whereas interconvertible forms should give a single component under these conditions. Our binding experiments did not reveal the presence of an appreciable concentration of a form with low affinity for leucine. Within an experimental error of about 10% all the binding by leucine binding protein could be explained by one binding site per molecule with a K_d of less than 1 μM. Thus we do not find any evidence for distinct conformational states as reported by the galactose binding protein.

Our results obviously do not rule out the possibility of small conformational disruptions of leucine binding protein that decrease the affinity for leucine to the extent necessary to account for active transport. Attempts to attain *in vitro* conditions to mimic a subtle *in vivo* conformation change that might occur in a membrane can obviously fail to disclose such a property even if it exists. But the tightness of the binding of leucine under a variety of stress conditions dampens our enthusiasm for hypotheses of active transport based on conformational change of binding proteins. An alternate possibility involving membrane conformational changes without any change in affinity of the binding protein for its ligand is currently under consideration (Boyer and Klein, 1972).

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Binding of Actin to Heavy Meromyosin in the Absence of Adenosine Triphosphate†

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ABSTRACT: Using the analytical ultracentrifuge, the binding of actin to heavy meromyosin (HMM) in the absence of ATP was studied under varied conditions of ionic strength and temperature. Under all conditions studied, the binding ratio was found to be 2 moles of actin monomer/mole of HMM suggesting that both heads of the HMM bind simultaneously to the actin. Furthermore under all conditions studied, the dissociation constant was found to be less than 6×10^{-7} M. This shows that at an ionic strength of 0.1 M, the binding in the absence of ATP is more than 200-fold stronger than the binding in the presence of ATP as estimated from kinetic measurements which in turn suggests that ATP has a remarkably strong influence on the binding of actin to HMM.

It is now generally established that molecules of myosin and its tryptic digestion product, heavy meromyosin (HMM)[1] each contain two active sites or "heads" (Stracher and Dreizen, 1966; Slayter and Lowey, 1967; Lowey *et al.*, 1969) whereas actin monomers are single polypeptide chains (Rees and Young, 1967). On this basis it might be expected that each myosin or HMM molecule would combine with two monomers in the F-actin filament. However, despite numerous studies on the binding of actin to myosin or HMM, it is still

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[1] Abbreviation used is: HMM, heavy meromyosin.

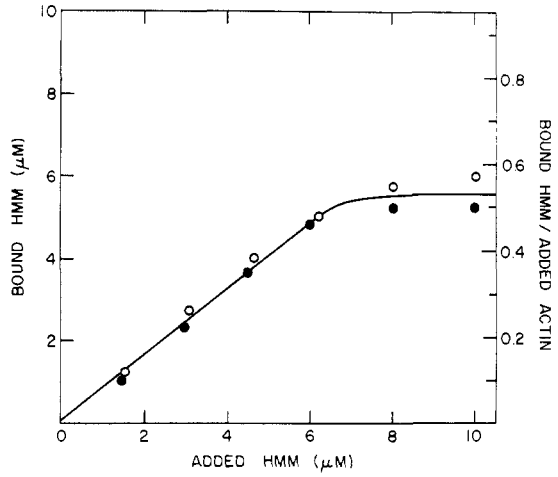

FIGURE 1: Binding of HMM to F-actin. Conditions: 0.1 M KCl, 1 mM $MgCl_2$, 10 mM imidazole, 5 mM P_i (pH 7.0), and 10.5 μM actin, 25°. (●, ○) Two different actin and HMM preparations.

TABLE I: Sedimentation of F-Actin in the Presence and Absence of P_i.[a]

P_i (mM)	Added Actin (μM)	Nonsedimentable Actin (μM)
0	2.3	1.5
0	11.6	1.9
0	46.0	2.0
5	4.1	0.54 (0.48)[b]
5	10.6	0.90 (0.68)[b]
5	42.4	0.71 (0.68)[b]

[a] Conditions same as Figure 1. [b] Concentrations given in parentheses are calculated from OD_{230} based on relationship $OD_{230} = 5 \times OD_{280}$.

unclear as to whether the molar binding ratio is 2:1 or 1:1. Many of these studies employed light scattering as a measure of binding (Gergely and Kohler, 1958; Tonomura *et al.*, 1962; Sekiya *et al.*, 1967; Finlayson *et al.*, 1969; Tawada, 1969) but studies using the preparative ultracentrifuge to sediment the F-actin have also differed in their conclusions (Tawada, 1969; Takeuchi and Tonomura, 1971) as have equilibrium studies employing the analytical ultracentrifuge. Using the latter technique Young (1967) found a binding ratio of one mole of actin per mole of HMM with relatively weak binding whereas more recently Lowey and her colleagues (Lowey, 1971; Lowey *et al.*, 1971) found a 2:1 binding ratio with unmeasurably strong binding between the actin and HMM.

In the present study we have used the analytical ultracentrifuge to reinvestigate the binding of column purified actin to HMM in the absence of ATP at varied salt and temperature. We find that under all conditions studied, the binding ratio of actin to HMM is 2:1 and the dissociation constant is less that 6×10^{-7} M.

Methods

Myosin was prepared by the method of Kielley and Harrington (1960) and HMM was prepared from the myosin as previously described (Eisenberg and Moos, 1968). G-Actin was prepared with a Sephadex G-200 column as described by Adelstein *et al.* (1963). It was then polymerized to F-actin with 0.1 M KCl, 1 mM $MgCl_2$, 5 mM P_i, and 5 mM imidazole and pelleted twice by centrifugation at 30,000 rpm. For use the pellets were homogenized into the same solvent with or without 5 mM P_i or into 3 mM Mg and 5 mM imidazole, with or without 5 mM P_i.

The binding experiments were performed in a Model E analytical ultracentrifuge equipped with a photoelectric scanner. In all cases the actin–HMM mixtures were stirred for 30 min prior to introduction into the centrifuge cell. A five-hole titanium rotor and 12-mm double-sector cells were used. Readings were taken at λ 280 or 230 mμ. It was found that an optical density error of *ca.* 0.04 occurred in the base-line readings. This error was found to be dependent on rotor velocity but independent of wavelength and it was therefore corrected by subtracting the optical density at λ 400 from the λ 280 or 230 readings. All protein concentrations were determined as previously by ultraviolet absorption at 280 mμ (Eisenberg and Moos, 1970). For purposes of calculation the molecular weights of myosin, HMM, and actin were taken to be 500,000 (Lowey *et al.*, 1969), 350,000 (Mueller, 1964), and 45,000 (Rees and Young, 1967), respectively.

Results

In determining the binding ratio of actin to HMM in the analytical ultracentrifuge, it is crucial that almost all of the added actin sediment so that we can be certain that at least 90% of the added actin is actively involved in binding HMM. However, as shown in Table I, in the absence of P_i, at 0.1 M KCl–1 mM $MgCl_2$, we found that 1.5–2.0 μM actin was nonsedimentable no matter how much actin was initially present. Since the amount of nonsedimentable actin was independent of the added actin concentration, it was apparently not simply denatured actin but rather was a "critical concentration" of G-actin in equilibrium with F-actin. Such a critical G-actin concentration has been postulated to occur by Oosawa and Kasai (1962) and has been demonstrated experimentally by other methods (Asakura *et al.*, 1960). In an effort to reduce the concentration of this nonsedimentable actin, we added 5 mM P_i which has been reported to increase the polymerization of actin (Grant, 1965). As can be seen in Table I, this reduced the nonsedimentable actin to about 0.7 μM, a value low enough to allow the binding studies to be done with reasonable accuracy.

Figure 1 shows the result of a typical experiment where increasing amounts of HMM are added to a fixed concentration of F-actin. As can be seen, the amount of binding leveled off at a ratio of bound HMM to added actin of 0.5–0.6. These data therefore suggest that 2 moles of actin bind per mole of HMM. The same result was obtained when this experiment was performed in the absence of P_i.

As can be seen in Figure 1 the binding between the actin and HMM was so strong that until the actin was completely saturated with HMM, almost all of the added HMM bound to the actin. For this reason the data could not be analyzed by the method of Scatchard (1949). However a minimum estimate for the dissociation constant could be roughly obtained by calculating the free HMM concentration present at the point where the actin is half-saturated with HMM. In Figure 1, this free HMM concentration is 0.6 μM. However, as shown in Table II, this cannot be a true equilibrium concen-

TABLE II: Effect of Increasing Actin Concentration on Residual Free HMM Concentration.[a]

Added Actin (μM)	Added HMM (μM)	Free HMM (μM)
10.5	4.65	0.64
42.0	4.65	0.76

[a] Conditions same as Figure 1.

tration of HMM, since when the actin concentration is quadrupled, the amount of this unbound HMM does not decrease. Because the optical density involved in this measurement is so low, it is difficult to determine exactly what it is due to—part of it may be due to contaminant trypsin and trypsin inhibitor which may make up 3% of the optical density of the added HMM. In addition part of it may be due to depolymerized actin or perhaps denatured HMM. At any rate these data show that the dissociation constant for the acto–HMM complex cannot be greater than 6×10^{-7} M and is probably even less.

To make certain that the results shown in Figure 1 are applicable under other conditions, we performed the experiments shown in Figures 2 and 3. As can be seen in Figure 2, both in the presence and absence of P_i, when less actin is present, the amount of HMM bound at saturation decreases proportionately, with the binding ratio remaining about 2 moles of actin/mole of HMM. Figure 3 shows that both at much lower ionic strength and at lower temperature, the binding ratio of actin to HMM remains 2:1. Furthermore lowering the temperature or the salt concentration does not appear to significantly change the strength of the binding between the actin and HMM.

Discussion

In the present study, we have found by using the analytical ultracentrifuge that the molar binding ratio of actin to HMM is 2:1. This result agrees with recent findings of Lowey (1971, Lowey *et al.*, 1971) but is in disagreement with a similar ultracentrifuge study by Young (1967) who found the binding ratio to be 1:1. We do not know why our results differ from those of Young. However since in both studies column purified actin was used, contaminated actin would not seem to be the problem. In any event, for the present we conclude that the molar binding ratio between actin and HMM is 2:1, a value which is in agreement with the structural finding that HMM has 2 active sites or heads whereas actin is a single polypeptide chain.

In addition to finding that the molar binding ratio of actin to HMM is 2:1, we also find that the dissociation constant is less than 6×10^{-7} M in a range of ionic strength from 0.01 to 0.1 M. This is in marked contrast to the situation in the presence of ATP where, using actin activation as a measure of binding, Eisenberg and Moos (1968) showed that the apparent dissociation constant of actin from HMM showed a marked increase with increasing ionic strength, and at 0.1 M was more than 10^{-4} M.

On the basis of a detailed steady-state kinetic analysis, Eisenberg and Moos (1970) showed that over a wide range of ionic strength, double-reciprocal plots of HMM ATPase *vs.* both actin and ATP concentration are linear and the binding

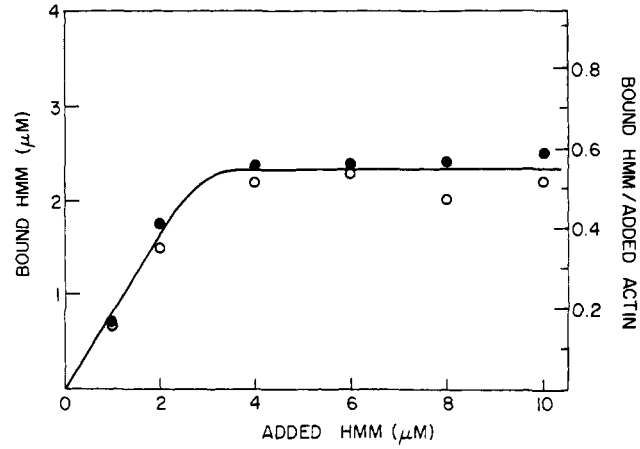

FIGURE 2: Binding of HMM to F-actin at low actin concentrations. Conditions: Same as Figure 1 except actin concentration = 4.25 μM. (○) 5 mM P_i; (●) no P_i added.

of actin to HMM increases the K_m of the HMM ATPase by a factor of 10. The simplest kinetic model which is consistent with these data leads to the prediction that, in an analogous way, the binding of ATP to HMM should increase the apparent dissociation constant of actin from HMM by about a factor of 10. In fact, as we noted above, at 0.1 M KCl there is at least a 200-fold difference in the binding of actin to HMM in the presence and absence of ATP. Therefore the simple kinetic model proposed by Eisenberg and Moos is not consistent with our present data.

It is also inconsistent with our recent work directly measuring the binding between actin and HMM in the presence of ATP (Eisenberg *et al.*, 1972). In this work we found that with ATP present, at any one time, a significant fraction of the HMM is apparently in a "refractory" state, unable to bind to actin even at very high actin concentrations. Furthermore, the remaining HMM which does bind might well bind with only one head at a time.

At this time we do not have enough data to devise a new kinetic model for the actin–HMM–ATP system which would explain all of our data. Nevertheless, it is interesting to speculate that perhaps the binding between actin and HMM is unusually strong in the absence of ATP because both heads

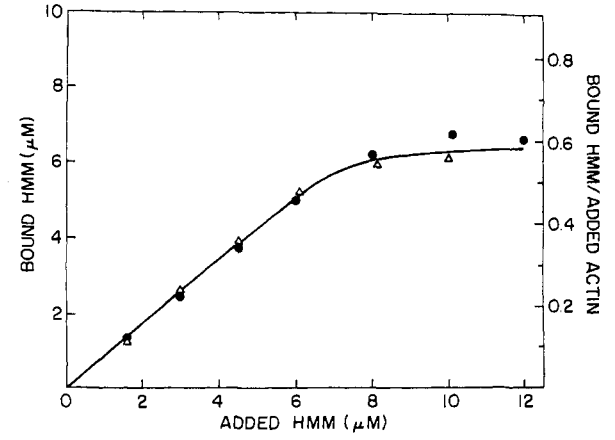

FIGURE 3: Binding of HMM to F-actin at low ionic stregnth and low temperature. Conditions: (Δ) 20 mM KCl, 10 mM imidazole, 1 mM $MgCl_2$, 5 mM P_i, and 11 μM actin, 25°; (●) 3 mM $MgCl_2$; 5 mM imidazole, 5 mM P_i, and 11 μM actin, 4°.

are binding simultaneously whereas in the presence of ATP, only one head binds at a time. In this regard it is of interest that subfragment 1, a further tryptic digestion product of HMM which has only one active site, has been reported to bind more weakly to actin than does HMM (Lowey *et al.*, 1971). To clarify this point we are presently carrying out further studies directly comparing the binding of actin to subfragment 1 in the presence and absence of ATP.

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Equilibria of Organic Phosphates with Horse Oxyhemoglobin†

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ABSTRACT: Organic phosphates, ATP, AMP, and 2,3-diphosphoglycerate (DPG) were interacted with horse oxyhemoglobin. Binding parameters were obtained by means of dialysis equilibrium in buffers at 5°, and from calorimetry at 5 and 25°, all in the pH range 6.5–7.3. The calorimetric results were evaluated assuming a single strong site, and assuming that the pH shifts which occur in the absence of added salt are due to electrostatic effects and not to changes in side-chain titration, upon mixing samples previously adjusted to the same pH. There was obtained for ATP and 2,3-diphosphoglycerate, respectively, $\Delta G^\circ = -6.5$ and -4.9 kcal mol^{-1}, $\Delta H^\circ = -5.2$ and -9.1 kcal mol^{-1}, and $\Delta S^\circ = +5$ and -14 cal deg^{-1} mol^{-1}, at pH 6.9 and 25°. These binding parameters are for the case of no added salt. In dialysis equilibrium experiments, buffer and supporting electrolyte were used. In that case, the results indicate strong competition by inorganic phosphate, lesser competition by chloride, while cacodylate possibly is noncompetitive or nearly so. Competition by salt ions also is manifest in calorimetry of organic phosphate binding. Equilibria of AMP, in the presence of orthophosphate, may engage in a somewhat different mechanism involving the protein. There appears to be chemical linkage between binding sites, when AMP is the substrate. There are indications that for organic phosphates, there exists more than one site available on oxyhemoglobin. However there is only one strong site. Certain of the pH-dependent phenomena were roughly accounted for by electrostatic effects using the smeared charge model. Calorimetry at 5°, compared to 25°, indicates a ΔC_P of binding of $+260$ cal deg^{-1} mol^{-1} for ATP-deionized oxyhemoglobin interaction, and a ΔC_P of about $+100$ cal deg^{-1} mol^{-1} for DPG interaction.

This paper reports on organic phosphate binding to horse oxyhemoglobin, HbO_2.[1] The binding of a variety of organic phosphates to both oxy and deoxy forms of hemoglobin has been rather widely studied and reviewed. However, there is considerable controversy regarding the extent of binding to both forms of hemoglobin. The actual difference in free energy between organic phosphate binding to the two forms of hemoglobin, *i.e.*, the amount of free energy that is available for control of oxygenation by these compounds, has not been satisfactorily determined.

The data presented here for organic phosphate binding

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[1] Abbreviations used are: HbO_2, oxyhemoglobin; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; DPG, 2,3-diphosphoglycerate.