SATURATION TRANSFER ELECTRON PARAMAGNETIC RESONANCE STUDY OF THE MOBILITY OF MYOSIN HEADS IN MYOFIBRILS UNDER CONDITIONS OF PARTIAL DISSOCIATION

SHIN'ICHI ISHIWATA,* BARBARA A. MANUCK,* JOHN C. SEIDEL,*‡ AND JOHN GERGELY*‡§
*Department of Muscle Research, Boston Biomedical Research Institute; ‡Departments of Biological Chemistry and Neurology, Harvard Medical School, Boston, Massachusetts 02114; and §Department of Neurology, Massachusetts General Hospital, Boston, Massachusetts, 02114

ABSTRACT The rotational motion of rigidly spin-labeled myosin heads of glycerinated myofibrils as reflected in saturation-transfer EPR spectra behaves to a first approximation as though the heads consist of two populations with different rotational motions. An immobilized fraction has a correlation time (τ_2) of ~0.5 ms, comparable to that of spin-labeled subfragment-1 (S1) bound to thin filaments, while a mobile fraction has a τ_2 of 10 μs, comparable to that of the heads of purified myosin filaments. The effects of nonhydrolyzable ATP analogues, potassium pyrophosphate (PP_i), or adenylyl imidodiphosphate, Ca^{2+}, temperature, or ionic strength on the spectra can be analyzed in terms of the fraction of myosin heads immobilized by attachment to thin filaments, without requiring changes in the motion of either attached or detached heads.

INTRODUCTION

Saturation-transfer spectroscopy (ST-EPR) has been used to measure submillisecond rotational motion of rigidly spin-labeled S1 or HMM or that of the myosin heads of purified myosin filaments or myofibrils (Thomas et al., 1975, 1980; Cooke et al., 1982). These motions may be particularly relevant to muscle contraction, occurring on the same time scale as molecular events associated with force generation (Huxley and Simmons, 1971, 1972). Use of fluorescent or paramagnetic probes has revealed motion of the heads in myosin filaments relative to the filament backbone, with rotational correlation times of the order of 10 μs (Mendelson et al., 1973; Thomas et al., 1975, 1980; Eads et al., 1984; Kinoshita et al., 1984), interpreted as an indication of flexibility of the myosin molecule at the junction of the S1 and S2 regions. Upon attachment of myosin heads to F-actin, τ_2 increases to ~0.5 ms, equal to that of a spin label rigidly bound to the actin filament, indicating a substantial degree of rigidity of the bond between the head and the actin filament.

The correlation time of the spin-labeled myosin heads in myofibrils decreases to ~10 μs during steady state hydrolysis of ATP, and when all ATP has been hydrolyzed it returns to the value observed in rigor. Although Ca^{2+} accelerates ATP hydrolysis, it has no detectable effect on the rotation of the heads during the steady state (Thomas et al., 1980), a finding consistent with the observation that Ca^{2+}, ATP, or ATP analogues do not affect the rotation of myosin heads in filaments of purified myosin (Mendelson and Cheung 1976, 1978).

In this study we observed that nonhydrolyzable ATP analogues, PP_i,[1] and AMPPNP, under conditions of partial dissociation, increase the rotational motion of myosin heads in myofibrils. These results are consistent with motion of myosin heads being determined primarily by whether or not they are attached to thin filaments, attached and detached heads having the rates of motion found for actomyosin and myosin filaments, respectively. Neither Ca^{2+} nor ATP analogues have any detectable, direct effect on the rotation of attached heads estimated by ST-EPR. A preliminary report of this work has been presented previously (Ishiwata et al., 1979).

MATERIALS AND METHODS

Preparation of Myofibrils

Rabbit skeletal muscle was homogenized for 30 s in a Waring blender in 4 volumes of a solution containing 60 mM KCl, 25 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 1 mM NaN_3, 1 mM EGTA, and 2 mM PP_i. The homogenate was centrifuged at 6,000 g for 15 min, again homogenized, and washed

Dr. Ishiwata's present address is Department of Physics, School of Science and Engineering, Waseda University, Tokyo 160, Japan.

[1]*Abbreviations used in this paper:* AMPPNP, adenylyl imidodiphosphate; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HMM, heavy meromyosin; MSL, N-(1-oxyl-2,2,6,6,-tetramethyl-4-piperidinyl) maleimide; PP_i, potassium pyrophosphate; S1, subfragment-1.

four times with 4 volumes of a solution containing 60 mM KCl, 25 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 1 mM NaN_3, and 1 mM EGTA (rigor buffer); the bottom layer of the pellet was discarded at each step. If necessary, homogenization and centrifugation were repeated until by phase-contrast microscopy the myofibrils appeared well dispersed with a regular sarcomere pattern. Myofibrils were stored in 50% glycerol at −20°C. The protein concentration was determined by the biuret method standardized with bovine serum albumin (Gornall et al., 1949).

Myofibrils were spin-labeled essentially as described previously, except for the use of PP_i in place of ATP (Thomas et al., 1980). After pretreatment with 10 μM MalNEt for 10 min, myofibrils were labeled with MSL, washed with rigor buffer to remove unreacted MalNEt or MSL, and incubated in 25 mM $K_3Fe(CN)_6$ for 40 h at 0°C to destroy any weakly immobilized components of the EPR spectra (Graceffa and Seidel, 1980). The myosin heads contained 90–95% of the bound label, virtually all of it attached to SH-1, as indicated by the extent of inhibition of the K^+-EDTA-activated ATPase activity. About 25% of the heads were labeled and the Mg^{2+}-activated ATPase activity was unchanged by the labeling. Rigid binding of the attached label was indicated by an L''/L of 1.08 for spin-labeled S1 immobilized on glass beads, which decreased to 1.03 and 1.04 on addition of PP_i or AMPPNP, respectively (Table I), indicating that local rotation of the label is small compared to that of myosin heads, with or without ATP analogues.

Preparation and Spin-labeling of Proteins

S1 and HMM labeled with MSL were prepared by digesting spin-labeled myosin with chymotrypsin (Weeds and Pope, 1977). MSL-labeled myosin was prepared by labeling purified myosin (Seidel et al., 1970; Thomas et al., 1980) or by extraction of spin-labeled myofibrils with Hasselbach-Schneider solution (Hasselbach and Schneider, 1951). Purified unlabeled myosin was prepared from rabbit skeletal muscle as described by Nauss et al. (1969). Actin was obtained from rabbit skeletal muscle as described by Spudich and Watt (1971). MSL-S1 was covalently coupled to glass beads as described previously (Bottomley and Trayer, 1975; Thomas et al., 1980). The molecular weights of myosin, HMM, S1, and actin were taken to be 4.8×10^5, 3.7×10^5, 1.15×10^5 and 4.2×10^4.

EPR Spectra

Conventional (V_1) and ST-EPR (V_2') spectra were recorded in a spectrometer (model E-109E; Varian Associates, Inc., Palo Alto, CA) as described previously (Thomas et al., 1980). The temperature of the sample was kept constant by flowing nitrogen gas of the appropriate temperature through the radiation slits of the E231 cavity. Rotational correlation times were estimated from the ratio of L and L'' (Fig. 1), using the signal intensities of two low-field spectral peaks (Thomas et al., 1976, 1980). Spectra were scanned once at modulation rates of 100 or 200 Gauss/h with time constants of 4 and 8 s, respectively. The concentration of Ca^{2+} was controlled with an EGTA buffer system (Potter and Gergely, 1975).

TABLE I

EFFECTS OF PP_i, AMPPNP, HIGH SALT AND TEMPERATURE ON MOTION OF MSL-S1 IMMOBILIZED ON GLASS BEADS

	Temperature	L''/L
	°C	
Rigor buffer	10	1.08
	27	0.95
+ 10 mM PP_i	10	1.03
0.2 M KCl	27	0.90
+ 4mM AMPPNP	10	1.04
0.2 M KCl	27	0.89
+ 0.44 M KCl	10	1.04
	27	0.95

MSL-labeled S1 covalently coupled to glass beads was suspended in solutions containing 60 mM KCl unless otherwise noted, 25 mM MOPS, pH 7.0, 5 mM $MgCl_2$, and 1 mM NaN_3. MSL-S1 was coupled to glass beads as described previously (Thomas et al., 1980; Bottomley and Trayer, 1975).

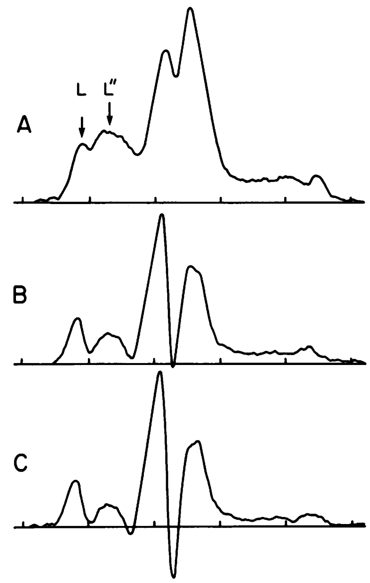

FIGURE 1 ST-EPR spectra of spin-labeled myofibrils in the presence and absence of PP_i and Ca^{2+}. Spectra were measured in suspensions containing 25 mM MOPS, pH 7.0, 5 mM $MgCl_2$, and 1 mM NaN_3 at 10°C. (*A*) 60 mM KCl, myofibrils (45 mg/ml); (*B*) 0.2 M KCl, 10 mM PP_i, 0.1 mM $CaCl_2$, and myofibrils (36 mg/ml); (*C*) 0.2 M KCl, 10 mM PP_i, 1 mM EGTA, and myofibrils (36 mg/ml).

To estimate the binding of MSL-S1 to F-actin, mixtures of MSL-S1 and unlabeled F-actin were centrifuged at 100,000 g for 30 min and the concentration of free MSL-S1 determined by double integration of the V_1 spectrum of the supernatant fraction.

RESULTS

Estimation of the Fraction of Immobilized Heads

On the assumption, discussed below, that in the presence of actin the heads assume one of two states having $\tau_2 = 10\ \mu s$ (mobile) or $\tau_2 = 0.5$ ms (immobilized), we have expressed the results in terms of a fraction of immobilized heads estimated from the ST-EPR motional parameter, L''/L (Thomas et al., 1976). The observed values of the spectral intensities, L_{obs} and L''_{obs}, for a mixture of mobile and immobilized labels at some concentration c can be expressed as

$$L_{obs} = f L_i + (1 - f) L_m \quad (1)$$

$$L''_{obs} = f L''_i + (1 - f) L''_m, \quad (2)$$

where f is the fraction of immobilized labels, L_i and L''_i are the spectral amplitudes of the fraction of immobilized labels, and L_m and L''_m are those of the mobile fraction, each referring to concentration c. Values of L_i and L''_i were obtained from the V_2' spectrum of labeled myofibrils in rigor and values of L_m and L''_m from myofibrils under relaxing conditions, at the same concentration c. In experiments with F-actin and MSL-S1, values of L_i and L''_i were obtained from the spectrum of acto-MSL-S1, and L_m and L''_m were obtained from that of MSL-S1 alone. To produce the calibration curve for estimating the fraction of immobilized heads (f) from L''/L, the above values of L_i, L''_i, L_m and L''_m were used to calculate values of L_{obs} and L''_{obs}, and a plot of L''/L against f was constructed (Fig. 2).

To verify the assumption that the motional parameters are weighted sums of the values for the mobile and immobilized states, we investigated the dependence of L and L'' on the concentration of actin monomers, in a model two-state system consisting of a mixture of MSL-S1 and unlabeled F-actin. As expected from Eqs. 1 and 2, both L and L'' increase linearly with actin concentration up to a molar ratio (actin/S1) of 1/1, with no further change at higher ratios, where all S1 is bound to actin (Fig. 3). The spectral amplitudes in the central region of the spectrum, C and C' (Thomas et al., 1976) also increased linearly with actin concentration and leveled off as did L and L'' (not shown).

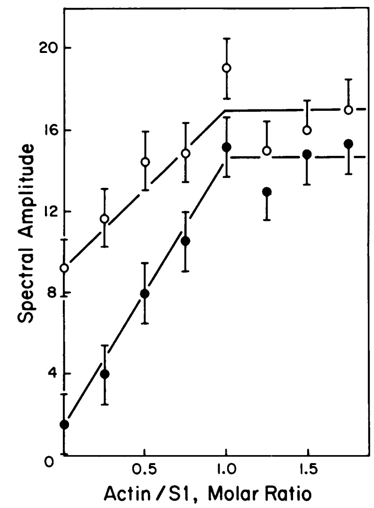

FIGURE 3 The intensities of ST-EPR signals of spin-labeled S1 at L (O) and L'' (O) on titration with F-actin. Spectra were obtained in solutions containing 60 mM KCl, 25 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 1 mM NaN_3, and S1 (1.9 mg/ml, 17 μM) at 20°C. Error bars reflect the average deviation of two or three scans obtained with the same sample.

Comparison of Immobilization and Binding of MSL-labeled S1 by F-actin

To determine how accurately the ST-EPR spectra reflect binding of S1 to actin, the effect of AMPPNP on the ST-EPR spectra of acto-MSL-S1 was compared with its effect on S1 binding determined by sedimentation. The fraction of MSL-S1 bound to F-actin was determined at increasing concentrations of AMPPNP by sedimentation and double integration of the V_1 spectrum of the supernatant solution. The fraction of immobilized MSL-S1 was estimated from the V_2' spectrum using a calibration curve similar to that in Fig. 2, constructed from the spectra of free MSL-S1 and acto-MSL-S1 at a 2/1 molar ratio of actin to S1. The fraction of immobilized heads varied from 0.2 to at least 0.9, and within the experimental error it did not differ from the fraction of bound S1 (Fig. 4).

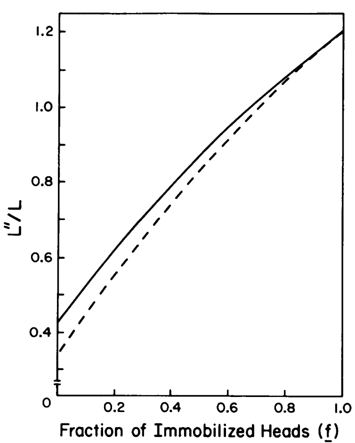

FIGURE 2 Relationship between L''/L and f used to estimate the fraction of immobilized heads in spin-labeled myofibrils. Values of L_m and L''_m were obtained from spectra of spin-labeled myofibrils under relaxing conditions, i.e., in the presence of ATP and EGTA, and L_i and L''_i were obtained from spectra of spin-labeled myofibrils in rigor, i.e., after the supply of ATP was exhausted. These spectra were obtained in the presence of 60 mM KCl, solid line or 0.5 M KCl, dashed line.

Effects of PP_i and Ca^{2+} on Rotational Motion of Myosin Heads

At low KCl concentrations, PP_i produces a small decrease in L''/L, but at higher KCl concentrations its effect becomes larger, approaching in magnitude that of ATP (Fig. 5); complete spectra in the presence and absence of PP_i are shown in Fig. 1. Under conditions where the concentration of KCl is not expected to affect the fraction of attached heads, viz., in the absence of nucleotides when

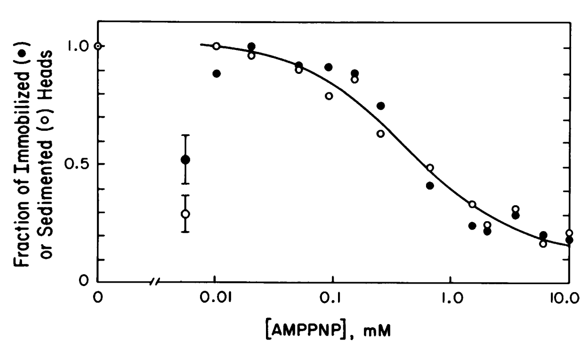

FIGURE 4 A comparison of the immobilization of spin-labeled S1 and its binding to F-actin. Spectra were recorded using MSL-labeled S1 (9.4 μM) and F-actin (60 μM) in a solution containing 0.2 M KCl, 50 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 1 mM NaN_3, and AMPPNP as indicated at 27°C. Error bars indicate the average range of f for eight different concentrations of AMPPNP using three values of f at each concentration.

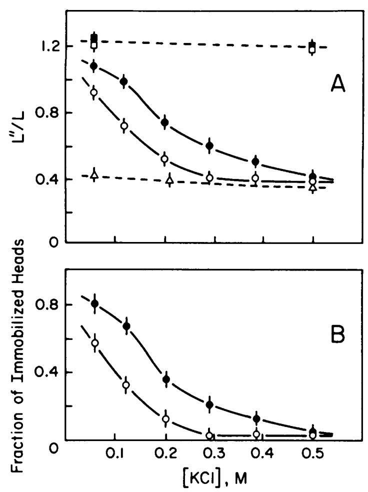

FIGURE 5 Effect of PP_i, Ca^{2+} and KCl concentration on the motion of myosin heads. Spin-labeled myofibrils (20 mg/ml) were suspended in solutions containing KCl as indicated, 22 mM MOPS, pH 7.0, 4.7 mM $MgCl_2$, and 0.9 mM NaN_3. Spectra were measured at 10°C. (*A*) L''/L; B, fraction of immobilized heads estimated from Fig. 2. (□, ■) no addition; (○, ●) 10 mM PP_i; (△) 5 mM ATP, 50 mM creatine phosphate, and 1 mg of creatine phosphokinase per ml. Open symbols, 1 mM EGTA; closed symbols, 0.1 mM $CaCl_2$.

all heads are attached or in the presence of MgATP when essentially all are detached, the concentration of KCl does not affect L''/L.

A Ca^{2+}-induced increase in L''/L is superimposed on the effect of PP_i. Ca^{2+} shifts the curve upwards, reflecting decreased motion of the heads as might be expected if the fraction bound to thin filaments were increased (Figs. 1, 5). Ca^{2+} has no effect at high concentrations of KCl in the presence or absence of PP_i, suggesting that Ca^{2+} does not affect the motion of heads in rigor or that of detached heads. Upon removing of PP_i or Ca^{2+} by washing the myofibrils with rigor buffer, L''/L returns to its initial value. The maximal decrease in L''/L induced by PP_i is independent of Ca^{2+}.

L''/L gradually increases from 0.7 to 0.9 with increasing Ca^{2+} concentration in the presence of PP_i, the change being half maximal at 2×10^{-6} M Ca^{2+} (Fig. 6). Plots of L''/L and plots of f have essentially the same dependence on pCa; since this is also true for the dependence on ionic strength (Fig. 5), concentration of PP_i, or temperature, only the fraction of immobilized heads is given in subsequent figures. Similar results are obtained with AMPPNP, the change induced by Ca^{2+} being half maximal at 6×10^{-7} M (Fig. 6). With 5 mM AMPPNP the concentration of Ca^{2+} producing a half maximal spectral change was the same with 4.5 or 10 mM $MgCl_2$. Together with the values of pCa at half maximal activation, this indicates that Ca^{2+} is acting at the Ca^{2+}-specific sites of troponin-C (Potter and Gergely, 1975). Ca^{2+} shifts the dependence of f on the concentration of PP_i toward higher values, the concentration producing half maximal change increasing from 15 to 50 μM (Fig. 7).

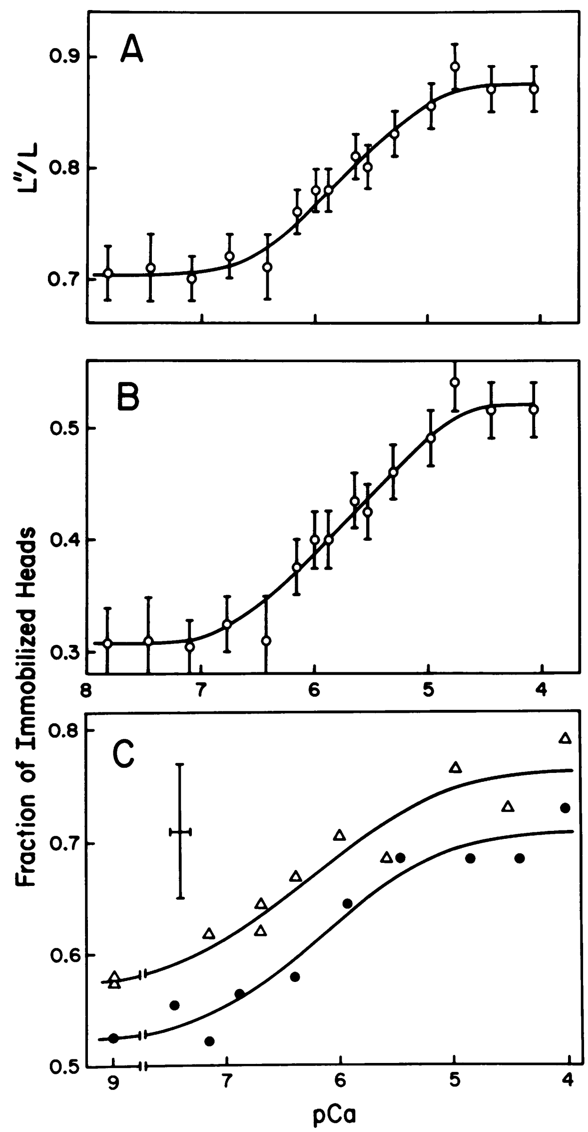

FIGURE 6 Effects of Ca^{2+} concentration in the presence of PP_i or AMPPNP, on the motion of myosin heads. (*A* and *B*) spin-labeled myofibrils (30 mg/ml) were suspended in solutions containing 45 mM KCl, 98 mM MOPS, pH 6.9, 5 mM $MgCl_2$, 10 mM PP_i, 1 mM EGTA, 0.75 mM NaN_3, and the indicated concentrations of $CaCl_2$ at 10°C. (*C*) spin-labeled myofibrils (28 mg/ml) were suspended in solutions containing 80 mM KCl, 5 mM AMPPNP 1 mM EGTA, 0.7 mM NaN_3 at 10°C. (△) 4.5 mM $MgCl_2$, 147 mM MOPS, pH 6.9; ●, 10 mM $MgCl_2$, 127 mM MOPS, pH 6.9. (*B* and *C*) fraction of immobilized heads estimated from Fig. 2.

Effect of Temperature

In the presence of 10 mM PP_i, increasing the temperature increases f with or without Ca^{2+}. Ca^{2+} shifts the curve upward without changing the shape (Fig. 8). Increasing the temperature could affect L''/L in two ways: decreasing the motion as more heads become bound, and increasing local motion of the label, viz., the rotation of the spin label relative to the protein (e.g., Johnson, 1978, 1979). The 10–15% decrease in L''/L between 10° and 27°C for MSL-labeled S1 immobilized on glass beads (Table I)

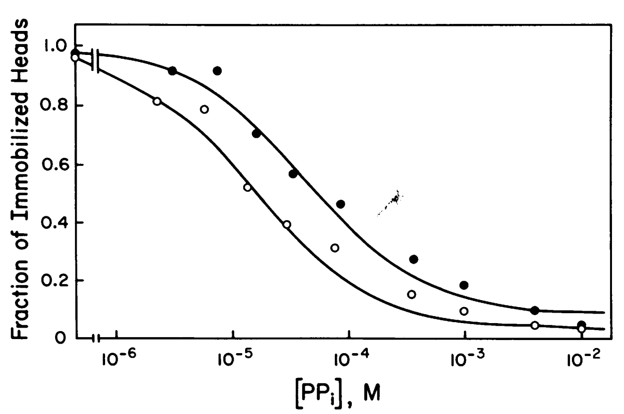

FIGURE 7 Effect of PP_i concentration on the fraction of immobilized myosin heads. Spin-labeled myofibrils (17.6 mg/ml) were suspended in solutions containing 0.5 M KCl, 21 mM MOPS, pH 7.0, 4.2 mM $MgCl_2$, and 0.85 mM NaN_3 at 10°C. Open symbols, 1 mM EGTA; closed symbols, 0.1 mM $CaCl_2$.

indicates that such local motion contributes little to the overall motion of the spin label. Temperature also had little influence on L''/L of attached heads in rigor, therefore changes in local rotation of the label appear to make only minor contributions to the spectra.

In analyzing the data in Fig. 8, we assume an equilibrium between attached (immobile) and detached (mobile) heads. An apparent equilibrium constant for attachment, defined as the ratio of immobile, attached heads to mobile, detached heads, determined from the V_2' spectra, was estimated at each temperature and plotted on a logarithmic scale against the inverse of the absolute temperature. From this plot, ΔH was estimated to be 80 ± 10 kJ/mol in the presence of Ca^{2+} and 135 ± 40 kJ/mol in its absence, suggesting that binding of myosin heads to thin filaments in myofibrils is endothermic as is the binding of S1 or HMM to actin in solution (Highsmith, 1978; Chantler and Gratzer, 1976; Smith et al., 1984).

Effect of Ionic Strength

The log of the association constant of actin and either S1 or HMM decreases linearly when plotted against the square root of the KCl concentration with slopes of −4 and −8, respectively (Highsmith 1978). A plot of the log of the fraction of immobilized heads in labeled myofibrils against the square root of the KCl concentration is also linear (Fig. 9), and the observed slope is nearly equal to that obtained for acto-S1 (Highsmith, 1978). The agreement between the estimated value of f and the binding of S1 to F-actin provides further support for our interpretation of the spectra in terms of a two-state model.

DISCUSSION

In previous work using spin labeled S1 and HMM (Thomas et al., 1975), or myofibrils specifically spin-labeled at the SH-1 groups of myosin (Thomas et al., 1980), the heads in the absence of nucleotides (i.e., in rigor) were shown to have a rotational correlation time of 0.5 ms. This value equals that of a label attached to F-actin, indicating essentially rigid attachment to the thin filament; the motion of the attached heads appears to be controlled by some internal mode of motion of the thin filament (Fujime and Ishiwata, 1971; Thomas et al., 1979). Under relaxing conditions (in the presence of MgATP and EGTA) or during Ca^{2+}-activated ATPase activity, the motion of the heads corresponds to that of heads in pure myosin filaments, indicating detachment of essentially all of the heads.

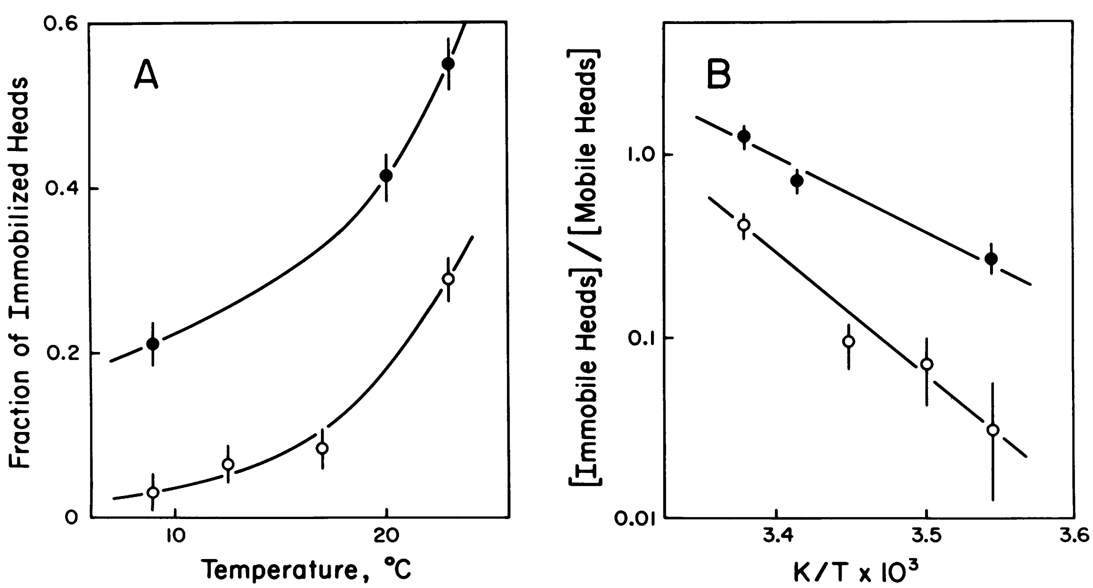

FIGURE 8 Effect of temperature and Ca^{2+} on the fraction of immobilized myosin heads. Spin-labeled myofibrils (46 mg/ml) were suspended in solutions containing 0.21 M KCl, 22 mM MOPS, pH 7.0, 4.5 mM $MgCl_2$, 0.9 mM NaN_3, and 10 mM PP_i. Open symbols, 5 mM EGTA; closed symbols, 5 mM EGTA and 5 mM $CaCl_2$.

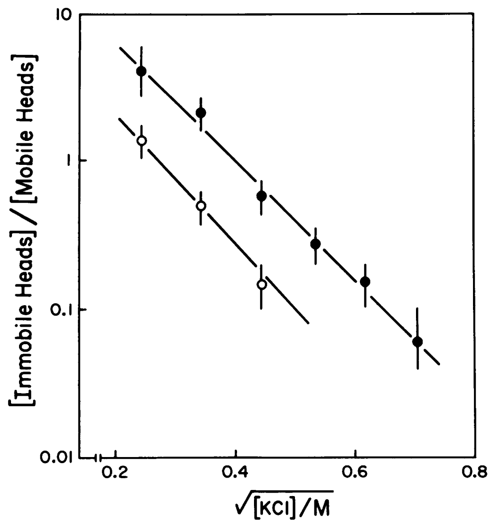

FIGURE 9 Dependence of the ratio of attached and detached myosin heads on the square root of KCl concentration. Data and symbols as in Fig. 5 *B*.

All of the data in the present work are consistent with a two-state model for rotational motion of myosin heads in which the attached heads are immobilized—their rotation being determined by internal motion of the actin filament—and the detached heads rotate at the same rate as do those of myosin filaments in the absence of actin. This view is supported by the linearity of L'' and L with the molar ratio of MSL-S1 to actin (Fig. 3), and by the correlation between the estimates of the fraction of immobilized heads obtained from ST-EPR and the fraction of bound heads from sedimentation experiments in which spin-labeled S1 is added to F-actin in the presence of varying concentrations of AMPPNP (Fig. 4).

In the present work we have studied the rotational properties of spin-labeled myosin heads of myofibrils under conditions of partial dissociation. Experimental spectra can be described as a weighted sum of spectra of relaxed and rigor myofibrils. These studies provide support for the view that various factors—including ionic strength, temperature, ATP analogues, and Ca^{2+}—that influence the binding of myosin heads to actin (Nauss et al., 1969; Highsmith et al., 1976; Highsmith, 1976; Greene and Eisenberg, 1978) affect the observed motion by increasing or decreasing f, without changing the motion of either attached or detached heads. This view is also supported by the agreement between the estimates of the fraction of attached heads obtained from ST-EPR and from sedimentation in experiments in which spin-labeled S1 is added to F-actin. Our results also confirm the earlier results obtained with fluorescent or paramagnetic probes rigidly attached to the SH-1 thiols of the myosin head, showing that ligands such as AMPPNP, PP_i, ATP, and Ca^{2+} do not affect the rotational motion of myosin heads of synthetic myosin filaments in the absence of actin (Mendelson and Cheung, 1976, 1978; Thomas et al., 1980).

The binding of myosin to actin in an organized system, such as muscle fibers or myofibrils, is governed by a first order equilibrium constant, so that direct comparison with the binding of S1 or HMM to actin, which is governed by a second order equilibrium constant, is not possible. Nevertheless, certain qualitative parallels are apparent in the effects of temperature and ionic strength in the presence of PP_i, because the concentration of PP_i, 10 mM, used in most of our experiments is sufficient to virtually saturate the PP_i binding sites (Nauss et al., 1969; Greene and Eisenberg, 1978) making the concentrations of unliganded M and AM very low; we therefore assume that the observed equilibrium constant and the value of ΔH, estimated from the data in Fig. 8, are mainly those of the binding of the head-PP_i complex to actin. The variation with temperature of the ratio of attached to detached heads indicates that the binding is endothermic, as is the binding of S1 or HMM to actin in solution (Highsmith, 1977, 1978; Chantler and Gratzer, 1976; Smith et al., 1984). The dependence on ionic strength of the ratio of attached/detached heads derived from the spectrum of spin-labeled myofibrils is also similar to that found for acto-S1 or acto-HMM (Highsmith et al., 1976; Highsmith, 1976, 1977, 1978) and suggests a strong electrostatic component in the interaction in both systems.

Regulation of ATPase activity of acto-S1 by Ca^{2+} under conditions where Ca^{2+} has little effect on S1 binding (Chalovich et al., 1981, 1982), suggests that one of the kinetic steps in the hydrolytic cycle involving the release of one or more products of ATP hydrolysis is also regulated. On the other hand, the binding of HMM to regulated F-actin during ATP hydrolysis shows some sensitivity to Ca^{2+} (Wagner and Giniger, 1981) although much less than the binding of S1-ADP (Greene and Eisenberg, 1980). Ca^{2+} produces about a fourfold increase in the binding constant of HMM to actin in the presence of ATP (Wagner and Stone, 1983). In the presence of PP_i, Ca^{2+} decreases the motion of labeled myosin heads (Fig. 5), indicating a small increase in the fraction of heads attached. In the presence of ATP, values of L''/L of spin-labeled myofibrils were not significantly different in the presence or absence of Ca^{2+} (Thomas et al., 1980). This result is consistent with a fourfold change in the binding constant being produced by Ca^{2+}; since the fraction of attached heads in the absence of Ca^{2+} is <0.025 (Thomas et al., 1980), Ca^{2+} will not produce a detectable change in the fraction of attached heads.

Studies on glycerinated muscle fibers, in which the myosin heads are selectively and rigidly labeled with MSL, also indicate the existence of two states reported by the spin label. Heads attached to thin filaments assume a well defined orientation, which is the same in rigor or contracting fibers, while detached heads are randomly oriented (Thomas and Cooke, 1980; Cooke, 1981; Cooke et al., 1982) and show Brownian motion on a microsecond time scale, as do those in MSL-labeled myofibrils (Thomas et al., 1980). Our present data on the effects of AMPPNP and PP_i complement those obtained on intact fibers (Thomas and Cooke, 1980), providing information on the dynamics of the heads in the attached and detached states. The value of L''/L of 1.2 for attached heads in myofibrils approaches the limit of sensitivity to slow motion of the method, and it is possible that τ_2 might be somewhat longer than 0.5 ms. On the other hand, estimation of correlation times by ST-EPR assumes unrestricted, isotropic rotation and the rotation of attached myosin heads must be strongly restricted, the half-width of the Gaussian distribution of bound heads being ~15°C (Thomas and Cooke, 1980). Since correlation times obtained by EPR are influenced both by diffusion rate and by amplitude of rotation (Gaffney, 1979; Lindahl and Thomas, 1982), a τ_2 of 0.5 ms for an attached head does not preclude more rapid rotation over a small angle. It has not yet been possible to estimate a value of τ_2 for attached spin-labeled heads during ATP hydrolysis or contraction, since the method gives a spectrum that is an average of those of attached and detached heads. Therefore, heads attached during ATP hydrolysis (Thomas et al., 1980) or "loosely attached" heads proposed on the basis of studies on relaxed fibers subjected to rapid stretches (Brenner et al., 1982), could possess more or less motion than those in rigor. Nevertheless, a value of 0.5 ms for τ_2 lies within the reorientation times of crossbridges of contracting muscles after rapid stretch or release (Huxley and Simmons 1971, 1972). More work is needed to distinguish effects of local motions and conformational changes in the vicinity of the probe from motions of the myosin head.

REFERENCES

Bottomley, R. C., and I. P. Trayer. 1975. Affinity chromatography of immobilized actin and myosin. *Biochem. J.* 149:365–379.

Brenner, B., M. Schoenberg, J. M. Chalovich, L. E. Greene, and E. Eisenberg. 1982. Evidence for cross-bridge attachment in relaxed muscle at low ionic strength. *Proc. Natl. Acad. Sci. USA.* 79:7288–7291.

Chalovich, J. M., P. B. Chock, and E. Eisenberg. 1981. Mechanism of action of troponin-tropomyosin. Inhibition of actomyosin ATPase activity without inhibition of myosin binding to actin. *J. Biol. Chem.* 256:575–578.

Chalovich, J. M., and E. Eisenberg. 1982. Inhibition of actomyosin ATPase activity by troponin-tropomyosin without blocking the binding of myosin to actin. *J. Biol. Chem.* 257:2432–2437.

Chantler, P. D., and W. B. Gratzer. 1976. The interaction of actin monomers with myosin heads and other muscle proteins. *Biochemistry.* 15:2219–2225.

Cooke, R. 1981. Stress does not alter the conformation of a domain of the myosin cross-bridge in rigor muscle fibres. *Nature (Lond.).* 294:570–571.

Cooke, R., M. S. Crowder, and D. D. Thomas. 1982. Orientation of spin labels attached to cross-bridges in contracting muscle. *Nature (Lond.).* 300:776–778.

Eads, T., D. D. Thomas, and R. Austin. 1984. Microsecond rotational motions of eosin-labeled myosin measured by time-resolved anisotropy of absorption and phosphorescence. *J. Mol. Biol.* 179:55–81.

Fujime, S., and S. Ishiwata. 1971. Dynamic study of F-actin by quasielastic scattering of laser light. *J. Mol. Biol.* 62:251–265.

Gaffney, B. J. 1979. Spin label-thiourea adducts. A model for saturation-transfer EPR studies at slow anisotropic rotation. *J. Phys. Chem.* 83:3345–3349.

Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177:751–766.

Graceffa, P., and J. C. Seidel. 1980. A reaction involving protein sulfhydryl groups, a bound spin-label, and $K_3Fe(CN)_6$ as a probe of sulfhydryl proximity in myosin. *Biochemistry.* 19:33–39.

Greene, L. E., and E. Eisenberg. 1978. Formation of a ternary complex: actin, 5′-adenylyl imidodiphosphate, and the subfragments of myosin. *Proc. Natl. Acad. Sci. USA.* 75:54–58.

Greene, L. E., and E. Eisenberg. 1980. Cooperative binding of myosin subfragment-1 to the actin-troponin-tropomyosin complex. *Proc. Natl. Acad. Sci. USA.* 77:2616–2620.

Hasselbach, W., and G. Schneider. 1951. Der L-Myosin- und Aktingehalt des Kaninchenmuskels. *Biochem. Z.* 321:462–475.

Highsmith, S., R. A. Mendelson, and M. F. Morales. 1976. Affinity of myosin S1 for F-actin, measured by time-resolved fluorescence anisotropy *Proc. Natl. Acad. Sci. USA.* 73:133–137.

Highsmith, S. 1976. Interactions of the actin and nucleotide binding sites on myosin subfragment-1. *J. Biol. Chem.* 251:6170–6172.

Highsmith, S. 1977. The effects of temperature and salts on myosin subfragment-1 and F-actin association. *Arch. Biochem. Biophys.* 180:404–408.

Highsmith, S. 1978. Heavy meromyosin binds actin with negative cooperativity. *Biochemistry.* 17:22–26.

Huxley, A. F., and R. M. Simmons. 1971. Proposed mechanism of force generation in striated muscle. *Nature (Lond.).* 233:533–538.

Huxley, A. F., and R. M. Simmons. 1972. Mechanical transients and the origin of muscular force. *Cold Spring Harbor Symp. Quant. Biol.* 37:669–680.

Ishiwata, S., J. Seidel, and J. Gergely. 1979. Regulation by calcium ions of crossbridge attachment in myofibrils studied by saturation transfer EPR spectroscopy. *Biophys. J.* 25(2, Pt. 2): 19*a*. (Abstr.)

Johnson, M. E. 1978. Librational motion of an "immobilized" spin label: hemoglobin spin labeled by a maleimide derivative. *Biochemistry.* 17:1223–1228.

Johnson, M. E. 1979. Spin label techniques for monitoring macromolecular rotational motion: empirical calibration under nonideal conditions. *Biochemistry.* 18:378–384.

Kinosita, K., S. Ishiwata, H. Yoshimura, H. Asai, and A. Ikegami. 1984. Submicrosecond and microsecond rotational motions of myosin heads in solution and in myosin synthetic filaments as revealed by time-resolved optical decay measurements. *Biochemistry.* 23:5963–5975.

Lindahl, K., and D. D. Thomas. 1982. Effect of limited rotational motion on simulated conventional and saturation transfer EPR spectra of nitroxide spin labels. *Biophys. J.* 37(2, Pt. 2): 71*a*. (Abstr.)

Mendelson, R. A., M. F. Morales, and J. Botts. 1973. Segmental flexibility of the S1 moiety of myosin. *Biochemistry.* 12:2250–2255.

Mendelson, R. A., and P. H. C. Cheung. 1976. Myosin crossbridges: absence of direct effect of calcium on movement away from the thick filaments. *Science (Wash. DC)* 194:190–192.

Mendelson, R. A., and P. H. C. Cheung. 1978. Intrinsic segmental flexibility of the S1 moiety of myosin using single-headed myosin. *Biochemistry.* 17:2139–2148.

Nauss, K. M., S. Kitagawa, and J. Gergely. 1969. Pyrophosphate binding to and adenosine triphosphatase activity of myosin and its proteolytic fragments. *J. Biol. Chem.* 244:755–765.

Potter, J. D., and J. Gergely. 1975. The calcium and magnesium binding sites on troponin and their role in the regulation of myofibrillar adenosine triphosphatase. *J. Biol. Chem.* 250:4628–4633.

Seidel, J. C., M. Chopek, and J. Gergely. 1970. Effect of nucleotides and pyrophosphate on spin labels bound to S_1 thiol groups of myosin. *Biochemistry.* 9:3265–3272.

Smith, S. J., H. D. White, and R. C. Woledge. 1984. Microcalorimetric measurement of the enthalpy of binding of rabbit skeletal myosin and heavy meromyosin to F-actin. *J. Biol. Chem.* 259:10303–10308.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin *J. Biol. Chem.* 246:4866–4871.

Thomas, D. D., J. C. Seidel, J. S. Hyde, and J. Gergely. 1975. Motion of subfragment-1 in myosin and its supramolecular complex: saturation transfer electron paramagnetic resonance. *Proc. Natl. Acad. Sci. USA.* 72:1729–1733.

Thomas, D. D., L. R. Dalton, and J. S. Hyde. 1976. Rotational diffusion studied by passage saturation transfer EPR. *J. Chem. Phys.* 65:3006–3024.

Thomas, D. D., J. C. Seidel, and J. Gergely. 1979. Rotational dynamics of spin-labeled F-actin in the sub-millisecond time range. *J. Mol. Biol.* 132:257–273.

Thomas, D. D., S. Ishiwata, J. C. Seidel, and J. Gergely. 1980. Submillisecond rotational dynamics of spin-labeled myosin heads in myofibrils. *Biophys. J.* 32:873–889.

Thomas, D. D., and R. Cooke. 1980. Orientation of spin-labeled myosin heads in glycerinated muscle fibers. *Biophys. J.* 32:891–906.

Wagner, P. D., and E. Giniger. 1981. Calcium-sensitive binding of heavy meromyosin to regulated actin in the presence of ATP. *J. Biol. Chem.* 256:12647–12650.

Wagner, P. D., and D. B. Stone. 1983. Calcium-sensitive binding of heavy meromyosin to regulated actin requires light chain 2 and the head-tail junction. *Biochemistry.* 22:1334–1342.

Weeds, A. G., and B. Pope. 1977. Studies on the chymotryptic digestion of myosin. Effects of divalent cations on proteolytic susceptibility. *J. Mol. Biol.* 111:129–157.