated in a highly ordered lattice, nevertheless, some aspects of the experiments reported here may have relevance for the actomyosin interaction in vivo. The large loss in free energy in forming a "rigor bond" between HMM and actin emphasizes the difficulties encountered in attaching a cross-bridge to actin; this problem will surely become even more formidable in an actively contracting muscle. A delicate balance of forces may exist in muscle such that one or two heads can become attached to actin at any given instance. In the presence of analogue, the predominant mode of binding may be through one head, and this explains why the X-ray pattern of muscle in AMP-PNP combines features of both the relaxed and rigor states. In contracting muscle, product release may favor the attachment of both heads to actin, resulting in the formation of the rigor bond. During this transition, some rearrangement of subunits within the head region may occur to facilitate attachment, and in so doing move the actin filament relative to the myosin filament (Huxley, 1969). One class of light chain which binds calcium and can be phosphorylated in many muscles has already been implicated in the regulation of the actomyosin interaction (Kendrick-Jones et al., 1976; Chacko et al., 1977). The other class of light chain, although less well understood, also appears to affect the interaction with actin (Wagner and Weeds, 1977). The point to be emphasized here is that the myosin molecule must possess an unusual degree of flexibility in order to adapt to the rapidly changing conditions in a muscle, and one should not think of this molecule in terms of the more conventional protein-protein interactions. These unusual demands may well be an adequate explanation for the two-headed nature of the molecule, without invoking a more direct interaction between the heads.

[2] We are indebted to Dr. D. M. Crothers for bringing this treatment of multivalent binding to our attention. A similar model has been proposed by Peller (1975).

References

Bagshaw, C. R., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., and Trentham, D. R. (1974), *Biochem. J. 141,* 351-364.

Barrington-Leigh, J., Holmes, K. C., Mannherz, H. G., Rosenbaum, G., Eckstein, F., and Goody, R. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 443-447.

Bergmeyer, H. U. (1962), Methoden der enzymatischen Analyse, Verlag chemie GmbH, Weinheim (Bergstrasse), Germany.

Chacko, S., Conti, M. A., and Adelstein, R. S. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 129-133.

Chantler, P. D., and Gratzer, W. B. (1976), *Biochemistry 15,* 2219-2225.

Crothers, D. M., and Metzger, H. (1972), *Immunochemistry 9,* 341-357.

Eisenberg, E., and Hill, T. L. (1978), *Prog. Biophys. Mol. Biol. 33,* 55-82.

Eisenberg, E., and Kielley, W. W. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 145-152.

Eisenberg, E., Dobkin, L., and Kielley, W. W. (1972), *Bio-*

chemistry 11, 4657-4660.
Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66,* 375-400.
Goody, R. S., Holmes, K. C., Mannherz, H. G., Barrington-Leigh, J., and Rosenbaum, G. (1975), *Biophys. J. 15,* 687-705.
Greene, L. E., and Eisenberg, E. (1978), *Proc. Natl. Acad. Sci. U.S.A. 75,* 54-58.
Highsmith, S. (1976), *J. Biol. Chem. 251,* 6170-6172.
Highsmith, S. (1977), *Arch. Biochem. Biophys. 180,* 404-408.
Highsmith, S. (1978), *Biochemistry 17,* 22-26.
Highsmith, S., Mendelson, R. A., and Morales, M. F. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 133-137.
Holmes, K. C., Goody, R., Mannherz, H. G., Barrington-Leigh, J., and Rosenbaum, G. (1976), 26 Colloquium Mosbach, 1975 (Heilmeyer, L., Rüegg, J. C., and Wieland, Th., Ed.) Springer-Verlag, Heidelberg.
Holtzer, A., and Lowey, S. (1959), *J. Am. Chem. Soc. 81,* 1370-1377.
Hornick, C. L., and Karush, F. (1972), *Immunochemistry 9,* 325-340.
Huxley, H. E. (1969), *Science 164,* 1356-1366.
Huxley, H. E., and Brown, W. (1967), *J. Mol. Biol. 30,* 383-434.
Huxley, A. F., and Simmons, R. M. (1971), *Nature (London) 233,* 533-538.
Jencks, W. P. (1975), *Adv. Enzymol. 43,* 219-410.
Kendrick-Jones, J., Szentkiralyi, E. M., and Szent-Györgyi, A. G. (1976), *J. Mol. Biol. 104,* 747-775.
Lowey, S. (1971), in *Subunits in Biological Systems,* (Timasheff, S. N. and Fasman, G. D., Ed.) Part A, p 201, Marcel Dekker, New York, N.Y.
Lowey, S., and Cohen, C. (1962), *J. Mol. Biol. 4,* 293-308.
Lowey, S., Slayter, H. S., Weeds, A. G., and Baker, H. (1969), *J. Mol. Biol. 42,* 1-29.
Lowey, S., Margossian, S. S., and Risby, D. (1971), *Biophys. J. 11,* 203a.
Lymn, R. W., and Huxley, H. E. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 449-453.
Lymn, R. W., and Taylor, E. W. (1971), *Biochemistry 10,* 4617-4624.
Margossian, S. S., and Lowey, S. (1973), *J. Mol. Biol. 74,* 313-330.
Margossian, S. S., and Lowey, S. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 671.
Margossian, S. S., and Lowey, S. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1580.
Margossian, S. S., Lowey, S., and Barshop, B. (1975), *Nature (London) 258,* 163-166.
Marston, S., and Weber, A. (1975), *Biochemistry 14,* 3868-3873.
Marston, S., Rodger, C. D., and Tregear, R. T. (1976), *J. Mol. Biol. 104,* 263-276.
Mendelson, R. A., Morales, M. F., and Botts, J. (1973), *Biochemistry 12,* 2250-2255.
Miller, A., and Tregear, R. T. (1972), *J. Mol. Biol. 70,* 85-104.
Moore, P. B., Huxley, H. E., and DeRosier, D. J. (1970), *J. Mol. Biol. 50,* 279-292.
Offer, G., and Elliott, A. (1978), *Nature (London) 271,* 325-329.
Oosawa, F., and Kasai, M. (1962), *J. Mol. Biol. 4,* 10-21.
Peller, L. (1975), *J. Supramol. Struct. 3,* 169-174.
Reedy, M. K. (1968), *J. Mol. Biol. 31,* 155-176.
Reedy, M. K., Holmes, K. C., and Tregear, R. T. (1965), *Nature (London) 207,* 1276-1280.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660-672.
Schachman, H. K., and Edelstein, S. J. (1966), *Biochemistry 5,* 2681-2705.
Seidel, J. C., and Gergely, J. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 187-193.
Spudich, J. A., and Watt, S. (1971), *J. Biol. Chem. 246,* 4866-4871.
Takeuchi, K., and Tonomura, Y. (1971), *J. Biochem. (Tokyo) 70,* 1011-1026.
Tawada, K. (1969), *Biochim. Biophys. Acta 172,* 311-318.
Thomas, D. D., Seidel, J. C., Hyde, J. S., and Gergely, J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1729-1733.
Wagner, P. D., and Weeds, A. G. (1977), *J. Mol. Biol. 109,* 455-473.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Weeds, A. G., and Taylor, R. S. (1975), *Nature (London) 257,* 54-56.
West, J. J., Nagy, B., and Gergely, J. (1967), *J. Biol. Chem. 242,* 1140-1145.
White, H. D., and Taylor, E. W. (1976), *Biochemistry 15,* 5818-5826.
Wray, J., Vibert, P., and Cohen, C. (1978), *J. Mol. Biol. 124,* 501-521.
Young, M. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 2393-2400.
Yount, R. G., Ojala, D., and Babcock, D. (1971), *Biochemistry 10,* 2490-2495.
Yphantis, D. A. (1964), *Biochemistry 3,* 297-317.