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# Interaction of Actin with Spin-Labeled Heavy Meromyosin in the Presence of Nucleotides†

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ABSTRACT: It has previously been shown that the mobility of spin labels attached to the fast reacting thiol groups of myosin (or its proteolytic subfragments) is decreased by the binding of F-actin and increased by nucleotide binding. In the present study the spin-labeling technique has been used to study the nature of the complex which exists when heavy meromyosin (HMM), F-actin, and nucleotide are simultaneously present. When actin is added to the spin-labeled HMM·ADP complex there is a decrease in spin-label mobility. This effect is dependent upon the actin concentration; saturation occurs at approximately 2 mol of actin monomer/mol of HMM. At low ADP concentrations the spectral effect of actin can be interpreted as a release of ADP from the nucleotide binding site and reversal of the nucleotide-induced conformational change. At high concentrations of ADP there is evidence for formation of a ternary actin·HMM·ADP complex in which the spectral change associated with nucleotide binding is reduced. In the presence of sufficient nucleoside triphosphate actin does not alter the mobility of the spin labels relative to that observed for the HMM·triphosphate complex alone. During ATP hydrolysis actin has no effect on the mobility of spin labels attached to HMM. However, spin-labeled HMM which has been affinity labeled with a 6-SH derivative of ATP (the resulting complex may mimic the predominant steady-state species) interacts weakly with actin as determined by spin-label mobility and viscosity.

The interaction of the thick filament protein (myosin) with the thin filament protein (actin) is a central event in the contractile cycle. Such interaction provides the mechanically continuous system requisite for tension development and also results in an acceleration of the energy yielding reaction of contraction, the myosin-catalyzed hydrolysis of ATP. From the work of Bárány and Bárány (1959) it is clear that the sites on myosin which bind nucleotide are distinct from those which bind actin. However, it is quite apparent that interaction between these sites occurs: ATP dissociates actomyosin; actin decreases the affinity of myosin for ADP (Kiely and Martonosi, 1969). Knowledge of this interaction on a molecular level is essential for a full understanding of the contractile mechanism.

The spin-labeling technique (Stone *et al.*, 1965; McConnell and McFarland, 1970) has proved useful for probing the conformation of discrete areas of the myosin molecule during interaction with nucleotides and actin. The $SH_1$ groups of myosin (whose modification results in an acceleration of the $Ca^{2+}$-moderated ATPase activity) are readily labeled with a paramagnetic derivative of iodoacetamide (Quinlivan *et al.*, 1969). Electron paramagnetic resonance (epr) spectra of the spin-labeled myosin indicate strong immobilization of the attached labels. Addition of actin causes a further small reduction in the mobility of the myosin-bound labels (Stone *et al.*, 1968; Seidel *et al.*, 1971; Tokiwa, 1971). Addition of nucleotides (in the absence of actin) has an opposite effect. The mobility of the labels is moderately increased when myosin is complexed with ADP or pyrophosphate (Seidel *et al.*, 1970; Stone, 1970) and markedly increased during steady-state hydrolysis of MgATP (Seidel and Gergely, 1971).

In the present study we have used the spin-labeling technique to study the nature of the complex which exists when

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HMM,[1] actin, and nucleotide are simultaneously present. HMM is the "head" end of the myosin molecule and possesses both the ATPase and actin-binding properties of myosin. It was chosen for these studies because, in contrast to myosin, HMM remains soluble at the low ionic strengths which promote actin–myosin interaction. Seidel *et al.* (1970) have demonstrated that the mobility of spin labels attached to the $SH_1$ groups of myosin or the myosin·ADP complex is essentially unaltered by tryptic digestion to HMM.

## Experimental Procedure

*Materials.* Previously published methods were followed for the extraction and purification of F-actin (Stone *et al.*, 1970) and myosin A (Stone, 1970) from rabbit skeletal muscle. HMM was isolated from a 5-min tryptic digest of myosin (1 mg of trypsin/300 mg of myosin) by the procedure of Lowey *et al.* (1969) and concentrated by ammonium sulfate precipitation (Young *et al.*, 1965). The spin label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, was purchased from Synvar Associates, adenylyl imidodiphosphate (AMP-PNP) was purchased from International Chemical and Nuclear Corp., and the $\alpha,\beta$-methylene analogs of ADP and ATP (AMP-CP and AMP-CPP, respectively) were purchased from Miles Laboratories, Inc. The affinity label, 6-mercapto-9-$\beta$-D-ribofuranosylpurine 5′-triphosphate (SH-TP), was prepared by the method of Murphy *et al.* (1970) and very kindly donated to us by J. A. Duke.

*Techniques of Spin Labeling and Recording Epr Spectra.* It is known from the work of others (Quinlivan *et al.*, 1969; Seidel *et al.*, 1970) that the iodoacetamide spin label reacts preferentially with the two fast-reacting thiol groups ($SH_1$) on myosin or HMM. Attachment to these sites strongly restricts the rotational motion of the nitroxide group. Additional label reacts with other less well-defined sites which only weakly immobilize the attached label. Preliminary studies in our laboratory indicated that labeling of sites other than the two $SH_1$ groups severely interferes with some aspects of acto-HMM (or actomyosin) interaction. Consequently, labeling of HMM was carried out with no more than 2 mol (usually 1.5) of iodoacetamide spin label/mol of HMM and only preparations which displayed homogeneous labeling of the $SH_1$ groups (as indicated by the epr spectra, see Results) were used in these studies. Labeling was carried out in a solvent consisting of 50 mM KCl and 100 mM Tris-Cl (pH 7.5). After a 48-hr incubation on ice, the unreacted spin label was removed by dialysis at 4° against 25 mM Tris-Cl–25 mM histidine chloride–5 mM KCl–1 mM $MgCl_2$ (pH 8.0). Epr spectra were recorded at room temperature with a Varian E-3 epr spectrometer as described previously (Stone *et al.*, 1970). The amount of spin label covalently bound to HMM was determined by comparison of the amplitude of the midpeak of the epr spectrum of a denatured sample of the labeled protein with that of known concentrations of free spin label in the same solvent. Denaturation (which was carried out by boiling for 1 min in 0.5 M KOH) caused no significant destruction of the paramagnetic species but did alter the spectrum to one indistinguishable from that given by free spin label.

[1] Abbreviations used are: HMM, heavy meromyosin; SH-TP, 6-mercapto-9-$\beta$-D-ribofuranosylpurine 5′-triphosphate; SL-HMM-S-S-DP, HMM which has been treated with both the iodoacetamide spin label and SH-TP; AMP-PNP, adenylyl imidodiphosphate; AMP-CP, $\alpha,\beta$-methyleneadenosine diphosphate; AMP-CPP, $\alpha,\beta$-methyleneadenosine triphosphate; $R_{21}$, ratio of the second and first peak heights, respectively, of the electron paramagnetic resonance (epr) spectrum.

*Reaction of Spin-Labeled HMM with SH-TP.* A sample of HMM labeled with 1.9 mol of the iodoacetamide spin label was dialyzed for 24 hr at 4° against 0.1 M $NaHCO_3$ (pH 8.2) and treated on ice in the same solvent with a 200-fold excess of SH-TP for 1 week as described by Murphy and Morales (1970). Unreacted label was removed by dialysis against 50 mM $NaHCO_3$ (pH 8.0), 5 mM KCl, and 1 mM $MgCl_2$. Complete labeling of the ATPase sites was confirmed by the finding that there was no measurable ATPase activity in a system containing 0.6 M KCl, 50 mM Tris-Cl (pH 8.0), 10 mM $CaCl_2$, 1 mM ATP, and 0.07 mg/ml of SL-HMM-S-S-DP. Under identical conditions a sample of the same spin-labeled HMM which was incubated with solvent in place of SH-TP, but otherwise treated the same, had an activity of 30.2 $\mu$mol of $P_i$ $g^{-1}$ $sec^{-1}$. The stoichiometry of labeling was determined from the difference in absorbance at 322 nm following reduction with excess $\beta$-mercaptoethanol assuming an $\epsilon_{322}$ for unbound SH-TP of 23,100 (Hampton and Maguire, 1961). The sample used in the studies reported below was labeled with 4.6 mol of SH-TP/mol of HMM indicating significant reaction with residues other than the thiol groups at the two active sites.

*Other Methods.* The velocity of the ATPase reaction was determined by measurements of $P_i$ production using the colorimetric technique of Fiske and Subbarow (1925) as modified by Morales and Hotta (1960). The viscosity of solutions of actin and HMM was measured in Ostwald viscometers at 25° according to the procedure described by Eisenberg and Moos (1967). Protein concentrations were determined either by the colorimetric procedure of Lowry *et al.* (1951) using bovine serum albumin as a standard or by ultraviolet absorption at 280 nm. The extinction coefficients used were 543 $cm^2/g$ for myosin A (Gellert and Englander, 1963), 647 $cm^2/g$ for HMM (Young *et al.*, 1964), and 1149 $cm^2/g$ for F-actin (Eisenberg and Moos, 1967). The molecular weights used in this work were 350,000 for HMM (Mueller, 1964) and 46,000 for actin monomer (Rees and Young, 1967).

## Results

*Effect of Spin Labeling on the Properties of the Actin·HMM Complex.* The ability of the $SH_1$-spin-labeled HMM to interact with actin was compared with unlabeled HMM in the presence of both ADP and ATP. Eisenberg and Moos (1967) have shown that during ATP hydrolysis the viscosity of a solution of actin and HMM is essentially that predicted for a noninteracting mixture of the two proteins. After the ATP is completely hydrolyzed (*i.e.*, in the presence of ADP and $P_i$) there is a large rise in the viscosity of the complex which is believed to be due to network formation. We have observed essentially similar results for our preparations of actin and HMM (Table I) with the exception that the initial viscosity was always slightly higher than that calculated for a noninteracting mixture of the proteins. Reaction of HMM with up to 2 mol of the iodoacetamide spin label did not significantly alter the viscosity of solutions of actin and HMM measured in the presence of either ATP or ADP. Since the latter determination (*i.e.*, in the presence of ADP) was made in the presence of excess actin (~3 mol of actin/mol of HMM) it is apparent that spin-labeled HMM retains the ability to form a viscous complex with F-actin.

It is well documented that reaction of the $SH_1$ groups of myosin activates the $Ca^{2+}$- or $Mg^{2+}$-moderated ATPase. Under the ionic conditions used in this study the MgATPase

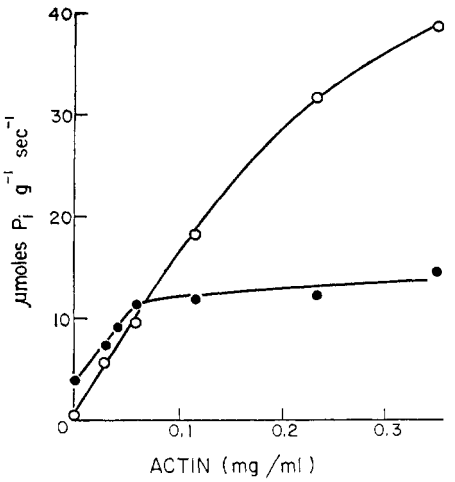


FIGURE 1: Effect of spin labeling on HMM ATPase in the presence of actin. Reaction conditions: 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0, 25°), 5 mM KCl, 1 mM MgATP, 0.072 mg/ml of unlabeled (○) or spin-labeled (●) HMM, and 0–0.35 mg/ml of actin.

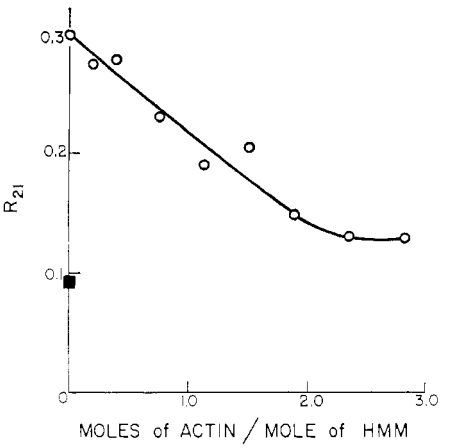


FIGURE 3: Spectral change as a function of actin concentration. Spectra were recorded for samples (○) containing 42 μM spin-labeled HMM, 13 mM Tris-Cl, 13 mM histidine chloride (pH 8.0), 21 mM KCl, 4 mM MgADP, and 0–119 μM actin. The spectral change was found to be independent of the order of addition of ADP and actin to spin-labeled HMM. Results are expressed as the ratio of the height of peak 2 to that of peak 1. The value of the parameter in the absence of both ADP and actin (■) is given for comparison.

of HMM was increased approximately tenfold by reaction with up to 2 mol of the spin label.

The effect of spin labeling on the actin-activated ATPase of HMM is shown in Figure 1. At low concentrations of actin (*i.e.*, at actin:HMM molar ratios of 6 or less) the degree of actin activation was approximately the same for unlabeled and spin-labeled HMM. At higher concentrations of actin the activity of unlabeled HMM continued to increase while that of spin-labeled HMM appeared to reach saturation. The maximal velocity of spin-labeled HMM approached 15 μmol of $P_i$ $g^{-1}$ $sec^{-1}$ while that of unlabeled HMM (estimated by extrapolation of double reciprocal plots according to Eisenberg and Moos (1968)) was 100 μmol of $P_i$ $g^{-1}$ $sec^{-1}$.

*Epr Spectrum of Spin-Labeled HMM in the Presence of ADP and Actin.* A typical spectrum of the spin-labeled HMM is shown in Figure 2A. The rotational motion of the spin label is very strongly restricted as indicated by the intensity of the outermost lines (labeled 1 and 5) of the spectrum. The near absence of weakly immobilized label (lines 2 and 4) indicates the label is almost exclusively attached to the $SH_1$ groups (see Seidel *et al.*, 1970). Addition of F-actin to this spin-labeled HMM in the absence of added nucleotide decreased the amplitude of the epr spectrum recorded at high microwave power in a manner similar to that described by Seidel and Gergely (1972) for the interaction of actin with spin-labeled myosin. However, in contrast to previous findings with spin-labeled myosin (Stone *et al.*, 1968; Tokiwa, 1971; Seidel and Gergely, 1972) the addition of actin to spin-labeled HMM in the present study did not significantly alter the relative heights of peaks 1 and 2 of the epr spectrum. It seems probable that this difference is due to the fact that in the present study labeling was restricted to the $SH_1$ groups. In the previously cited studies with myosin the spectra indicate significant labeling of sites other than the $SH_1$ groups. Seidel and Gergely (1972) have reported that the actin effect is confined to the regions of the spectrum arising from labels attached to $SH_1$ groups.

Spectrum B of Figure 2 shows spin-labeled HMM in the presence of saturating ADP. Binding of the nucleotide produces an increase in the mobility of the spin label as reported

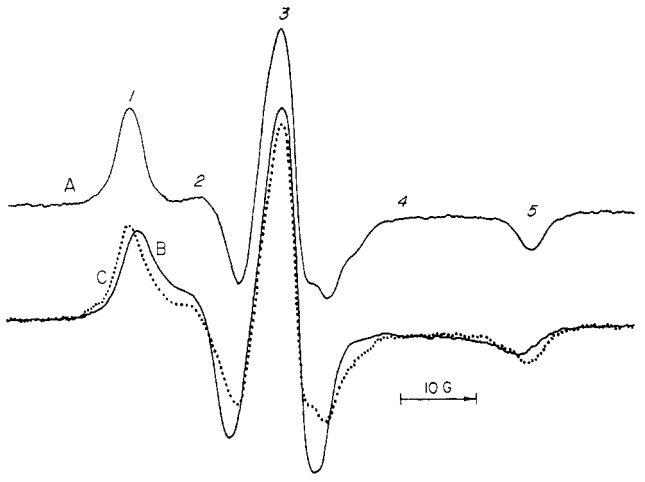


FIGURE 2: Effect of ADP and actin on the epr spectrum of spin-labeled HMM. Each sample contained 43 μM spin-labeled HMM in 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0), 5 mM KCl, 4 mM $MgCl_2$, and the following additions: (A) no addition; (B) 4 mM ADP; and (C, broken line) 4 mM ADP plus 110 μM actin. Gain settings were $10^5$ for spectra A and B and $1.5 \times 10^5$ for spectrum C; microwave power was 100 mW.

TABLE I: Effect of Spin Labeling on the Viscosity of Solutions of Actin and HMM.[a]

| Time of Measurement | Log $\eta_{rel}$ (Multiple of Theor Value)[b] | |
|---|---|---|
| | Unlabeled HMM | Spin-Labeled HMM |
| Initial Reading | 1.10 | 1.17 |
| Final (after all ATP hydrolyzed) | 2.91 | 2.91 |

[a] The samples contained 1.06 mg/ml of HMM and 0.45 mg/ml of actin in 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0), 5 mM KCl, and 5 mM MgATP. [b] The theoretical value of log $\eta_{rel}$ (= 0.123) is the sum of the log $\eta_{rel}$ for actin and HMM measured individually.

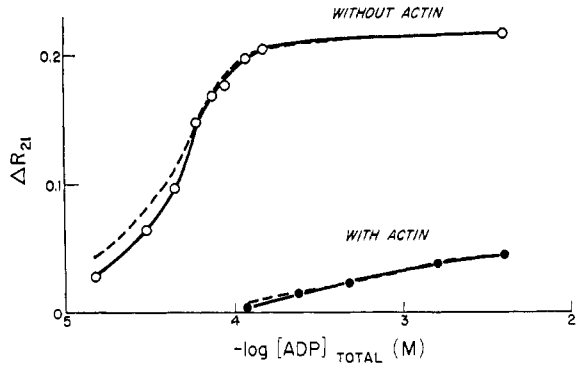


FIGURE 4: Spectral change as a function of ADP concentration. Conditions: 32 $\mu$M spin-labeled HMM in 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0), 5 mM KCl, 4 mM $MgCl_2$, and 0–4 mM ADP; (○) without actin; (●) 70.8 $\mu$M actin added. The F-actin sample used had been dialyzed against nucleotide-free solvent to remove unbound nucleotide. The change in the ratio of the height of peak 2 to that of peak 1 is plotted *vs.* the logarithm of the total ADP concentration. $R_{21}$ at 0 M ADP was 0.086 in the absence of actin and 0.080 in the presence of actin. The broken lines give the theoretical binding curves calculated with the constants obtained from Figure 5 (see text).

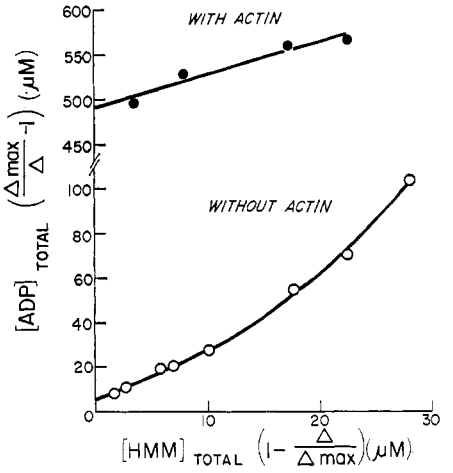


FIGURE 5: Graphical estimation of the number of binding sites and the dissociation constant for the binding of ADP to spin-labeled HMM. See text for explanation of symbols. Data are taken from Figure 4. Values for $\Delta_{max}$ are 0.216 in the absence of actin and 0.050 in the presence of actin.

previously for both myosin and HMM (Seidel *et al.*, 1970; Stone, 1970). This effect is observed as an increase in the ratio ($R_{21}$) of the heights of the second (representing weakly immobilized label) and first (strongly immobilized label) peaks, respectively, and a decrease in the separation of peaks 1 and 5. When F-actin is added to the complex of spin-labeled HMM with ADP, there is a decrease in spectrum amplitude as well as a decrease in $R_{21}$ and an increase in the separation of peaks 1 and 5 (see spectrum C of Figure 2 which has been recorded at 1.5 times the gain used for spectra A and B). The magnitude of this decrease in spin-label mobility depends on the concentration of actin. In Figure 3 it is shown that $R_{21}$ decreases linearly with increasing actin; saturation is observed at ~2 mol of actin monomer/mol of HMM. The spectrum amplitude (measured in the absence of nucleotide) displays a similar dependence on actin concentration (Seidel and Gergely, 1972).

In Figure 4 the dependence of the spectral parameter, $R_{21}$, on ADP concentration was determined in the presence and absence of saturating actin. The effect of actin is twofold: higher concentrations of ADP are required to produce an increase in $R_{21}$ and the magnitude of the spectral change at saturating ADP is markedly reduced. If the assumption is made that the change in $R_{21}$ (designated $\Delta$) is a linear measure of ADP binding to HMM then the data of Figure 4 can be used to determine the number of independent[2] binding sites per mole of HMM ($n$) and the dissociation constant ($K_d$). From the mass law

$$K_d = \frac{(n[HMM]_T - [ADP]_B)([ADP]_T - [ADP]_B)}{[ADP]_B}$$

where the subscripts T and B denote total and bound concentrations, respectively. The concentration of bound ADP is given by the expression $n[HMM]_T(\Delta/\Delta_{max})$ where $\Delta_{max}$ is the change in $R_{21}$ occurring at infinite ADP concentration (obtained by extrapolation from plots of $1/\Delta$ *vs.* $1/[ADP]_T$). Substituting for $[ADP]_B$ and rearranging yields $[ADP]_T \cdot [(\Delta_{max}/\Delta) - 1] = n[HMM]_T[1 - (\Delta/\Delta_{max})] + K_d$. A plot of $[ADP]_T[(\Delta_{max}/\Delta) - 1]$ *vs.* $[HMM]_T[1 - (\Delta/\Delta_{max})]$ should be linear with a slope of $n$ and an intercept on the ordinate of $K_d$. Figure 5 shows such a plot using the data of Figure 4. The data for HMM in the absence of actin show some deviation from linearity for the points obtained at low ADP concentrations. (It is possible that this is due to some heterogeneity in the sites labeled by the iodoacetamide spin label.) At higher concentrations of ADP, as $\Delta$ approaches $\Delta_{max}$, the plot becomes linear with a slope of 2.2 and an intercept of 5 $\mu$M. In the presence of actin the extrapolated value for $K_d$ is increased nearly 100-fold to 492 $\mu$M; the value for $n$ given by the slope is also increased to 3.5. The theoretical binding curves obtained with these constants are given by the broken lines in Figure 4.

*Effect of Actin on SL-HMM-S-S-DP*. The affinity label, SH-TP, has been shown to form a disulfide link with a cysteine residue at the active site of myosin (Murphy and Morales, 1970). Tokiwa (1971) showed that spin-labeled myosin can be affinity labeled with SH-TP and that the resulting complex does not suffer any epr spectral changes upon addition of actin in a high ionic strength medium. We have carried out the analogous experiment with HMM in a low ionic strength medium. The epr spectrum (Table II) of the resulting SL-HMM-S-S-DP had an $R_{21}$ of 1.24 compared with 0.422 for a similar sample of spin-labeled HMM which had not been treated with SH-TP. Nearly complete labeling of the ATPase sites with SH-TP is indicated by the complete loss of catalytic activity (see Experimental Procedure) and the very small spectral effect of added ADP relative to that observed with spin-labeled HMM (Table II). The increase in spin-label mobility produced by affinity labeling with SH-TP far exceeds that produced by ADP or by steady-state hydrolysis of ATP, as shown in Table II. When added to SL-HMM-S-S-DP, actin had no effect on the spectral parameter, $R_{21}$, but did cause an 8% decrease in the amplitude of the epr spectrum as measured from the height of peak 3. (Under similar conditions the addition of actin to spin-labeled HMM caused a 27% decrease in the height of peak 3.) Viscosity measurements were also carried out in an attempt to determine whether physical interaction between actin and SL-HMM-S-S-DP occurs. The results (Table III) indicate that the interaction

[2] The possibility of interacting sites was ruled out by the finding that Hill plots of the data from Figure 4 gave slopes close to 1.

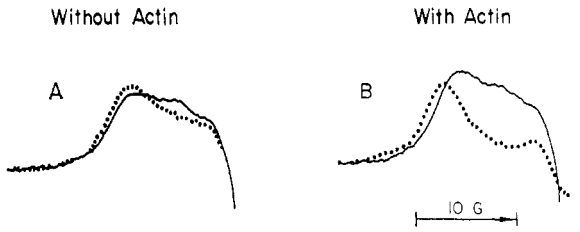


FIGURE 6: Effect of actin on spin-labeled HMM during and after steady-state hydrolysis of ATP. The samples contained 31 $\mu$M HMM in 15 mM Tris-Cl, 15 mM histidine chloride (pH 8.0), and 13 mM KCl: (A) without actin; (B) 63 $\mu$M actin added. Low-field lines of the spectrum were recorded 2 min (—) and 15 min (· · ·) after the addition of 20 mM MgATP.

between actin and SL-HMM-S-S-DP (log $\eta_{rel}$ = 1.54 times theoretical value) is much less than that between actin and spin-labeled HMM either in the presence (3.70) or absence (3.24) of ADP, but still somewhat greater than that measured for the same system during steady-state hydrolysis of ATP (1.15).

*Effect of Actin on Spin-Labeled HMM during ATP Hydrolysis.* During steady-state hydrolysis of ATP the predominant species is currently believed to be a myosin*ADP·P complex (where the asterisk denotes a state different from that obtained by equilibration of myosin with products) (Viniegra-Gonzalez and Morales, 1972; Trentham *et al.*, 1972). Seidel and Gergely (1971) have reported that the mobility of spin labels attached to the $SH_1$ groups of myosin is greatly increased in this complex. With complete hydrolysis of ATP the spectrum decays to one identical with that obtained by adding MgADP. We have observed essentially similar results with HMM at low ionic strength (Figure 6A). The addition of actin does not alter the spectrum observed during ATP hydrolysis (Figure 6B, solid line) but does decrease the mobility of the labels following exhaustion of ATP (Figure 6B, broken line). The latter spectrum is identical with that obtained by adding MgADP and actin to spin-labeled HMM. Although actin did not alter the mobility of the labels during steady-state hydrolysis of ATP, it did reduce the lifetime of this state indicating that the actin used in this study was producing the normal acceleration of the HMM-catalyzed hydrolysis of ATP.

*Effect of Actin on Spin-Labeled HMM in the Presence of Nucleoside Triphosphates.* In order to study possible effects of actin on the HMM·ATP complex two synthetic analogs have been employed. AMP-PNP binds well to HMM but is not hydrolyzed (Yount *et al.*, 1971b). The spectral change induced by the binding of this analog to spin-labeled HMM (Figure 7) is indistinguishable from that produced by ADP (Seidel and Gergely, 1972). The addition of actin (Figure 7) was found to decrease the mobility of spin labels attached to the HMM·AMP-PNP complex when the total AMP-PNP concentration was 5 mM. As the concentration of AMP-PNP was increased the spectral effect of actin was decreased. At 20 mM AMP-PNP the mobility of the spin labels attached to HMM was nearly the same in the presence or absence of actin.

The other analog, AMP-CPP, is a substrate for myosin. However, in contrast to ATP, the rate-limiting step in the dephosphorylation of AMP-CPP is the hydrolysis step

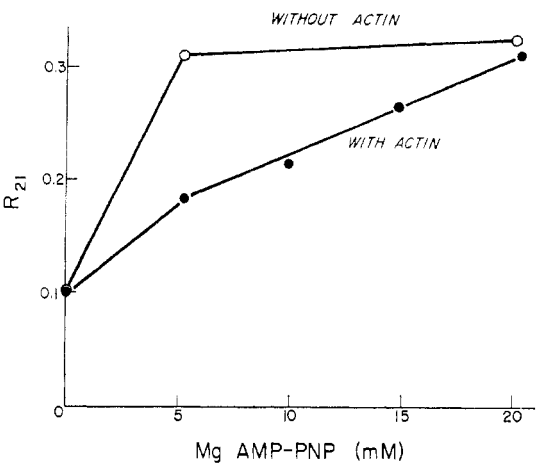


FIGURE 7: Effect of AMP-PNP on the epr spectrum of spin-labeled HMM in the presence and absence of actin. Conditions: 34 $\mu$M spin-labeled HMM in 110 mM Tris-Cl, 20 mM histidine chloride (pH 8.3), 4 mM KCl, and 0–20 mM MgAMP-PNP; (○) without actin; (●) in the presence of 74 $\mu$M actin (dialyzed *vs.* nucleotide-free solvent). The pH of 8.3 was chosen to assure complete dissociation of the terminal phosphate proton of AMP-PNP (see Yount *et al.*, 1971a). Under identical conditions the addition of 4 mM MgADP to the spin-labeled HMM (in the absence of actin) resulted in a value for $R_{21}$ of 0.314.

TABLE II: Effect of Actin on Affinity Labeled HMM.[a]

| Additions | Ratio of Peak Heights, 2:1 | |
|---|---|---|
| | Spin-Labeled HMM | SL-HMM-S-S-DP |
| None | 0.422 | 1.24 |
| 4 mM ADP | 0.740 | 1.38 |
| 4 mM ADP + 81 $\mu$M actin | 0.645 | |
| 81 $\mu$M actin | | 1.25 |
| 4 mM ATP | 0.856[b] | |

[a] The samples contained 25 $\mu$M HMM in 36 mM $NaHCO_3$ (pH 8.0), 16 mM KCl, 4 mM $MgCl_2$, and the indicated additions. The two samples of HMM were from the same preparation and were treated identically except for the addition of SH-TP (see Experimental Procedure). [b] Recorded during steady-state hydrolysis of ATP.

TABLE III: Effect of Affinity Labeling on the Viscosity of Solutions of Actin and HMM.[a]

| Additions | Log $\eta_{rel}$ (Multiple of Theor Value)[b] | |
|---|---|---|
| | Spin-Labeled HMM | SL-HMM-S-S-DP |
| None | 3.24 | 1.54 |
| 5 mM ATP | | |
| Initial | 1.15 | |
| Final | 3.70 | |

[a] The samples contained 0.71 mg/ml of HMM and 0.30 mg/ml of actin in 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0), 5 mM KCl, and 5 mM $MgCl_2$. Preparation of the HMM samples is described under Experimental Procedure. [b] The theoretical value of log $\eta_{rel}$ is the sum of log $\eta_{rel}$ for actin and HMM measured individually. This value was 0.071 for spin-labeled HMM and 0.070 for SL-HMM-S-S-DP.

TABLE IV: Rate of Hydrolysis of AMP-CPP.[a]

| Additions | μmol of $P_i$ $g^{-1}$ $sec^{-1}$ | |
|---|---|---|
| | Unlabeled HMM | Spin-Labeled HMM |
| None | 0.98 | 0.45 |
| Actin | 0.55 | 0.52 |

[a] Assay conditions: 25 mM Tris-Cl, 25 mM histidine chloride (pH 8.0, 25°), 5 mM KCl, 1 mM $MgCl_2$, 1 mM AMP-CPP, 0.38 mg/ml of unlabeled or spin-labeled HMM, and (where indicated) 0.12 mg/ml of F-actin.

TABLE V: Effect of Actin on Spin-Labeled HMM in the Presence of α,β-Methylene Analogs of ADP and ATP.[a]

| Nucleotide (5 mM) | Ratio of Peak Heights, 2:1 | |
|---|---|---|
| | Without Actin | With Actin |
| None | 0.087 | 0.111 |
| ADP | 0.326 | 0.174 |
| AMP-CP | 0.106 | 0.107 |
| AMP-CPP (initial) | 0.260[b] | 0.247 |
| AMP-CPP (final) | 0.137[b] | 0.085 |

[a] The samples contained 34 μM spin-labeled HMM in 110 mM Tris-Cl, 20 mM histidine chloride (pH 8.2), 4 mM KCl, and 5 mM $MgCl_2$; where indicated 5 mM nucleotide and 74 μM actin (dialyzed *vs.* nucleotide-free solvent) were added. [b] Nucleotide concentration was 1 mM in these samples.

(Mannherz *et al.*, 1973). Consequently, the predominant species during steady-state hydrolysis should be a complex of HMM with the triphosphate form of this analog. Since spin labeling has only a small effect on the overall rate of AMP-CPP hydrolysis (Table IV) it seems reasonable to assume that the same HMM·AMP-CPP species predominates during hydrolysis by the spin-labeled enzyme. The spectral change observed immediately after addition of AMP-CPP to spin-labeled HMM is close to that observed for ADP (Table V). This is consistent with the presumption that HMM·AMP-CPP (and not, for example, HMM*AMP-CP·P) is the predominant species during steady-state hydrolysis of this compound. Following complete hydrolysis of AMP-CPP the spectrum decayed to one similar to that obtained by adding AMP-CP. The latter change was small and could not be increased by increasing the concentration of AMP-CP to 20 mM. It is known that AMP and adenosine have little or no effect on the spectrum of spin-labeled myosin (Stone, 1970). Consequently, it is possible that substitution of the methylene bridge for an oxygen bridge prevents the α- and/or β-phosphates from binding to the enzyme surface. Actin does not accelerate the hydrolysis of AMP-CPP by either unlabeled (Mannherz *et al.*, 1973; Table IV) or spin-labeled (Table IV) HMM. The addition of actin had only a slight effect on the change in spin-label mobility induced by binding of AMP-CPP to spin-labeled HMM (Table V).

## Discussion

Before interpreting data obtained from molecular probes it is essential to fully understand any perturbations created in the protein under study by the introduction of the probe. The iodoacetamide spin label, like other sulfhydryl reagents, modifies the catalytic activity of myosin. Both the $Ca^{2+}$- (Quinlivan *et al.*, 1969) and $Mg^{2+}$- (this study) moderated activities are increased while the $K^+$-moderated activity (Seidel *et al.*, 1970) is lost following reaction with 2 mol of spin label/mol of myosin or HMM. The enhancement of the $Mg^{2+}$-moderated, low ionic strength activity is not accompanied by any significant alteration in the affinity of HMM for ADP. From the spectral change induced by the binding of ADP to spin-labeled HMM (Figure 5) we obtained an apparent dissociation constant of 5 μM. Recent determinations of ADP binding to unmodified HMM by the method of equilibrium dialysis have yielded $K_d$ values of 3.3–10 μM for similar ionic conditions (Lowey and Luck, 1969; Malik and Martonosi, 1971). It was previously reported (Stone, 1970) that the affinity of spin-labeled myosin for MgADP was considerably weaker than that determined by equilibrium dialysis for unlabeled myosin. Since the studies with myosin were carried out in a high ionic strength medium it seems possible that the effect of spin labeling on nucleotide affinity depends on the ionic strength of the medium.

The formation of an actin·HMM complex appears to be little affected by reaction of the HMM $SH_1$ groups with the iodoacetamide spin label. Under conditions of excess actin there was no perceptible change in the viscosity attained in the presence of ADP. Nearly complete dissociation of the complex by ATP was also observed. The spectral change observed upon addition of actin to the spin-labeled HMM·ADP complex (Figure 3) saturates at approximately 2 mol of actin monomer/mol of HMM. Assuming a low $K_d$ for the actin·HMM complex, this value may be taken as the binding ratio and agrees well with results obtained with unmodified HMM by the light scattering technique (see, for example, Takeuchi and Tonomura, 1971). More direct estimates of the binding ratio made from measurements of the small amount of unbound HMM remaining following centrifugation of mixtures of actin and HMM have yielded values of both 1 (Young, 1967) and 2 (Takeuchi and Tonomura, 1971) actin monomers per HMM. Although the formation of the actin·HMM complex is not affected by spin labeling, the catalytic activity of the complex is impaired. The velocity of ATP hydrolysis is only one-sixth that observed for unlabeled HMM. A similar effect has recently been reported for *N*-ethylmaleimide-treated HMM (Silverman *et al.*, 1972).

In the absence of nucleotide actin binds to spin-labeled HMM (Table III) producing a decrease in spin-label mobility. When labeling is restricted to the $SH_1$ groups the decreased mobility is manifested in a decrease in spectrum amplitude; no significant changes in $R_{21}$ occur. (Occasionally an increase in $R_{21}$ (indicating an increase in spin-label mobility) was observed upon addition of actin to spin-labeled HMM. An example of this appears in Table V. It seems probable that this arises from small amounts of free ADP in the actin samples. Despite complete removal of unbound nucleotide by exhaustive dialysis against nucleotide-free solvent, small amounts of nucleotide are always present in these solutions due to a slow release from the binding sites on the actin polymer (Kasai and Oosawa, 1969).)

In the presence of ADP actin decreased the mobility of the spin labels relative to that observed for the HMM·

diphosphate complex alone. Both the spectrum amplitude and the value of $R_{21}$ were decreased. Since actin has no effect on $R_{21}$ in the absence of nucleotide, the effect observed in the presence of ADP may be interpreted as a reversal of ADP binding. This interpretation is substantiated by our finding of a marked increase in the apparent dissociation constant for the HMM·ADP complex in the presence of actin (Figure 5). The latter finding is consistent with previous reports by Kiely and Martonosi (1968, 1969) and Lymn and Taylor (1971) that actin facilitates the dissociation of ADP from myosin and HMM. It has been argued previously (Seidel *et al.*, 1970; Stone, 1970) that the increase in spin-label mobility associated with nucleotide (or pyrophosphate) binding is due to an alteration in protein conformation in the area of the $SH_1$ groups. The results reported here suggest that binding of actin opposes this ligand-induced conformational change.

In the presence of sufficient nucleoside triphosphate (*e.g.*, AMP-PNP, AMP-CPP) actin caused no change in the mobility of the spin labels relative to that observed for the HMM·triphosphate complex alone. These results are consistent with dissociation of actin and HMM by nucleoside triphosphates and suggest that dissociation by ATP does not require hydrolysis of the terminal phosphate bond. Dissociation by AMP-PNP was observed previously with intact fibers (Chaplain and Frommelt, 1968; dos Remedios *et al.*, 1972) and with purified actomyosin at high ionic strength (Yount *et al.*, 1971b). The very high concentration of AMP-PNP required for dissociation in the present study seems surprising and is possibly due to modification of HMM by the spin label. It is perhaps pertinent that Silverman *et al.* (1972) have observed an increase in the apparent binding constant of HMM·ATP to actin following reaction of the $SH_1$ groups of HMM.

During steady-state hydrolysis of ATP, when the predominant species is believed to be an HMM*ADP·P intermediate (Viniegra-Gonzalez and Morales, 1972; Trentham *et al.*, 1972), actin had no effect on spin-label mobility. Seidel and Gergely (1972) have recently reported a similar finding. Under these conditions the viscosity of the solutions of actin and HMM was low, but always slightly higher than that calculated for a noninteracting solution of the two proteins. It has been suggested by Viniegra-Gonzalez and Morales (1972) that myosin treated with SH-TP is a model of myosin*-ADP·P. The greatly enhanced mobility observed for SL-HMM-S-S-DP is consistent with this species being a pure form of that which predominates during steady-state hydrolysis. Addition of actin to SL-HMM-S-S-DP did not alter the value of $R_{21}$. However, there was a slight decrease in spectrum amplitude and a significant increase in viscosity suggesting some interaction between actin and SL-HMM-S-S-DP. It is an attractive speculation that actin recombines with myosin when the latter is in the M*ADP·P state (dos Remedios *et al.*, 1972). Our data provide some support for a weak physical interaction. In the case of the affinity-labeled protein, no effect on $R_{21}$ is observed since nucleotide release is prevented by covalent binding. The lack of effect on spin-label mobility during ATP hydrolysis suggests that the steps subsequent to actin rebinding are fast and therefore not observed in a steady-state measurement.

Although the nucleotide and actin binding sites on myosin are clearly separate, attachment of ligand to these two sites appears in some cases to be competitive; *i.e.*, in the presence of both nucleoside triphosphate and actin only one ligand binds and the resulting complex is determined by the relative affinities and concentrations of ligands. In the presence of relatively low concentrations of ADP ($\leq$0.1 mM) actin appears to compete successfully with ADP with the result that HMM is bound largely to actin. At higher ADP concentrations there was some increase in spin-label mobility suggesting formation of HMM·ADP in lieu of actin·HMM. However, even with ADP concentrations as high as 20 mM (Figure 6) this effect was only partial and extrapolation of the data in Figure 4 indicates a greatly reduced value for $\Delta R_{21}$ at saturating ADP in the presence of actin. Since there is no evidence for a decrease in the number of binding sites (in fact, an increase is indicated in Figure 5) it appears that the magnitude of the spectral change associated with binding at each site is reduced. It seems probable that under these conditions a ternary actin·HMM·ADP complex is formed.

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# Exchangeability of Bicarbonate Specifically Bound to Transferrin†

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ABSTRACT: The Fe(III)- and anion-binding functions of transferrin are strongly interdependent, with specific binding of either dependent on the presence of the other. Under physiologic conditions, bicarbonate is the anion preferentially bound by transferrin, although a variety of other anions are also capable of occupying the specific anion-binding site of the protein. Because of its possible role in the uptake of iron from transferrin by the reticulocyte, studies were undertaken of the exchangeability of transferrin-bound bicarbonate with bicarbonate free in solution. The rate of exchange depends on the anionic composition of the medium. At physiological pH and ambient $p$-$CO_2$, bicarbonate exchange is detectable but slow, with a half-time of about 20 days. The presence of millimolar concentrations of citrate or nitrilotriacetate increases the exchange rate by two orders of magnitude. Increasing the bicarbonate concentration also increases the exchange rate in an approximately proportional manner. The exchange of bicarbonate from monoferric transferrin prepared by isoelectric focusing is describable by a simple first-order plot. However, exchange from diferric transferrin is more complex and requires two exponential terms to fit the data satisfactorily. In every case studied the half-time for exchange in monoferric transferrin has a value intermediate to the two half-times for exchange in differric transferrin. These results point to an interaction between the two specific anion-binding sites of the protein. They may account, in part, for the observed difference in the rates at which iron is taken up from the two iron-binding sites of transferrin by the reticulocyte.

The transferrin molecule consists of a single polypeptide chain of mol wt ~80,000 (Greene and Feeney, 1968; Mann *et al.*, 1970), on which are disposed two specific metal-binding sites with identical, or nearly so, thermodynamic and spectroscopic properties (Aasa *et al.*, 1963; Aisen *et al.*, 1966). For each metal ion bound, an anion must also be bound. For a time it was thought that anion binding was facultative and that specific binding of ferric ions could occur in the absence of anions (Aasa and Aisen, 1968). However, recent studies indicate that for specific binding of Fe(III) to occur, binding of a suitable anion is obligatory (Price and Gibson, 1972). On the basis of present information, bicarbonate (or, possibly, carbonate (Williams and Woodworth, 1973)) seems to be the anion most favored by the protein. In its absence, however, specific binding of Fe(III) will occur if oxalate, malonate, EDTA, nitrilotriacetate, thiogylcollate, or other suitable anions are available to occupy the anion-binding site of transferrin (Aisen *et al.*, 1967; Aisen and Pinkowitz, 1973). The stoichiometries of specific anion binding and Fe(III) binding are identical, and neither metal ion nor anion is tightly bound in the absence of the other (Schade and Reinhart, 1966; Aisen and Leibman, 1973). Thus, the anion- and metal-binding functions of transferrin may be described as showing strong positive cooperativity.

Recently, evidence has been presented indicating that the mechanism of iron transfer from transferrin to the reticulocyte involves the anion-binding site of the protein. It has been suggested that disruption of the anion–protein linkage must precede delivery of iron to the cell (Aisen and Leibman, 1973; Egyed, 1973). The present studies of the exchange of transferrin-bound bicarbonate were undertaken to gain understanding of some of the factors which affect the stability of the

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