Phosphorus-31 Nuclear Magnetic Resonance Evidence for Two Conformations of Myosin Subfragment-1·Nucleotide Complexes†

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ABSTRACT: ^{31}P NMR has been used to study intermediate complexes in the myosin subfragment-1 (S-1) ATPase reaction. We report here the ^{31}P NMR spectra and interpretation of S-1 complexes of ADP and AMP·PNP (a nonhydrolyzable ATP analogue). At 25 °C in 0.1 M KCl and pH 7 the β-phosphate resonance of bound ADP is shifted 3.7 ppm downfield from the free. The bound β-phosphate titrates negligibly from pH 6.6 to pH 8.0, whereas the free β-phosphate titrates significantly downfield. An analysis of the line width of the β-phosphate of bound ADP using the principal elements of the chemical shift tensor of pyrophosphate is consistent with a rigid binding site with a rotational correlation time expected for a protein the size of S-1. At 0 °C the bound β-phosphate resonance intensity is decreased, and there is a new resonance of nearly equal intensity 0.7 ppm downfield, indicating two forms of bound ADP with a highly temperature-dependent equilibrium constant. AMP·PNP also binds to S-1 to give two complexes with a temperature-dependent equilibrium. Thus, the binding of a nucleotide (N) to S-1 (M) may be represented by M + N ⇌ M·N ⇌ M#N ⇌ N*N, where M·N is an initial recognition complex in fast exchange with free nucleotide and M#N and M*N are the two nucleotide complexes observed by NMR. Comparison of the ^{31}P NMR results with the temperature-dependent fluorescence data from the literature [Bechet, J. J., Breda, C., Guinand, S., Hill, M., & d'Albis, A. (1979) *Biochemistry 18*, 4080, and Trybus, K. M., & Taylor, E. W (1979) *Biophys. J. 75*, M-AM-D11] allows determination of the rate constants for interconversion of the two forms for bound ADP and AMP·PNP and allows us to assign the most shifted bound β-phosphate resonances of both ADP and AMP·PNP to the most fluorescent of the two complexes M*N. At room temperature only the M#·ADP form is significantly populated, whereas AMP·PNP, and presumably ATP, causes a specific endothermic conformational change in S-1 that is not induced by ADP. The addition of these two new complexes to the myosin ATPase scheme results in a mechanism with two ATP complexes, two products complexes, and two ADP complexes and implies that there are two fundamental myosin conformations independent of actin both of which may hydrolyze ATP. An ATPase scheme is presented, along with supporting evidence from the literature from a broad range of experiments, in which myosin is viewed as an allosteric protein which acts on its "substrate" actin due to a time-dependent nucleotide effector in the enzymatic site and the R → T conformational change is expressed as the "power stroke".

The myosin ATPase[1] mechanism has been the focus of numerous researchers' efforts over the last 10 years using primarily the methods of transient and steady-state kinetics [Bagshaw & Trentham (1974), Eisenberg et al. (1972), Lymn & Taylor (1970), Taylor (1979), Tonomura & Inoue (1974), and references cited therein]. The presently accepted mechanism contains at least six intermediates. Summarizing the work to this point the mechanism can be written as

$$\mathrm{M + ATP \rightleftharpoons M{\cdot}ATP \rightleftharpoons M^{*}ATP \rightleftharpoons M^{**}ADP{\cdot}P \rightleftharpoons M^{*}ADP{\cdot}P \rightleftharpoons M^{*}ADP + P \rightleftharpoons M{\cdot}ADP + P \rightleftharpoons M + ADP + P} \quad (1)$$

(where * denotes qualitatively the relative fluorescence intensity). Recent work has indicated that the mechanism is even more complicated than shown here. Evidence for an additional state following M**ADP·P has been presented (Stein et al., 1979). In addition, the binding of both ADP and the nonhydrolyzable ATP analogue AMP·PNP to myosin subfragment-1 (S-1) has been reported to occur in three steps (Trybus & Taylor, 1979). Garland & Cheung (1979) have suggested that ATP also may bind to S-1 in three steps. A similar mechanism to eq 1 exists for the hydrolysis of ATP by actomyosin (White & Taylor, 1976; Stein et al., 1979).

It is important to obtain a better understanding of the relationship between the detailed chemical mechanisms observed in solution and the power stroke observed during muscle contraction. Some of the observed intermediates may have more relevance than others to the molecular mechanism of muscle contraction, and some may represent only transient protein isomers which are not populated to any great extent and/or have a minor function in muscle mechanics. Of particular interest is the significance of the three-step binding processes of nucleotides.

In an effort to further understand the relative importance of the intermediates of the myosin ATPase, we have begun a ^{31}P NMR study of S-1·nucleotide complexes. ^{31}P NMR offers a unique method for observing many of the intermediates of the myosin ATPase. Each intermediate contains at least two natural, nonperturbing phosphorous probes which serve as discrete monitors of the myosin active site in various stages of the ATP hydrolysis reaction. We have shown that the ^{31}P

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[1] Abbreviations used: S-1, myosin subfragment-1; AMP·PNP, β,γ-imidoadenosine 5′-triphosphate; AP_5A, diadenosine pentaphosphate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; P, phosphate; ATPase, adenosine triphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Pipes, piperazine-*N,N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide.

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NMR resonances of phosphorous nuclei bound to a protein the size of S-1 are sufficiently narrow to serve as sensitive indicators of structural differences without problems due to prohibitive line widths (Shriver et al., 1979). ^{31}P NMR has recently been used to determine equilibrium and rate constants for enzyme-bound intermediate interconversions and to monitor structural changes of enzyme–phosphate complexes under various conditions including addition of allosteric effectors (Feldmann & Hull, 1977; Hull et al., 1976; Rao et al., 1976, 1978, 1979 Rao & Cohn, 1977a,b; Withers et al., 1979).

^{31}P NMR studies of the S-1·ADP complex were performed by mixing ADP and myosin in the absence of ATP and P, i.e., "backing up" the reaction in the absence of orthophosphate. A significant amount of M*ATP is present in the steady state; however, high concentrations of ATP, or a precursor such as creatine phosphate, would be required to maintain the steady state during accumulation of NMR spectra. An analogue of this intermediate can be observed by using the nonhydrolyzable ATP analogue AMP·PNP (Yount et al., 1971).

The results presented herein show that ADP binds to S-1 to form an S-1·ADP complex at 25 °C, which is observable with ^{31}P NMR. However, at 0 °C there are two complexes of S-1·ADP present in nearly equal concentrations. Experiments with AMP·PNP show that at least two S-1·AMP·PNP complexes are significantly populated at 25 °C. The equilibrium between the two is also sharply temperature dependent and at 4 °C only one form is observed. The ability of myosin S-1·nucleotide complexes to exist in two conformations, in addition to an initial recognition complex, is supported by other reports in the literature (see Discussion). These results imply that it is an intrinsic property of S-1 to exist in two forms, and we propose that the apparently complicated binding of nucleotides to S-1 may be easily understood in terms of the mechanism

$$\begin{array}{ccccc} M_T + N & \rightleftharpoons & M_T{\cdot}N & \rightleftharpoons & M_T{*}N \\ \uparrow\downarrow & & \uparrow\downarrow & & \uparrow\downarrow \\ M_R + N & \rightleftharpoons & M_R{\cdot}N & \rightleftharpoons & M_R{*}N \end{array} \qquad (2)$$

This mechanism incorporates our NMR results presented here and is consistent with the transient kinetic results of Trybus & Taylor (1979). Two interconvertible forms of free myosin have been considered by Bagshaw et al. (1974) and Chock et al. (1979); however, binding to both forms does not appear to have been considered. The presence of two S-1·nucleotide complexes both before and after hydrolysis has important implications for designing models of contraction. Marston et al. (1979) have recently presented evidence that cross-bridge–nucleotide complexes may also exist in two conformations in a muscle fiber.

Materials and Methods

ADP, AMP·PNP,and AP_5A were obtained from Sigma Chemical Co. and checked for purity by ^{31}P NMR. Nicotinamide riboside was obtained by hydrolyzing nicotinamide mononucleotide (Sigma) with 5′-nucleotidase (Sigma) (J. W. Shriver and B. D. Sykes, unpublished results). Nucleoside phosphorylase and α-chymotrypsin were obtained from Sigma. Special enzyme grade ammonium sulfate was obtained from Schwarz/Mann. D_2O was obtained from Bio-Rad Laboratories and stored over Chelex-100 prior to use to remove trace paramagnetic ions. In addition, all NMR samples contained 1 mM EDTA. Ultra-pure Pipes was obtained from Calbiochem. All other chemicals were reagent grade.

Myosin was isolated by standard procedures (Lowey et al., 1969; Perry, 1955). S-1 was prepared from myosin by a procedure modified from that of Weeds & Taylor (1975). Myosin was extracted from ground rabbit back and leg muscle (both frozen muscle from Pel-Freeze and fresh muscle were used) with 0.3 M KCl, 0.075 M K_2HPO_4, 0.075 M KH_2PO_4, 2.5 mM $MgCl_2$, 1 mM EGTA, and 0.5 mM ATP at pH 6.5 at 4 °C for 10 min with gentle stirring. After removal of cellular debris with a low-speed centrifugation, myosin was precipitated by diluting to 0.02 M KCl with 14 volumes of cold deionized water. The myosin precipitate was removed by centrifugation and dissolved in 0.5 M KCl, 0.075 M K_2HPO_4, 0.075 M KH_2PO_4, 2.5 mM $MgCl_2$, 1 mM EGTA, and 0.5 mM ATP at pH 6.7. The myosin was precipitated by dilution and taken up in 0.5 M kCl, 0.075 M K_2HPO_4, 0.075 M KH_2PO_4, 2.5 mM $MgCl_2$, and 1 mM EGTA at pH 6.7. The myosin was precipitated again by dilution and the precipitate taken up in enough distilled water and solid NaCl to make 600 mL of a 0.5 M NaCl solution. The solution was centrifuged at 20000 rpm in a Beckman 50.2 Ti rotor for 30 min and then dialyzed against 0.12 M NaCl, 0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, and 1 mM EDTA, at pH 7. S-1 was obtained by limited proteolysis of the myosin filaments with 0.05 mg/mL α-chymotrypsin at 23 °C. The cleavage was stopped after 15 min with enough PMSF in 1 mL of 95% ethanol to make a final concentration of 0.5 mM and stirred for 15 min at room temperature. The resultant solution was dialyzed against 0.04 M NaCl, 0.003 M NaH_2PO_4, 0.003 M $NaHPO_4$, 0.33 mM EDTA, 5 mM $MgCl_2$, and 1 mM K_4PP at pH 7 and then centrifuged at 20000 rpm in a Beckman 50.2 Ti rotor for 30 min. The resulting supernatant was dialyzed against 0.05 M Tris, 1 mM EDTA, and 0.1 mM NaN_3 at pH 8, and the S-1 was purified by DEAE-cellulose column chromatography by eluting with a 0.0–0.2 M KCl gradient. The S-1(A1) and S-1(A2) fractions were isolated and further purified by $(NH_4)_2SO_4$ fractionation below 54% saturation. The S-1 precipitate was collected by centrifugation, dissolved in a minimal amount of 0.05 M Tris, 1 mM EDTA, and 0.1 mM NaN_3, pH 8, buffer, and dialyzed against the same. It was concentrated to ~300 mg/mL with a Minicon B-15 concentrator just prior to use. Generally 200 g of muscle yielded ~600 mg of S-1(A1) and S-1(A2). S-1 was generally used within 5 days; however, no loss of activity was detected over a period of several weeks. Occasionally, material was reused after extensively dialyzing it against 0.05 M Tris, pH 8.0, 1 mM EDTA, and 0.1 mM NaN_3.

The purity of S-1 was checked by $NaDodSO_4$ gel electrophoresis and showed an intense heavy chain and the A1 and A2 light chains with little or no DTNB light chain present, as shown by Weeds & Taylor (1975). Concentration was determined by using an extinction coefficient of 0.75 mL/(mg·cm) (at 278 nm) and a molecular weight of 115000 (Weeds & Pope, 1977).

ATPase activities were determined by using a Radiometer pH stat. Ca^{2+}–ATPase activity was measured in 0.6 M KCl, 10 mM $CaCl_2$, and 5 mM ATP, at 25 °C and pH 7.9. K^+-EDTA–ATPase activity was measured in 0.6 M KCl, 5 mM EDTA, and 5 mM ATP, at 25 °C and pH 7.9. Typically, the Ca^{2+}–ATPase activity was 1.1 s^{-1} and the K^+EDTA activity was 13 s^{-1}. The Mg^{2+}–ATPase activity was measured in 0.1 M KCl, 40 mM ATP, and 40 mM $MgCl_2$, pH 7.9 and 4 °C, by ^{31}P NMR spectroscopy. The typical activity was 0.01 s^{-1}.

^{31}P NMR spectra were collected on a Bruker HXS-270 instrument operating in the Fourier-transform mode with quadurature detection. Flat-bottomed 10-mm polished NMR tubes (Wilmad) were used throughout with a Teflon vortex suppression plug. Sample volume was typically 1 mL. Sweep

Table I: ^{31}P NMR Chemical Shifts of Phosphate Resonances of Free and Bound Nucleotides[a]

nucleotide	pH	temp (°C)	α	β	γ
MgADP	6.6	25	−9.44	−5.54	
	7.0	25	−9.40	−5.45	
	7.0	0	−9.83	−5.84	
	7.5	25	−9.45	−5.35	
	8.0	25	−9.38	−5.30	
S-1·MgADP	6.6	25	−9.4	−1.78	
	7.0	25	−9.4	−1.78	
	7.0	0	−9.90[b]	−1.89	
				−1.24	
	7.5	25	−9.4	−1.78	
	8.0	25	−9.4	−1.83	
MgAMP·PNP	7.0	25	−9.66	−5.77	−0.90
	7.0	5	−9.76	−5.60	−0.90
S-1·MgAMP·PNP	7.0	25	−7.35	−2.34	0.21
			−9.05	−3.28	
	7.0	5	−9.05	−3.47	0.49

[a] See figure captions for experimental details for spectra. Chemical shifts are referenced relative to an external capillary of 85% H_3PO_4. [b] Due to interference by the free α-phosphate it was not possible to determine if there were two bound α-phosphate resonances at 0 °C. The estimated error in the chemical shift of the bound α-phosphate resonance at 0 and 25 °C is ±0.2 ppm.

widths of ±2500 Hz were used with a acquisition time of 0.46 s and a pulse delay of 1.6 s. The typical flip angle was 75° (17-μs pulse angle). Generally 5000 transients were required to obtain a reasonable signal to noise ratio in the spectrum. The C(6) methylene protons of ribose were routinely decoupled in all spectra. Chemical shifts were measured relative to an external standard of 85% H_3PO_4 in a capillary fixed in the center of the NMR tube.

Results

Myosin S-1 Preparation and Properties. The S-1 prepared as described under Materials and Methods was highly pure as indicated by ATPase activities, $NaDodSO_4$ gel electrophoresis, and sedimentation studies. ATPase activities were similar to those reported in the literature: 1.1 s^{-1} for the Ca^{2+}–ATPase and 13 s^{-1} for the K^+EDTA–ATPase. $NaDodSO_4$ gel electrophoresis showed only three major bands: the heavy chain and the A1 and A2 light chains. There was normally little or no evidence of the DTNB light chain even under heavy loading of the gel. Sedimentation studies indicated only one component with no evidence for aggregation of the S-1 up to 34 mg/mL. An intrinsic sedimentation coefficient of 5.80×10^{-13} s was obtained which agrees with the value determined by Lowey et al. (1969). The rate of hydrolysis of MgATP at 115 mg/mL of S-1 was monitored by observing the rate of decay of ATP by ^{31}P NMR at 4 °C. The observed rate of 0.01 s^{-1} compares favorably with the rate observed at low S-1 concentrations in the pH stat. This indicates no unusual behavior, such as aggregation of S-1, at concentrations on the order of 1 mM.

^{31}P NMR Spectroscopy. (1) MgADP. The ^{31}P NMR spectrum of free MgADP at pH 7 and 25 °C is shown in Figure 1A. The upfield doublet at −9.40 ppm can be assigned to the α-phosphate of MgADP due to the presence of long-range proton coupling in the absence of proton decoupling. The low-field doublet at −5.45 ppm can therefore be assigned to the β-phosphate. (See Table I.) The two phosphates have a scalar coupling constant of 17.8 Hz [compare with Rao & Cohn (1977a)]. Resonances due to trace amounts of AMP and orthophosphate appear at 3.45 ppm and 1.84 ppm, respectively. Resonances at −4.9, −10.1, and −18.4 ppm are due to the γ, α, and β resonances, respectively, of contaminating ATP in the ADP.

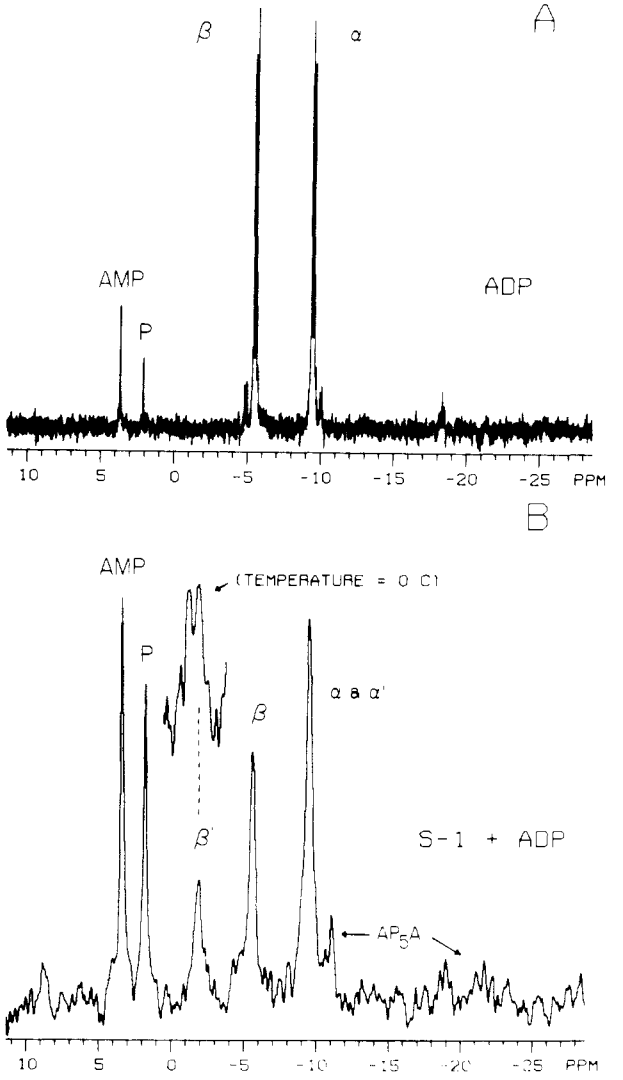

FIGURE 1: (A) ^{31}P NMR spectrum of MgADP. 1.7 mM ADP, 6.8 mM $MgCl_2$, 0.5 mM EDTA, 0.1 M KCl, and 50 mM Pipes, in 50% D_2O at 25 °C and pH 7 (pH not corrected for D_2O). Chemical shifts relative to external capillary of 85% H_3PO_4. 3000 transients, 75° pulse angle, 0.42-s acquisition time, 1.6-s pulse delay, 0.5-Hz line broadening. (B) ^{31}P NMR spectrum of mixture of MgADP and myosin S-1. 0.5 mM S-1 (A1, A2), 1 mM ADP, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 M KCl, 50 mM Pipes, 20 μM AP_5A, and 50% D_2O at 25 °C and pH 7 (pH not corrected for D_2O). 3000 transients, 75° pulse angle, 0.41-s acquisition time, 1.6-s pulse delay, 20-Hz line broadening. (Insert) Similar conditions except at 0 °C and 30 000 transients. α′ and β′ refer to the α- and β-phosphate resonances of bound ADP.

(2) MgADP and S-1. The spectrum of a mixture of S-1-(A1,A2) and MgADP at 25 °C is given in Figure 1B. AP_5A has been added to inhibit trace contaminating adenylate kinase, which coupled with the ATPase activity of S-1 leads to the hydrolysis of ADP to AMP and orthophosphate. Even with the high concentrations of AP_5A used here (0.02 mM) there was still a significant ADP hydrolysis rate, indicating that S-1 might function as an ADPase. An excess of ADP over the myosin S-1 sites was used to maintain a sufficient concentration of ADP. Resonances due to AP_5A appear at −11.0 and −21.7 ppm. Due to the low concentration of AP_5A relative to ADP, the upfield resonance at −21.7 ppm is not obvious in this spectrum. Also, an unassigned resonance appears at −11 ppm and is present in all myosin S-1 spectra including those without nucleotides. It has a chemical shift characteristic of

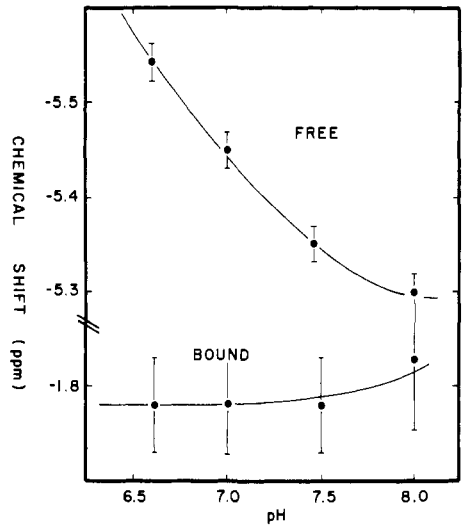

FIGURE 2: Titration of the free and bound β-phosphate resonances of MgADP. Conditions are as described in Figure 1B.

a pyrophosphate dinucleotide, e.g., FAD or NAD (Moon & Richards, 1973). AMP and orthophosphate are seen at 3.5 and 2.0 ppm.

In addition to the above assigned peaks for free ADP, AP_5A, AMP, and orthophosphate, two further resonances are present at 25 °C. The one at −9.4 ppm (under the free ADP α-phosphate resonance) can be seen when less than stoichiometric amounts of MgADP are used and is indicated in Figure 1B by a difference in intensity between the two free MgADP resonances. This resonance is assigned to the α-phosphate resonance of bound MgADP. The peak at −1.78 ppm is assigned to the β-phosphate resonance of bound MgADP. These assignments are consistent with spectral assignments of other protein-bound nucleotides in that the α-phosphate chemical shift is not often affected by binding, whereas the β-phosphate resonance shifts significantly upon binding (Rao & Cohn, 1977a). In this paper we focus primarily on the β-phosphate resonance for an analysis of bound MgADP.

Figure 2 shows the effect of variation of pH from 6.6 to 8.0 on the chemical shift of the β-phosphate of bound MgADP at 25 °C. Whereas the free β-phosphate resonance titrates significantly over this range, the bound β resonance titrates negligibly upfield. This indicates that binding of MgADP to S-1 perturbs the β-phosphate and significantly modifies its pK. The apparent partial upfield titration of the bound β-phosphate resonance at high pH is similar to that observed for bound MgADP on arginine kinase (Rao & Cohn, 1977a).

At 0 °C the β-phosphate resonance of bound MgADP is split into two resonances with a new peak appearing 0.7 ppm downfield from the peak observed at 25 °C (Figure 1B). This indicates that there are two bound forms of MgADP at 0 °C in slow exchange with free MgADP. Removal of orthophosphate by nucleoside phosphorylase catalyzed phosphorolysis of nicotinamide riboside did not change the spectrum indicating that binding of phosphate was not responsible for the second resonance. The two resonances at 0 °C are observed with both S-1(A1) and S-1(A2) indicating that the second resonance is not due to differences in light chains. The DTNB light chain is not present in the S-1 preparations used in these experiments.

Powder Pattern of Pyrophosphate. To determine a rotational correlation time for the β-phosphate of bound ADP, we require the principal elements of the chemical shift tensor of the β-phosphate (Abragam, 1961; Hull & Sykes, 1975). These may be obtained from the powder pattern (spectrum of the solid powder) of pyrophosphate, which we use as a model of the β-phosphate since the powder pattern of ADP would contain both the α- and β-phosphates. The powder pattern for tetrasodium pyrophosphate is given in Figure 3. Values for the principal elements, δ_{ii}, of the chemical shift tensor, $\bar{\sigma}$, were obtained by fitting the observed spectrum with a calculated spectrum. The best fit was obtained with $\delta_{xx} = -42$ ppm, $\delta_{yy} = -42$ ppm, and $\delta_{zz} = 85$ ppm, where chemical shifts are referenced relative to (1/3) trace $\{\bar{\sigma}\}$. The chemical shift anisotropy contribution to the ^{31}P NMR line width for large proteins at high fields is given by (Hull & Sykes, 1975; Abragam, 1961)

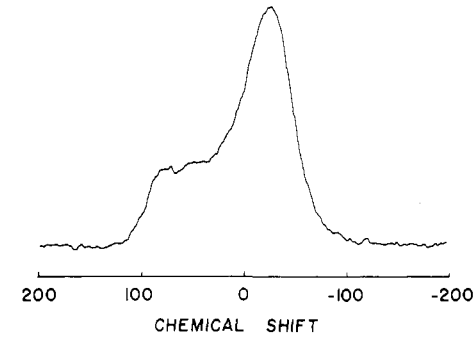

FIGURE 3: ^{31}P NMR spectrum (powder pattern) of solid tetrasodium pyrophosphate at 25 °C. Na_4PP was twice lyophilized from D_2O. 15 transients, 30° pulse angle, 0.02-s acquisition time, 60-s pulse delay, 250-Hz line broadening. Chemical shift referenced relative to (1/3) trace $\{\bar{\sigma}\}$.

$$\Delta\nu_{csa} = 1/(5\pi)\omega_0^2\delta_{zz}^2(1 + \eta^2/3)\tau_c \qquad (3)$$

where ω_0 is the resonance frequency of the phosphorus nucleus, τ_c is the rotational correlation time, and

$$\eta = (\delta_{xx} - \delta_{yy})/\delta_{zz}$$

MgAMP·PNP. Figure 4A shows the spectrum of MgAMP·PNP at pH 7 (0.1 M KCl, 25 °C) [see Tran-dinh & Roux (1977)]. The three dominant peaks at −9.66, −5.77, and −0.90 ppm are due to MgAMP·PNP. All commercial samples investigated contained significant quantities of impurities. The AMP·PNP used in these experiments was the purest obtained. The most noticeable impurity in these samples was assigned to AMP·PN [see Yount et al. (1971)], with resonances at −0.36 ppm and −10.32 ppm. AMP·PN showed no sign of binding and did not interfere with the bound β-phosphate resonance which was used for most of the analysis of these experiments. No attempt was made to remove it since it arises during the purification of AMP·PNP in triethylammonium bicarbonate buffer (Yount et al., 1971). The only other impurity appears at −8.3 ppm and is unassigned. Orthophosphate appears at 2.0 ppm. The most upfield resonance of the three AMP·PNP resonances is assigned to the α-phosphate by comparison to MgADP and due to the large coupling constant (17.2 Hz) similar to the α–β coupling in ADP. Coupling between the β- and γ-phosphates through nitrogen is not as strong (9.4 Hz), and the low-field doublet at −0.90 ppm is assigned to the γ-phosphate. The quartet at −5.77 ppm is obviously due to the β-phosphate ($J_{\alpha\beta} = 17$ Hz and $J_{\beta\gamma} = 9.2$ Hz).

MgAMP·PNP and S-1. Figure 4B shows the spectrum of a mixture of MgAMP·PNP and S-1(A1,A2) at pH 7 and 25 °C. At the low-field end of the spectrum are free AMP and orthophosphate. Peaks due to AMP·PN are more prevalent than above since AMP·PN is not bound whereas AMP·PNP is divided into free and bound populations. The free AMP·PN and excess AMP·PNP which is unbound can be easily assigned.

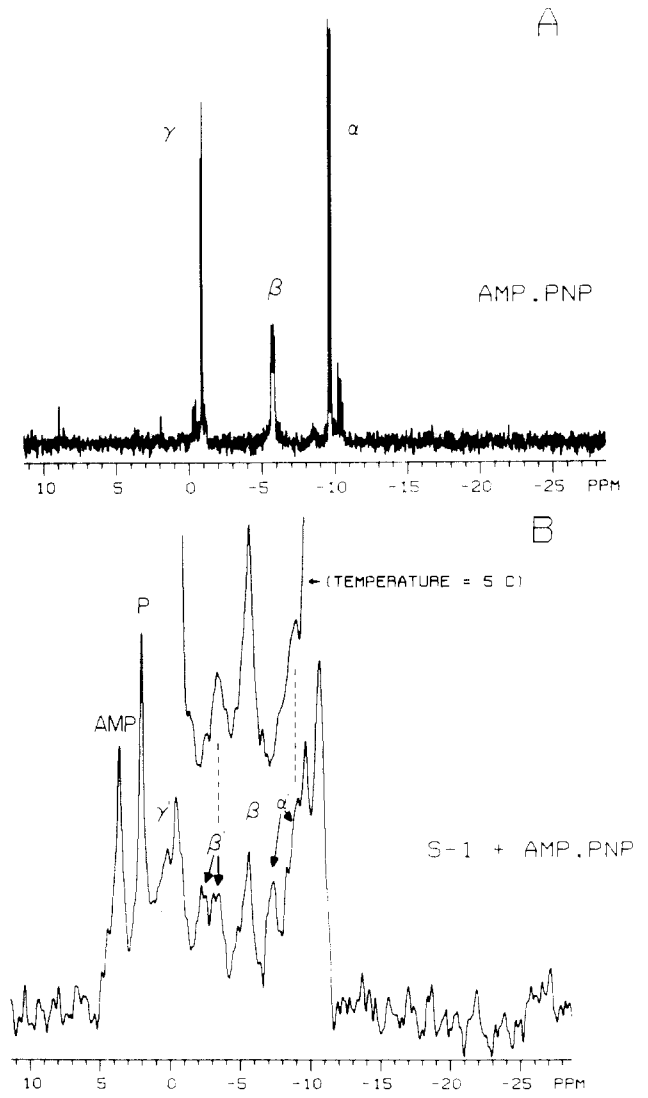

FIGURE 4: (A) ^{31}P NMR spectrum of MgAMP·PNP. 1 mM AMP·PNP, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 M KCl, and 50 mM Pipes, in D_2O at 25 °C and pH 7 (pH not corrected for D_2O). Chemical shift relative to external 85% H_3PO_4. 7500 acquisitions, 75° pulse angle, 0.41-s acquisition time, 1.2-s pulse delay, 1-Hz line broadening. (B) ^{31}P NMR spectrum of a mixture of MgAMP·PNP and myosin S-1. Same conditions as in (A) except 40 000 transients, 30-Hz line broadening, and 0.7 mM S-1. (Insert) Similar conditions, as above except temperature was 5 °C. α', β', and γ' refer to the α-, β-, and γ-phosphate resonances of bound AMP·PNP.

The remaining peaks, except for the one at −8.3 ppm, can be assigned to S-1 bound AMP·PNP. This leaves more than three resonances for bound AMP·PNP and indicates that multiple bound forms exist. The two resonances at −2.34 and −3.28 ppm are assigned to the bound β-phosphate, and the two resonances at −7.35 and −9.05 ppm are assigned to the bound α-phosphate. The resonance at 0.8 ppm is assigned to bound γ-phosphate. The structure of the bound γ-phosphate resonance is not obvious due to overlapping neighboring resonances. This assignment is justified by comparison with results from other protein–nucleotide complexes and variable temperature results. A decrease in temperature leads to a decrease in intensity of the lower field member of the bound α and β bound resonance pairs (see Figure 4B). Temperature effects will be more fully presented in a forthcoming paper. Therefore, AMP·PNP binds to S-1 in at least two forms similar to ADP. The equilibrium constant describing the interconversion of these forms is significantly temperature dependent, and at 25 °C it is equal to 1. The relative intensities of the two β-phosphate resonances are considered a more reliable indicator of the equilibrium constant than the two α-phosphate resonances due to interfering resonances overlapping with the upfield peak of the bound α-phosphate pair.

Discussion

In this paper we present evidence for myosin S-1 existing in two conformations in complexes with both AMP·PNP and ADP. The equilibrium constants describing the interconversion of the two forms are highly temperature dependent between 0 and 25 °C. In the discussion which follows we first discuss the NMR results and relate these to the rate constants for nucleotide binding obtained from transient kinetics experiments. These results confirm other experiments, including work with muscle fibers, which have implied that myosin–nucleotide complexes may exist in at least two conformations. We then present a myosin ATPase scheme which incorporates these results and may be expanded into an actomyosin ATPase scheme. The reaction scheme presented simplifies the presently accepted ATPase mechanisms and, most importantly, explicitly includes a conformational change which may be expressed as the power stroke leading to force production in the muscle fiber.

The ^{31}P NMR spectrum of MgADP bound to S-1 at 25 °C (0.1 M KCl, pH 7) indicates that MgADP is tightly bound and is in slow exchange with free MgADP, which is consistent with the published binding constant for MgADP of 10^6 M^{-1} (Lowey & Luck, 1969). This is also true at 0 °C where there is an additional bound β resonance in the ^{31}P NMR spectrum shifted downfield from the resonance seen at 25 °C. The relative narrowness of the bound resonances at both temperatures implies that exchange broadening is negligible. Orthophosphate is present in these experiments due to the hydrolysis of ADP by coupling contaminating traces of adenylate kinase with the S-1 ATPase. At pH 8.0 and 4 °C orthophosphate can be rapidly removed by coupling its formation with the phosphorolysis of nicotinamide riboside (NR^+, R-1-PO_4^{2-} = ribose 1-phosphate)

$$NR^+ + HPO_4^{2-} \rightleftharpoons \text{nicotinamide} + \text{R-1-}PO_4^{2-} + H^+$$

catalyzed by nucleoside phosphorylase (J. W. Shriver and B. D. Sykes, unpublished results). At pH 8.0 the equilibrium of the phosphorolysis is far to the right because of the H^+ production (Rowen & Kornberg, 1951), and phosphate is continuously removed from the NMR sample. The ^{31}P NMR spectra show complete conversion of phosphate to ribose 1-phosphate. Under these conditions the bound line widths are unchanged indicating that phosphate exchange is not contributing to the line width, nor is it responsible for the additional peaks observed at low temperature. These results agree with the published binding constant of orthophosphate to S-1*ADP, i.e., $K \geq 50$ mM (Goody et al., 1977).

As discussed above, ^{31}P NMR indicates there are two bound forms of ADP significantly populated at low temperature. Trybus & Taylor (1979) have recently reported that MgADP binds to S-1 in three steps

$$M + ADP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M\cdot ADP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M\#ADP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M^*ADP \quad (4)$$

At 3 °C the fluorescence transient observed upon mixing ADP and S-1 is significantly biphasic with M*ADP having a higher fluorescence than M#ADP. The first step in (4) is presumably in rapid equilibrium with K_1 on the order of 1000 M^{-1}. The first-order rate constant k_2 is approximately 150–200 s^{-1}, and $k_3 + k_{-3} = 15$ s^{-1} at 3 °C and pH 7.0. Our NMR data

show only one complex at 25 °C, where the fluorescence transient is not biphasic, and since the relative fluorescence of myosin·ADP is known to decrease slightly with increasing temperature (Bechet et al., 1979), we tentatively assign the high-temperature complex as M#ADP, the low-fluorescence form, and the low-temperature complex with the more shifted β-phosphate resonance (−1.24 ppm) to the more fluorescent form M*ADP. This assignment is more fully substantiated below. At 3 °C K_3 ($=k_3/k_{-3}$) is equal to 0.5 (J. W. Shriver and B. D. Sykes, unpublished experiments); thus, k_3 is $\sim 5\ s^{-1}$ and k_{-3} is $\sim 10\ s^{-1}$.

The values for k_3 and k_{-3} are consistent with M*ADP being in slow exchange on the NMR time scale with M#ADP around 0 °C and demonstrate that the observed line widths of the β-phosphate of M*ADP and M#ADP are essentially the natural line widths. From the line widths of the bound phosphates, we can calculate a rotational correlation time for S-1 assuming that the predominant relaxation mechanism for phosphate in a protein of molecular weight 115 000 and at a field of 63 350 Gauss is due to chemical shift anisotropy. Indeed, from the results of Rao et al. (1978) on the magnetic field dependence of the line width of ATP bound to 3-phosphoglycerate kinase (M_r = 47 000), we may estimate the magnitude of the dipole–dipole contribution to line widths of protein-bound phosphates by extrapolating their observed field-dependent line widths to zero magnetic field. The chemical shift anisotropy relaxation contribution to the line width is field dependent (see eq 3 under Results), and the dipole–dipole relaxation contribution is field independent. The line width remaining after extrapolating to zero magnetic field is the dipole–dipole relaxation contribution. Thus, $\Delta\nu_{dd}$ proves to be on the order of 5–10 Hz for a phosphate on a protein of molecular weight 115 000.

Calculation of the rotational correlation time for S-1 from the bound ADP β-phosphate resonance line width requires the principle elements of the chemical shift tensor of the β-phosphate. It is difficult to obtain these elements from the powder pattern of ADP due to the overlap of the α-phosphate resonance. However, the chemical shift tensor of the β-phosphate of ADP should be nearly identical with that of pyrophosphate. The symmetry of PP leads to only one resonance in the powder pattern and unambiguous assignment of the principal elements: δ_{xx} = −42 ppm, δ_{yy} = −42 ppm, and δ_{zz} = 85 ppm for Na_4PP [chemical shifts relative to (1/3) trace $\{\bar{\sigma}\}$]. These values are similar to the values reported by Terao et al. (1977) for the disodium salt of AMP, a phosphate ester in a similar state of ionization: δ_{xx} = −36 ppm, δ_{yy} = −36 ppm, and δ_{zz} = 71 ppm. Both tensors are axially symmetric, i.e., $\delta_{xx} = \delta_{yy}$. Using the δ_{ii} values for pyrophosphate and an observed line width for each line of the doublet of 15–20 Hz (after removal of 5–10 Hz for the dipole–dipole relaxation contribution), we calculate a rotational correlation time of 8 (±2) × 10^{-8} s.

The calculated correlation time of 8 (±2) × 10^{-8} s for the β-phosphate of bound ADP is consistent with an ADP bound to a spherical, isotropically rotating protein of molecular weight 100 000. It is significantly less than the correlation times for S-1 determined by EPR and fluorescence depolarization techniques, i.e., 1.6 × 10^{-7}–2.2 × 10^{-7} s (Thomas, 1978; Mendelson et al., (1973), which have been recognized to be high for a protein of molecular weight 115 000 and have been attributed to the asymmetry of the S-1 molecule which is known to be significantly elongated (Elliot & Offer, 1978). The value determined by ^{31}P NMR may be small due to some motion in the binding site, or the principal axis of the chemical shift tensor of the bound β-phosphate of ADP may be closer to being perpendicular to the long axis of S-1, and therefore the relaxation time would be more sensitive to motions about the long axis than previously used probes. Since the two bound forms observed at 0 °C have essentially identical line widths and do not differ from that observed at 25 °C, the principal axes of the β-phosphate chemical shift tensor in the two forms would appear to be oriented similarly with respect to the principal axis of the diffusion tensor. Similarly, the line widths of the phosphates of bound AMP·PNP are consistent with a rigid active site and a diffusion tensor similar to that observed for the subfragment with bound MgADP.

In contrast to ADP at 25 °C, the binding of AMP·PNP to S-1 at 25 °C and pH 7 leads to a much more complicated spectrum. Both the α-phosphate and β-phosphate resonances indicate that MgAMP·PNP binds to S-1 in two different conformations at 25 °C. However, similar to MgADP complexes, the equilibrium between the two bound forms is very temperature dependent, and at 0 °C only one form is significantly populated. The β-phosphate resonance allows a calculation of the equilibrium constant for these two forms of one at 25 °C. As with ADP under the conditions in which we observe two bound forms, a biphasic fluorescence response upon mixing S-1 and AMP·PNP at 25 °C has been observed by Trybus & Taylor (1979). They have reported that the binding of AMP·PNP to S-1 takes place in at least three steps at 20 °C, 0.1 M KCl, and pH 7

$$\mathrm{M} + \mathrm{AMP{\cdot}PNP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{M{\cdot}AMP{\cdot}PNP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{M\#AMP{\cdot}PNP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{M^*AMP{\cdot}PNP} \quad (5)$$

with k_2 = 300 s^{-1} and $k_3 + k_{-3}$ = 20 s^{-1}. The temperature dependences of both the equilibrium constant K_3 observed by ^{31}P NMR and the amplitude of enhancement of the intrinsic fluorescene of heavy meromyosin observed upon binding AMP·PNP (Bechet et al., 1979) support the assignment of the two forms of S-1·AMP·PNP observed by NMR to M#-AMP·PNP and M*AMP·PNP. The fluorescence experiments of Bechet et al. (1979) indicate the titration of one myosin·AMP·PNP complex into another with a change in temperature from −15 to 20 °C, with the high-fluorescence form being favored by high temperature. From the transient experiments we know that the high-fluorescence form seen at high temperature is the third complex, M*AMP·PNP. In the ^{31}P NMR spectra the most shifted bound β-phosphate resonance (at −2.34 ppm) is favored by high temperature. We therefore assign the resonance at −2.34 ppm in the ^{31}P NMR spectra to the β-phosphate of bound AMP·PNP in the M*AMP·PNP form, i.e., the most fluorescent form gives rise to the most shifted β-phosphate resonance, as is seen with the M·ADP complex. If Trybus and Taylor have correctly assigned the observed rates to the above mechanism (see below for a different mechanism), then $k_3 = k_{-3}$ = 10 s^{-1} at 25 °C and pH 7, in 0.1 M KCl. It should be noted that this assignment of rate constants is consistent with the observation of two distinct β-phosphate resonances, i.e., the two forms are in slow exchange.

Swenson & Ritchie (1979) have shown that the binding enthalpy of ADP is approximately −8 kcal (exothermic) and, in contrast, that of AMP·PNP is near 0. The difference in enthalpies of binding for ADP and AMP·PNP at 15 °C has been interpreted as AMP·PNP binding like ADP, but with a further endothermic conformational step for myosin·AMP·PNP which leads to a total zero enthalpy change in contrast to the exothermic process observed for ADP binding (Swenson

& Ritchie, 1979). The results presented here show that indeed the binding of AMP·PNP at high temperatures results in a conformational change which is not observed for ADP and that this transition is endothermic.

The above evidence from AMP·PNP binding experiments indicates that the binding of ATP to myosin may be represented by a minimum of three steps, with a conformational change occurring with the M#ATP → M*ATP transition. A specific conformational change that takes place with the binding of ATP has been proposed to account for the high binding constant of ATP relative to ADP (Bagshaw & Trentham, 1974). The data for ADP and AMP·PNP would indicate that tight nucleotide binding is associated with an increased population of the M* form and therefore the M*ATP would be much more favored than the M#ATP form. This is consistent with ATP leading to an intrinsic fluorescence enhancement greater than that observed for AMP·PNP binding. The enhancement for ATP is difficult to measure due to the hydrolysis step, but Garland & Chueng (1979) show that it may be around 30%. Trybus & Taylor (1979) report that the AMP·PNP enhancement is ~15%. Garland & Cheung (1979) have recently stated that a three-step binding mechanism for ATP is consistent with their transient kinetic results.

In addition to the NMR results presented here and the work of Trybus and Taylor, a number of other reports have indicated that myosin–nucleotide complexes may exist in two conformations. First, there is the obvious pair of isomers M**ADP·P and M*ADP·P. The large negative ΔC_p for binding MgADP to myosin has been interpreted as arising from myosin or myosin·ADP existing in two different conformations with a sharply temperature-dependent equilibrium such that the relative population of the two states is very different at 0 and 12 °C (Kodama & Woledge, 1976), which is in agreement with our ADP results presented here. Bechet et al. (1979) have recently presented results indicating that there are two forms of the heavy meromyosin·AMP·PNP complex which have different fluorescence enhancements. The equilibrium between two forms is very temperature dependent, with a midpoint around 5 °C. Also, the apparent stoichiometry of ADP bindng to heavy meromyosin decreases with decreasing ionic strength which may be explained by assuming two sites with different ADP binding constants (Kodama et al., 1977). Proton-release measurements also indicate two classes of sites which differ in ADP affinity and protons released (Kardami et al., 1979). Arrhenius plots of myosin and actomyosin ATPase activities often show a sharp break in slope at ~5 °C (Bechet et al., 1979) which, as pointed out by Gutfreund (1975), cannot be explained by the temperature dependence of two different rate constants in a sequential reaction mechanism or by preequilibria. It is possible to obtain the sharp break by assuming that ADP may dissociate from two myosin·ADP forms, the relative population of which is highly temperature dependent (unpublished results).

Marston et al. (1979) have recently presented evidence for two actomyosin conformations in the muscle fiber with different nucleotide and actin affinities. They have observed changes in muscle fiber tension upon binding MgADP and MgAMP·PNP. Their results indicate that when MgAMP·PNP binds to a cross-bridge in the rigor state, the cross-bridge swings from the rigor state toward the conformation of a cross bridge prior to the power stroke, i.e., toward a 90° conformation relative to the actin and myosin fiber axes. This leads to a drop in tension without dissociation of the actomysoin. In addition, they have pointed out that there is a direct relationship between the effectiveness of a nucleotide leading to a tension drop and its ability to dissociate actomyosin. They have proposed that the actomyosin complex exists in two states which differ in nucleotide and actin affinities and angle of attachment of the cross-bridge:

$$\begin{array}{ccc} AM_r & \rightleftharpoons & AM_p \\ \downarrow\uparrow & & \downarrow\uparrow \\ AM_rL & \rightleftharpoons & AM_pL \end{array} \qquad (6)$$

The AM_r state is the actomyosin rigor state observed in the absence of nucleotides. An "effective nucleotide", i.e., one which leads to a change in rest length of a fiber, binds preferentially to the AM_p state and leads to a weakening of the "interprotein affinity and induces a length change". MgADP is not an effective nucleotide and therefore presumably binds preferentially to the AM_r state. They propose that the $AM_pL \rightarrow AM_rL$ transition may be analogous to the release of products from AM**ADP·P and contraction may be visualized as travelling clockwise around the mechanism shown in eq 6 (Marston et al., 1979). This is similar to the models of Eisenberg and Hill in which a chemical change is accompanied by a mechanical change. [See, for example, Figure 6 of Eisenberg & Hill (1978).] This mechanism has been reiterated by Morales & Botts (1979) in which they choose to write the actomyosin reaction as

$$AM^1N(1) \rightleftharpoons AM^2N(2) \rightleftharpoons \ldots \qquad (7)$$

indicating that a change in the nucleotide, $N(i)$, is directly coupled to a change in the actin binding site, M^i.

Equation 7 summarizes the mechanisms for coupling ATP hydrolysis and force production which have been presented in the past. In these mechanisms there is a particular sequence of events which must occur following the essentially irreversible binding of ATP. The power stroke is normally viewed as resulting from the release of products from the AM**ADP·P complex, i.e., the preferred cross-bridge orientation in AM**ADP·P is 90° and in AM*ADP it is 45°.

The NMR and transient kinetic data indicate that the myosin ATPase mechanism may be represented as

$$\begin{aligned} &M + ATP \rightleftharpoons M\cdot ATP \rightleftharpoons \boxed{M\#ATP \rightleftharpoons M^*ATP} \rightleftharpoons \\ &\boxed{M^{**}ADP\cdot P \rightleftharpoons M^*ADP\cdot P} \rightleftharpoons \\ &\boxed{M^*ADP + P \rightleftharpoons M\#ADP + P} \rightleftharpoons \\ &M\cdot ADP + P \rightleftharpoons M + ADP + P \end{aligned} \qquad (8)$$

The NMR results indicate that both forms of the ATP and ADP complexes may be significantly populated. The boxes emphasize that the present data is not sufficient to justify a linear mechanism. However, there is good reason to believe that the highly fluorescent complexes M*ATP and M**ADP·P are directly connected. While this mechanism may appear initially to be even more complicated than (1), the addition of two new intermediates results in a symmetry which was lacking in (1) and which may be important in the mechanism of muscle contraction since a similar symmetry is obvious in (6), a result of fiber binding experiments. Indeed, Marston and Tregear's postulate of two forms of the myosin cross-bridge demands an expansion of the Bagshaw–Trentham mechanism into a scheme similar to (8). Our ^{31}P NMR results have provided direct evidence for two new myosin intermediates which allows (1) to be expanded into a format similar to the Marston and Tregear scheme. The existence of two bound ATP forms, two bound products forms, and two bound ADP forms implies that there are two fundamental myosin con-

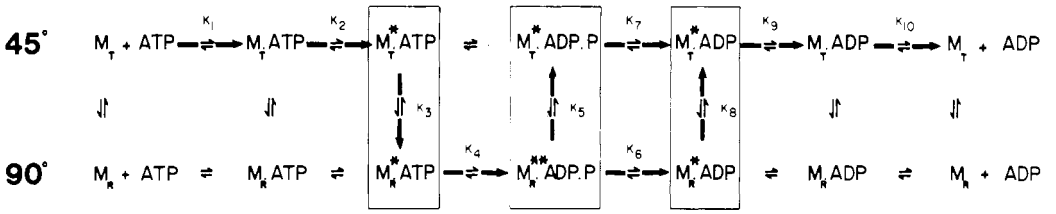

FIGURE 5: A proposed mechanism for the myosin ATPase which is consistent with the data presented in this work and a wide variety of other results (see Discussion). There are two myosin conformers, R and T, both of which can hydrolyze ATP. The predominant myosin conformer at 25 °C is labeled M_T. The predominant ATP complex at 25 °C is M_R*ATP and the predominant ADP complex is M_T*ADP. Note that the R forms of the ATP (as indicated by AMP·PNP), ADP·P, and ADP complexes have higher intrinsic fluorescence intensities than the T form. The kinetically most favored pathway of hydrolysis is emphasized by a bold line. Following the arguments of Marston et al. (1979), M_T binds to actin in approximately a 45° conformation with high affinity and has a low ATP affinity. M_R binds to actin in approximately a 90° conformation with low affinity and has a high ATP affinity.

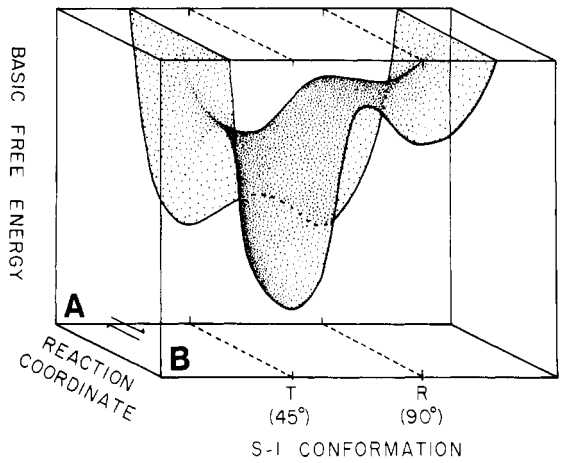

FIGURE 6: An idealized representation of a segment of the free energy surface describing the proposed myosin ATPase reaction scheme presented in Figure 5. Only two reaction intermediates are displayed here, A and B. The free energy "profile" for each intermediate contains two wells, labeled T and R, which are expressed as approximately 45° and 90° conformations, respectively, when bound to actin. Only chemistry may occur along the reaction coordinate and mechanics along the conformation coordinate. With appropriate barrier heights between wells, chemistry may be "coupled" with mechanics.

formations as seen with other energy transducing ATPases (Hill, 1977; Boyer, 1977) and each form may hydrolyze ATP. That is, the multiplicity of intermediates and complicated nature of (8) may be due to the presence of two interconvertible ATPase conformations, and the myosin ATPase scheme may be written as shown in Figure 5. In this scheme for each intermediate in the hydrolyisis of ATP there are *two* potential minima in the free energy surface (Figure 6) with the preferred conformation being a function of temperature and nucleotide in the binding site. The two forms corresponding to the two minima are labeled R and T to be consistent with the Monod et al. (1965) formalism for allosteric proteins. It should be noted that although the existence of these two states was implied by the experiments of Marston et al. (1979), their existence is not a function dependent on actin but is an inherent property of S-1. The form observed in the absence of nucleotide and actin at 25 °C is labeled the T form.

A mechanism similar to that shown in Figure 5 would be expected for actomyosin in another dimension with each myosin species connected to a corresponding actomyosin species by a reversible actin association reaction. The resulting actomyosin ATPase mechanism is a logical extension of the Michaelis–Menten formalism to enzymes involved in energy transduction, i.e., there are two interconvertible Michaelis–Mentin-type hydrolysis schemes and the conformational change responsible for the different pathways may be responsible for the "power stroke" in muscle energy transduction. The similarity between the actomyosin scheme analogous to that in Figure 5 and the Marston–Tregear mechanism (6) adds support to this argument.[2] Our NMR experiments appear to be entirely consistent with the experiments of Marston et al., (1979). At 25 °C the binding of AMP·PNP to S-1 results in significant population of the R form [M*AMP·PNP in (5)], whereas ADP binding results in population of essentially only the T form [M#ADP in (4)]. This implies that the R form (M*N) is the 90° form in Marston and Tregear's scheme and the T form (M#N) is the 45° conformation, i.e., the R form is expressed as approximately a 90° conformation in actomyosin and the T form is expressed as the 45° conformation. The transition from R to T results in force generation and tension, i.e., a power stroke can be viewed as an R → T transition. The likelihood of such a transition and the relative populations of the R and T forms is dependent on the intermediate and conditions such as temperature.

This presents a different view of the actomyosin ATPase scheme from the normal one in which actin is an enzyme activator. Here actin is the "substrate" of myosin and the ATPase site is an effector site which determines the relative populations of the two myosin cross-bridge conformations. This formalism is very similar to the Monod et al. (1965) model of allosteric phenomena except that the effector site is an enzymatic site which changes the effector from one favoring the R state to one favoring the T state and therefore a time dependence is introduced into the effector. A high concentration of ATP relative to ADP and P and the low actin affinity of the R state serve to drive the cross-bridge through repeated power stroke dissociation cycles.

Both the NMR experiments reported here and the fiber binding experiments of Marston et al. (1979) indicate that AMP·PNP serves as an ATP analogue and differs from ADP by being able to significantly populate the R form at higher temperatures. However, the inability of AMP·PNP to force the T ⇌ R equilibrium far to the right is consistent with its lower binding constant relative to ATP and its decreased efficiency at dissociating actomyosin.

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[2] To be consistent with the Monod et al. (1965) formalism, we prefer to label the myosin form which preferentially hydrolyzes ATP in Figure 5 the R form, i.e., the relaxed or active form. Note that AM_R would be analogous to AM_p in (6) and AM_T would be analogous to AM_r.

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