Modification of the interactions of myosin with actin and 5'-adenylyl imidodiphosphate by substitution of ethylene glycol for water

Steven B. MARSTON* and Richard T. TREGEAR†

*Cardiothoracic Institute, 2 Beaumont Street, London W1N 2DX, U.K., and †ARC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

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We studied the effect of replacing water by ethylene glycol as solvent on the properties of skeletal muscle myosin, myosin subfragment-1 (S1) and heavy meromyosin. Ethylene glycol (50%, v/v) had no detectable effect on the affinity of myosin or actomyosin for the substrate analogue 5'-adenylyl imidodiphosphate (AMPPNP). However, the rate constants for formation and dissociation of the myosin·Mg-AMPPNP complex were reduced 200-fold; the logarithm of the dissociation rate was roughly proportional to the fractional concentration of ethylene glycol. Nucleotide dissociation was accelerated at least 300-fold by pure actin but remained slow with regulated actin in the absence of Ca^{2+} . Ethylene glycol substitution reduced the affinity of S1 and the S1·MgAMPPNP complex for actin equally (100-fold at 50% ethylene glycol). These results show that ethylene glycol has specific effects on myosin's enzymic mechanism, which can account for its effect on the tension and stiffness of glycerinated muscle fibres.

The interaction of myosin with MgATP and F-actin is the basis of muscular contraction. The system has been extensively studied over the last 20 years, usually by manipulating the substrate or the proteins. Less attention has been paid to the solvent. The substitution of organic solvents for part of the solvent water has a powerful effect on the enzymic properties of many proteins (Douzou, 1975). Comparative studies indicate that their principal effect is due to the hydrophobicity of the solvent acting to restrict the surface area of the protein (Maurel, 1978; Gekko & Timasheff, 1981). Studies of myosin ATPase in the presence of organic solvents such as ethylene glycol were initiated as a preliminary to studying myosin's reactions below 0°C where an antifreeze solvent is required (Travers & Hillaire, 1979; Bechet *et al.*, 1979). Although ethylene glycol was regarded as an inert solvent there was evidence that it changed some of myosin's ATPase properties (Travers & Hillaire, 1979; Barman *et al.*, 1983). Ethylene glycol also affected demembranated muscle fibres: in the absence of nucleotide it reduced the tension held, and in the presence of the unhydrolysed substrate analogue AMPPNP it caused the fibres to relax (Clarke *et al.*, 1980a). Once relaxed in this

way the bound MgAMPPNP was only very slowly released; it became 'trapped' at the enzymic site (Tregear *et al.*, 1982).

The effects on demembranated muscle fibres could be due to changes either in the filament lattice or in the individual protein molecules. In order to distinguish between these two possibilities we have examined the effect of ethylene glycol on the nucleotide and actin-binding properties of isolated vertebrate skeletal muscle myosin and subfragment-1. We find that the solvent has highly specific effects on myosin's enzymic properties which can account for the observations in demembranated fibres. Preliminary reports of our results have been published (Clarke *et al.*, 1980a,b; Marston & Tregear, 1982; Tregear *et al.*, 1982).

Materials and methods

Materials

All proteins were prepared from rabbit fast skeletal muscle. Myosin, F-actin and regulated actin were prepared as described by Marston & Weber (1975). S1 and HMM were prepared by chymotryptic digestion of myosin (Weeds & Taylor, 1975) and labelled with iodo[^{14}C]acetamide (Marston & Weber, 1975).

AMPPNP was supplied by Sigma; the purity, as determined by poly(ethyleneimine)-cellulose chro-

Abbreviations used: AMPPNP, 5'-adenylyl imidodiphosphate (adenosine 5'-[β,γ -imidotriphosphate]); HMM, heavy meromyosin; S1, myosin subfragment-1.

matography was 95%. [^3H]AMPPNP, supplied by ICN, was 70–80% pure by the time it was sampled; where necessary it was purified by chromatography on poly(ethylene)imine-cellulose thin layers, eluted with formic acid (5%) and lyophilized before use.

Preparation and handling

All media contained low ionic strength, neutral, Ca^{2+} -free buffer: concentrations (in mM) KCl, 50; MgCl_2 , 5; Pipes (1,4-piperazinediethanesulphonic acid), pH 7.0, 5; EGTA, 5; dithiothreitol, 1; NaN_3 , 1. Solvent mixtures varied from 0 to 50% (v/v) ethylene glycol (ethanediol, AnalaR grade from BDH). Except where otherwise stated experiments were performed at 10°C in 50% ethylene glycol. Concentrated aqueous proteins were adjusted to the desired solvent either by dilution or by dialysis. F-actin and myosin were mixed in 0.6M-KCl prior to dialysis. Proteins were stored in 50% glycol buffer at –20°C. Volumetric handling was by positive displacement wherever possible, as the mixtures were often very viscous.

ATP hydrolysis

MgATP hydrolysis by myosin and actomyosin was measured in the solution described above. Reactions were started by the addition of MgATP and terminated by mixing with an equal volume of 5% (w/v) trichloroacetic acid. The precipitated protein was sedimented; in 50% glycol prolonged centrifugation (5 min at 12000g) was required. Phosphate in the supernatant was estimated by phosphomolybdate formation (Taussky & Schorr, 1953). Addition of ethylene glycol had no perceptible effect on the calibration of the assay.

Equilibrium measurements

Binding of S1 to F-actin. A sedimentation method was used (Marston & Weber, 1975). ^{14}C -labelled S1 at a concentration considerably less than the expected dissociation constant of acto-S1 was mixed with 8–12 different concentrations of F-actin ranging from 0.2 to 10 times the dissociation constant. The mixtures were centrifuged at 50000g for 2 h and the unbound [^{14}C]S1 in the supernatant was assayed. Approx. 15% of the radioactivity did not sediment when double-reciprocal plots were extrapolated to infinite actin concentration; the unsedimented fraction was assumed to represent inactive [^{14}C]S1 and free iodo[^{14}C]acetamide (see Marston & Weber, 1975; Marston *et al.*, 1979; Marston, 1980). The data were analysed by subtraction of this fraction and fitting to a power law (Hill plot). The best fit order was not significantly different from unity ($h = 1.05 \pm 0.06$, $n = 35$), indicating simple binding. The calculated

K_d varied between 10^3 and 10^8 M^{-1} , dependent on conditions.

In the presence of MgAMPPNP plus 30% or more ethylene glycol most of the S1 remained in the supernatant at the highest F-actin concentration we could obtain (0.2 mM). In these conditions 5 mM-[^3H]glucose was also added to the mixture and the ^3H and ^{14}C contents of the pellet and supernatant were measured after centrifugation. The S1 bound to F-actin in the pellet was estimated by subtraction of the free S1 equivalent to the trapped glucose volume. In comparative tests the single- and double-label methods gave results from which the calculated K_d varied by less than a factor of 2.

Binding of MgAMPPNP to S1. The amount of MgAMPPNP bound to S1 was determined by a rapid gel filtration method (Fig. 2). A mixture (200 μl) containing 1 μM -S1 and 0.9 μM -[^3H]MgAMPPNP was applied to a column (0.8 cm $\times$ 20 cm) of Sephadex G-25 at 6°C and eluted at 60 ml/h. Two peaks of ^3H radioactivity corresponding to excluded and retained volume were obtained and assigned to bound and free MgAMPPNP respectively. The concentrations were chosen so that bound and free MgAMPPNP were roughly equal since this permits the most accurate measurement. The viscosity of 50% ethylene glycol solutions resulted in asymmetric elution peaks. The asymmetry was determined by separations of S1 and nucleotide alone and accounted for as shown in Fig. 2. The binding constant was estimated from the bound and free data assuming simple binding and 100% activity and purity of the components.

Kinetics of nucleotide binding and release

The rate of reaction of MgAMPPNP with myosin in 50% ethylene glycol was measured indirectly from the inhibition of myosin MgATPase. MgAMPPNP was added to 10–20 μM (sites) myosin, HMM or S1 and incubated in ethylene glycol buffer for 0–60 s. At the end of the preincubation MgATP was added to 1 mM and after a further 5 min the reaction was stopped with an equal volume of 5% trichloroacetic acid and the phosphate released was assayed as described above. The degree of MgAMPPNP binding was calculated from the inhibition of MgATPase activity, on the assumption that it was constant during the MgATP incubation.

The release of MgAMPPNP bound to myosin, HMM or S1 was very slow and was also measured by the resultant MgATPase. The protein was incubated for 30–60 min with 50–500 μM -MgAMPPNP to saturate the nucleotide binding sites. MgATP was then added to 5 mM and the time course of phosphate release assayed over 5–60 min.

Controls were run omitting the preincubation stage. The instantaneous MgATPase activity was estimated from the timed series of experiments and hence the proportion of nucleotide binding sites from which MgAMPPNP had been released was calculated (Fig. 4). These experiments were repeated at different ethylene glycol concentrations, temperatures and in the presence of F-actin.

Results

Effect of ethylene glycol on equilibria

Actin binding equilibria with and without MgAMPPNP. Actin-S1 binding in ethylene glycol/water mixtures could be measured by the sedimentation method (Marston & Weber, 1975) since it was found that F-actin and F-actin-S1 complex sediment in ethylene glycol/water mixtures at least as rapidly as in aqueous buffer. Binding of actin to S1 was measured over a range of ethylene glycol/water fractions up to 50% ethylene glycol. The binding constant was reduced by the substitution of ethylene glycol for water. The plot of $\log(K_A)$ against ethylene glycol concentration showed a downward curvature (Fig. 1). At 50% glycol the binding constants measured in

separate protein preparations were 6.1×10^5 , 2.3×10^5 and $1.7 \times 10^5 \text{ M}^{-1}$ (mean 3.4×10^5). Substitution of 50% ethylene glycol for water thus reduced K_{A-M} by a factor of approx. 100.

In aqueous solution MgAMPPNP binding to S1 is known to reduce the affinity of actin for S1 by a factor of between 100 and 1000 (Marston *et al.*, 1979; Marston, 1982; Greene & Eisenberg, 1980; Konrad & Goody, 1982); in the present experiments the factor was approx. 300 (Fig. 1). Substitution of ethylene glycol for water further reduced the actin-S1-MgAMPPNP affinity; the effect was similar to that on actin-S1 without nucleotide (Fig. 1). The affinity of myosin or myosin-nucleotide for actin is thus greatly reduced by substitution of ethylene glycol for water in the solvent (Table 1).

Nucleotide binding equilibrium. The binding constant of MgAMPPNP to S1 in 50% ethylene glycol was determined by rapid gel filtration, which separated bound and free MgAMPPNP (Fig. 2). This method can be used because the S1-MgAMPPNP complex has a long lifetime (approx. 500 min at 6°C) relative to the time taken for separation. The estimated binding constants from two pairs of experiments using $1 \mu\text{M}$ - and $0.7 \mu\text{M}$ -S1 respectively were 3.6×10^6 , 3.4×10^6 , 5.2×10^6 and $5.3 \times 10^6 \text{ M}^{-1}$ (mean 4.4×10^6). Possible systematic errors, such as the presence of inactive S1 or dissociation of nucleotide during separation, would cause these figures to be underestimates. Similar binding constants have been reported for S1 in aqueous solution (Table 1).

Fig. 1. Effect of varying ethylene glycol fraction on the affinity of S1 (○) and S1-MgAMPPNP complex (●) for actin

Conditions: 10°C, 50mM-KCl, 5mM-MgCl₂, 5mM-EGTA, 5mM-Pipes, pH7.0, 1mM-Na₂SO₄, 0.5mM-dithiothreitol, 0-50% ethylene glycol. ○, No added nucleotide; ●, 0.5mM-MgAMPPNP. Binding was measured by the sedimentation method.

Table 1. Equilibria between Mg-AMPPNP (P), S1(M) and actin (A)

Standard conditions were 10°C, 50mM-KCl, 5mM-MgCl₂, 5mM-Pipes, 5mM-EGTA, 1mM-Na₂SO₄, 0.5mM-dithiothreitol, pH7.0. Measurements made under different conditions are placed in parentheses. References: ^aMarston *et al.*, 1979: conditions the same as in this work. ^bKonrad & Goody, 1982; 15°C, $I = 0.095$, pH8.0. ^cBagshaw *et al.*, 1972; 21°C, 100mM-KCl, pH8.0. ^dMarston, 1980; 20°C, 5mM-KCl, pH7.0.

Step	Binding constant (M ⁻¹) in:	
	50% ethylene glycol	Aqueous solution
A + M ⇌ AM	3 × 10 ⁵	3 × 10 ⁷
K_{A-M}		2 × 10 ^{7a}
A + MP ⇌ AMP	2 × 10 ³	2 × 10 ⁵
K_{A-MP}		2 × 10 ^{5a}
M + P ⇌ MP	4 × 10 ⁶	(11 × 10 ^{6b})
K_{M-P}		(3 × 10 ^{6c})
AM + P ⇌ AMP	3 × 10 ^{4*}	3 × 10 ^{4a*}
K_{AM-P}		(0.7 × 10 ^{4b})
		(2.5 × 10 ^{4d})

* Calculated from $K_{M-P} \times K_{A-MP} / K_{A-M}$.

Fig. 2. Separation of S1·MgAMPPNP complex from free MgAMPPNP by gel filtration

A mixture (200 μ l) of S1 (1 μ M) and [3 H]MgAMPPNP (0.9 μ M) was applied to a column (0.8 cm $\times$ 20 cm) of Sephadex G-25 and eluted at 60 ml/h. Conditions: 6°C, pH 7.0, 50% ethylene glycol. Control experiments gave a ratio of 1:2.4 of material eluting before and after the peak maximum in this column; the boundary of the S1. The MgAMPPNP peak was estimated on that basis (broken line). Some 60% of radioactivity was in the S1·MgAMPPNP peak; hence $K_{M-P} = 3.6 \times 10^6 \text{ M}^{-1}$.

The affinity of MgAMPPNP for actin-S1 complex in 50% ethylene glycol was calculated to be $2 \times 10^4 \text{ M}^{-1}$ from the known values of K_{A-M} , K_{M-P} and K_{A-MP} (Table 1). This value is similar to that determined in aqueous solution (Table 1) and in demembrated muscle fibres (Marston *et al.*, 1976; $3 \times 10^4 \text{ M}^{-1}$). The affinity of myosin or actomyosin for MgAMPPNP is thus little altered by substitution of ethylene glycol for water in the solvent.

Effect of ethylene glycol on rates

At 10°C myosin MgATPase activity was found to be about 0.02 s^{-1} in aqueous buffer at pH 7. Substitution of up to 50% ethylene glycol for water had little effect on the MgATPase activity (Fig. 3), as already shown (Travers & Hillaire, 1979; Bechet *et al.*, 1979).

Rate of dissociation of myosin·MgAMPPNP complex. When myosin in 50% ethylene glycol was preincubated with sufficient MgAMPPNP to saturate myosin, addition of a large excess of MgATP produced initially a low rate of MgATP hydrolysis. This gradually accelerated to reach the

Fig. 3. MgATP hydrolysis by S1 at 10°C in 50% ethylene glycol buffer

S1 concentration was 17 μ M. ●, Control: MgATPase rate is 0.017 s^{-1} . ○, Preincubated for 30 min in 50 μ M-MgAMPPNP. In both cases the reaction was started by adding MgATP to 5 mM. Preincubation in MgAMPPNP results in inhibition of the ATPase which is only very slowly relieved by displacement with MgATP.

Fig. 4. Determination of the rate of release of MgAMPPNP from S1 based on data in Fig. 3

Uninhibited activity, V_{∞} , 0.017 s^{-1} . Activity at time t , v_t , is obtained from the time course for each successive pair of points. Assuming $V_{\infty} - v_t/V_{\infty} = \text{proportion of sites occupied by MgAMPPNP}$, the slope of plot $\ln(V_{\infty} - v_t)/V_{\infty}$ against time gives the rate constant for MgAMPPNP release. In this experiment for S1 at 10°C with 50% glycol it is 0.0001 s^{-1} .

activity of myosin which had not been preincubated (Fig. 3). On the assumption that the MgATP hydrolysis rate was inhibited due to bound

Table 2. Rates of association and dissociation of myosin (M) with MgAMPPNP (P) and actin (A)
Standard conditions were as for Table 1. M, myosin; H, HMM; S, S1.

	Temperature (°C)	$k_+ (M^{-1} \cdot s^{-1})$ in:		$k_- (s^{-1})$ in:	
		50% ethylene glycol	Water	50% ethylene glycol	Water
$M + P \xrightleftharpoons[k_-]{k_+} MP$	10	$2.6 \times 10^3 (M)$ $1.8 \times 10^3 (H)$ $1.6 \times 10^3 (S)$	$5 \times 10^5 (S) \ddagger$	$0.00016 (M)$ $0.0001 (S)$	—
	20	—	$2 \times 10^5 (S) \dagger$	$0.0006 (M)$	$0.02 (S) \dagger$
$A + M \xrightleftharpoons[k_-]{k_+} AM$	10	$5 \times 10^5 (S) *$	$3 \times 10^6 (S) *$	$0.3 (S) *$	$0.01 (S) *$

* From Marston, 1982; conditions as for this work.
† From Trybus & Taylor, 1982; pH 7.0, 100mM-KCl.
‡ Tryptophan fluorescence increase (S. B. Marston, unpublished work).

MgAMPPNP the instantaneous MgATPase activity enabled us to determine the time course of MgAMPPNP release (see Fig. 4). Goodno & Taylor (1982) report a similar effect for the myosin·MgADP·vanadate complex.

We conclude that MgAMPPNP leaves myosin very slowly in 50% ethylene glycol. At 10°C it takes 50–100min for the MgATPase activity to reach half its maximum value (Fig. 3). Similar rates were obtained from myosin, HMM and S1. The time course of MgAMPPNP release was fitted adequately by a first-order reaction equation with a rate constant of about $10^{-4} s^{-1}$ (Fig. 4), which is much lower than the rate reported in aqueous solution (Table 2).

The rate constant was highly temperature-sensitive; the results were linear on an Arrhenius plot in the range 10–30°C. From the slope an activation energy of 130 kJ/mol was estimated. Reduction of the ethylene glycol content of the solution increased the rate of release of MgAMPPNP (Fig. 5). The relationship between log(rate constant) and glycol fraction appeared to be uniform over the range investigated (15–50%) and extrapolated to a value in aqueous buffer similar to the reported values. This form of relationship has been observed with other enzymes in organic solvents (Travers *et al.*, 1978; Maurel, 1978).

When myosin was preincubated with 1mM-MgADP in 50% ethylene glycol at 10°C, the subsequent MgATP hydrolysis time course was indistinguishable from that of untreated myosin even when measurements were made within the first 30s following MgATP addition. For MgADP, therefore, the rate constant of release exceeds $0.03 s^{-1}$ at 10°C in 50% ethylene glycol.

Rate of formation of myosin·MgAMPPNP complex. The formation of complexes between myosin

Fig. 5. Dependence of the rate of release of MgAMPPNP from myosin on the ethylene glycol fraction
Measurements were made at 10°C using 17 μM-myosin. Note logarithmic scale; the vertical bar represents the range of values published for release rate in aqueous solutions (Konrad & Goody, 1982; Trybus & Taylor, 1982).

and MgAMPPNP in 50% glycol was measured by inactivation of the MgATPase.

The time course of formation of the slowly released complex was followed by measuring initial myosin MgATPase activity after 0–60s preincubation with low MgAMPPNP concentrations. On this basis MgAMPPNP binding to

Fig. 6. Inhibition of myosin MgATPase activity in 50% ethylene glycol, 10°C, following preincubation with MgAMPPNP

MgAMPPNP (25 μM) was mixed with 17 μM -myosin (sites). After a given preincubation time MgATP was added to 1 mM. MgATP hydrolysis was measured after a 5 min incubation. Inhibition was assumed to be due to myosin sites being occupied by MgAMPPNP. The rate constant for MgAMPPNP binding was determined using a standard second order rate equation (Moore, 1963). In this experiment the rate constant was estimated as $2.6 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. Solid line gives theoretical time course using this rate constant.

myosin in 50% glycol at 10°C is slow but complete (Fig. 6). The most accurate binding time course was obtained with high myosin concentration and low MgAMPPNP concentration. The time course was fitted to a second-order reaction equation, since enzyme and substrate concentrations were similar; the rate constant obtained was $(2.6 \pm 0.4) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ (mean $\pm$ S.E.M.; $n = 7$). Single experiments performed with S1 and HMM gave similar values for the rate constant (Table 2). Binding was also measured under pseudo-first-order conditions, with the substrate in more than 4-fold excess. The determinations were less accurate but showed an increase in the observed rate constant with increasing nucleotide concentration up to 100 μM . The slopes of the plots obtained with three separate myosin preparations were similar (3.1×10^3 , 1.6×10^3 and $3.0 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$). The bulked data gave a slope of $2.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 7) which is close to the second-order rate constant estimated in Fig. 6. The ratio of formation and dissociation constants ($2.6 \times 10^3 / 1.6 \times 10^{-4} = 16 \times 10^6 \text{ M}^{-1}$) is four times the binding constant determined from equilibrium measurements (Table 1).

Effect of F-actin on rates. Myosin MgATPase activity was activated by F-actin in 50% glycol,

Fig. 7. Dependence of the apparent rate constant of MgAMPPNP binding to myosin (k_+) on the concentration of free MgAMPPNP in 50% ethylene glycol at 10°C. Myosin was mixed with MgAMPPNP [5 μM (sites) with 20 or 35 μM -nucleotide, 8–10 μM with 50–100 μM -nucleotide] and preincubated for 0–20 s (or more than 10 min). MgATP was then added and the free myosin site fraction (ϕ) determined as in Fig. 6. k_+ was calculated from the variation of ϕ with preincubation time: $k_+ = 1/t \ln \phi$. The points shown are each the mean ($\pm$ S.E.M.) of 4–16 assays. The regression line through the means is also shown. Its slope is $2.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$.

10°C (Fig. 8). The activation appeared to saturate at actin concentrations in excess of the myosin site concentrations. The maximum degree of activation was approx. 4–8-fold at 10°C.

When actomyosin in 50% ethylene glycol was preincubated with 500 μM -MgAMPPNP the subsequent time course of MgATP hydrolysis differed from those with myosin (Fig. 8). At an actin : myosin site ratio of only 0.3 the rate of hydrolysis increased much more rapidly than with myosin alone; at a 1.5 ratio the rate following preincubation equalled the control rate after 4 min and at ratios above 3 preincubated and control time courses were indistinguishable. These observations indicate that the release of MgAMPPNP from myosin is speeded up by actin from 10^{-4} s^{-1} to at least 0.03 s^{-1} .

The experiments were repeated using regulated actin, in the absence of free Ca^{2+} . In this case the myosin MgATPase was only very slightly (20%)

Fig. 8. Effect of F-actin on MgATP hydrolysis of myosin in 50% ethylene glycol at 10°C (a) Time course of MgATP hydrolysis for 15 μM-myosin (sites) plus (●) no actin, (□) 5 μM-actin, (○) 20 μM-actin, (Δ) 80 μM-actin. (b) Time course of MgATP hydrolysis of the same preparations following 30 min preincubation with 0.5 mM-MgAMPPNP.

activated by addition of actin, and the release of preincubated MgAMPPNP was not accelerated ($k = 2 \times 10^{-4} \text{ s}^{-1}$).

Discussion

We have found that replacement of water by ethylene glycol as solvent for myosin has several specific effects on its enzymic properties. In 50% ethylene glycol neither myosin's MgATPase activity nor its affinity for the substrate analogue MgAMPPNP is changed (Table 1) but there is a large kinetic effect; the rate of formation and dissociation of the myosin·MgAMPPNP complex is slowed about 200-fold (Table 2). The effect of glycol on myosin's interaction with actin is different: the interprotein affinity is reduced by up to 100-fold in 50% ethylene glycol, whether or not the myosin nucleotide binding site is occupied and the rates of actin–myosin binding and dissociation are both changed (Marston, 1982). Thus ethylene glycol is far from being an inert solvent.

Effect of ethylene glycol on nucleotide binding

Study of the kinetics of nucleotide binding to myosin have revealed a common pattern of events, summarized as a three-step process:

in which step 1 is a rapid equilibrium, step 2 is a rapid forward step with a large equilibrium constant and step 3 is less rapid with a small equilibrium constant (Trybus & Taylor, 1982; Johnson & Taylor, 1978). In aqueous solution the rate of MgAMPPNP dissociation is equal to the rate of $\text{PM}_1 \rightarrow \text{PM}_0$, which is at least 100-fold slower than $\text{PM}_2 \rightarrow \text{PM}_1$ (Trybus & Taylor, 1982). In 50% ethylene glycol at 10°C the rate of MgAMPPNP dissociation is reduced 200-fold, as judged by either the dissociation rate directly (Fig. 5) or the association rate (Table 2) together with the equilibrium constant (Table 1). Comparisons of measurements made by Trybus & Taylor (1982) in water and by ourselves in 50% ethylene glycol at 20°C indicate a smaller change; when adjusted for the different ionic strength employed (Konrad & Goody, 1982) a factor of 70-fold is estimated (0.04 versus 0.0006 s^{-1}). Thus k_{-2} is probably reduced substantially when ethylene glycol is substituted for water.

The observed second-order rate constant for association of MgAMPPNP and myosin in aqueous solution equals $K_1 k_{+2}$ (Trybus & Taylor, 1982). In 50% ethylene glycol this rate constant is reduced (Table 2) which could be due to a decrease in either K_1 or k_{+2} . We have no way of dissecting these factors from our observations. The linear

increase of observed rate constant with nucleotide concentration (Fig. 7) shows that K_1 is not greater than 10^4 M^{-1} but sets no lower limit to its value, which in aqueous solution is in the region of 10^3 M^{-1} (Trybus & Taylor, 1982).

The simplest interpretation of our observations is that ethylene glycol decreases k_{+2} and thus acts at a single point: it raises the activation energy for the transition between PM_0 and PM_1 . The higher activation energy of MgAMPPNP dissociation in 50% ethylene glycol (130 kJ/mol) than in aqueous solution (93 kJ/mol; Konrad & Goody, 1982) fits with this idea. The similarity between the affinity for MgAMPPNP for myosin, which is a measure of $K_1 K_2 K_3$, and the ratio of association to dissociation rates, which is a measure of $K_1 K_2$, shows that K_3 is near unity in 50% ethylene glycol. Values of K_3 near unity have been shown in aqueous buffer (Shriver & Sykes, 1981; Konrad & Goody, 1982).

The kinetics of association of MgATP with myosin in 40% ethylene glycol have recently been studied by Barman *et al.* (1983). They find that K_1 increases and k_{+2} decreases by about two orders of magnitude when water is replaced by 40% ethylene glycol; $K_1 k_{+2}$, $k_{+3} + k_{-3}$ and k_{cat} remain roughly constant. A large increase in K_1 is incompatible with our results, so ethylene glycol appears to have a different effect on the binding properties of the two nucleotides.

We have no evidence that ethylene glycol affects the kinetics of MgADP's interaction with myosin. The dissociation rate of MgADP from myosin in 50% ethylene glycol at 10°C exceeds 0.03 s^{-1} compared with approx 0.2 s^{-1} in aqueous solution (deduced from the values given at 4 and 20°C by Trybus & Taylor, 1982).

Effect of ethylene glycol on actin binding

The actin–myosin binding reaction has not been fully analysed, but it appears to have at least three

steps (collision followed by two conformational transitions) both for S1 itself and for the S1·MgAMPPNP complex (Trybus & Taylor, 1980, 1982; Konrad & Goody, 1982; Geeves & Gutfreund, 1982). The observed effect of replacing water with up to 50% ethylene glycol on the S1–actin interaction is similar to the effect of binding MgAMPPNP to aqueous S1: K_{A-M} decreases 100-fold, k_+ decreases 10–15-fold and k_- increases 20–100-fold (Fig. 1, Tables 1 and 2; Marston, 1982). It is thus possible that ethylene glycol alters the kinetics of interaction in a similar manner to MgAMPPNP. We cannot tell from our data whether ethylene glycol alters the structure of actin or myosin or both.

The parallelism of ethylene glycol and MgAMPPNP binding effects can be extended to demembranated muscle fibres. Both treatments lead to lengthening of the muscle and specific changes in the X-ray diffraction pattern from the crossbridge without net detachment of the myosin from the actin (Tregear *et al.*, 1982). The biochemical results with ethylene glycol (Table 1) confirm the relationship between magnitude of mechanical and structural change in muscle fibres and weakening of actin–myosin links previously shown with a series of different nucleotides (Marston *et al.*, 1979). The process underlying these coupled events therefore does not require binding of nucleotide to myosin.

Effect of ethylene glycol on the ternary interaction actin–myosin–nucleotide

Replacement of solvent water with 50% ethylene glycol results in weakening of the actin to myosin·MgAMPPNP linkage by about 100-fold whilst the affinity of actin·myosin for MgAMPPNP remains constant (Fig. 1, Table 1). It is noteworthy that the principle of tight coupling between sites whereby weaker actin–myosin binding is coupled to stronger myosin–nucleotide

Table 3. Comparison of the properties of the steady-state myosin·MgADP· P_i complex with those of two equilibrium complexes

Complex	K_{A-MP} (M^{-1})	Nucleotide dissociation rate (s^{-1}) from:			References
		Myosin	Actomyosin	Regulated actomyosin in the absence of Ca^{2+}	
Myosin·MgADP· P_i in water	3×10^3	10^{-4} *	6	–	Sleep & Hutton, 1978; Cardon & Boyer, 1978 Chalovich & Eisenberg, 1982
		0.05†	20	0.8	
Myosin·MgAMPPNP in 50% ethylene glycol	10^3	10^{-4}	>0.03	2×10^{-4}	Present work
Myosin·MgADP·vanadate in water	1.5×10^3	10^{-5}	0.5	0.02	Goodno & Taylor, 1982

* Released as ATP.

† Released as ADP + P_i .

binding (Marston *et al.*, 1979; Greene & Eisenberg, 1980; Konrad & Goody, 1982; Trybus & Taylor, 1982) is not observed here. Ethylene glycol acts on interprotein binding independently of its coupling to myosin's nucleotide-binding site and the effects of ethylene glycol and nucleotide are additive (Fig. 1).

The state myosin·MgAMPPNP in 50% ethylene glycol appears analogous both to the steady state intermediate of MgATP hydrolysis in aqueous solution, myosin·MgADP·P_i, and to the stable analogue complex myosin·MgADP·vanadate (Goodno, 1979; Goodno & Taylor, 1982). The biochemical properties of the complexes are compared in Table 3; K_{A-MN} is low and nucleotide release is slow but can be accelerated by actin, itself under regulatory control. The analogy is also true for the intact muscle matrix, which is mechanically and structurally relaxed; the tension is zero, the stiffness low and the X-ray diffraction pattern (of insect flight muscle, in which it has been studied) characteristic of detached crossbridges (Miller & Tregear, 1972; Goody *et al.*, 1980; Tregear *et al.*, 1982). Thus relaxation, however obtained, correlates with 'trapping' of nucleotide at the enzymic site.

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