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The influence of 2,3-butanedione 2-monoxime (BDM) on the interaction between actin and myosin in solution and in skinned muscle fibres

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Summary

2,3-butanedione 2-monoxime (BDM) inhibits muscle contraction and actomyosin ATPase both in fibres and in solution. It is potentially useful as a tool for exploring weak interactions between actin and myosin. We have examined the effect of BDM on several key steps of the myosin subfragment-1 and actomyosin subfragment-1 ATPase in solution. These studies show that BDM shifts the equilibrium between two actomyosin states towards a more weakly bound form when the acto.myosin complex has ADP alone or ADP and phosphate bound. We also confirm the findings of Herrmann and colleagues (1993, *Biochemistry*, **31**, 12227–32) that the main effect of BDM on the myosin subfragment-1 ATPase is to slow the release of phosphate following ATP hydrolysis. Skinned fibre studies show that the effects of BDM and phosphate on the steady isometric tension of the fibres are additive. This is consistent with the interpretation that BDM is reducing fibre tension either by increasing phosphate binding or by a direct effect on the crossbridge. Tension transients induced by rapid pressure release were examined in single muscle fibres; they showed that BDM reduces the rate of tension generation following pressure release. This result suggest that BDM directly affects the force generating event in the crossbridge.

Introduction

A detailed understanding of the relationship between the interaction of actin and myosin and the hydrolysis of ATP by myosin is central to the understanding of energy transduction in muscle. A physical or chemical effector which can reversibly alter the mechanism of energy transduction is potentially a powerful tool for studying the molecular processes underlying muscle contraction.

2,3-butanedione 2-monoxime (BDM) has been shown to inhibit contraction in both intact and skinned muscle fibre preparations from rabbit and frog (Mulieri & Alpert, 1984; Fryer *et al.*, 1988; Horiuti *et al.*, 1988; Bagni *et al.*, 1992) although it has little effect on lobster muscle (Silva *et al.*, 1993). In skinned muscle fibres, in which the effects on calcium release and uptake are eliminated, BDM reduces isometric tension, shortening speed and instantaneous stiffness. It inhibits the ATPase activity of HMM and acto.HMM in solution and also that of myofibrils (Higuchi & Takemori, 1989). Herrmann and colleagues (1993) have characterized the effect of BDM on the myosin subfragment-1 (S-1) ATPase in solution using the pathway described by *scheme I* (Bagshaw & Trentham, 1974).

$$M + T \underset{K_1}{\rightleftharpoons} MT \underset{K_2}{\rightleftharpoons} M^*T \underset{K_3}{\rightleftharpoons} M^{**}DP \underset{K_4}{\rightleftharpoons} M^*DP \underset{K_5}{\rightleftharpoons}$$

$$M^*D \underset{K_6}{\rightleftharpoons} MD \underset{K_7}{\rightleftharpoons} M \quad \textit{Scheme I}$$

where M indicates myosin and T, D, and P indicate that ATP, ADP and phosphate respectively are bound, K_i is

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the forward constant (k_{+i}/k_{-i}) for step i. Herrmann and colleagues (1993) showed that at 4°C BDM reduces the rate limiting release of phosphate (Pi) from S-1 which increases the ratio of [M**.ADP.Pi]:[M*.ADP] in the steady state. No studies to date have examined the effect of BDM on the interaction of actin and myosin in detail although Higuchi and Takemori (1989) concluded that the main cause of suppression of the acto.HMM and myofibrillar ATPase is the formation of a myosin-BDM complex rather than an effect on acto.myosin. Belknap and colleagues (1993) have reported that BDM has similar effects on the hydrolysis of a range of nucleoside triphosphates in solution and on the mechanical properties of muscle fibres using different nucleoside triphosphates. We report here a detailed study of the effect of BDM on key events in the ATPase cycle of S-1 and acto.S-1 in solution and in skinned muscle fibres.

The interaction between actin and S-1 can be described by a three-step mechanism as follows (see Geeves, 1991 for review):

$$A + M.N \overset{K_0}{\rightleftharpoons} A \sim M.N \overset{K_I}{\rightleftharpoons} A\text{-}M.N \overset{K_{II}}{\rightleftharpoons} A.M.N.$$

collision complex — A-state — R-state

Scheme II

where N is nucleotide (ATP, ADP or ADP + phosphate) bound to the S-1 active site. In this scheme, actin first forms a collision complex with S-1 and then undergoes two sequential isomerizations. After the first, actin is bound relatively weakly to S-1 and the nucleotide site remains almost unaffected. Following the second isomerization, actin is bound tightly to S-1 and any nucleotide is bound more weakly. While $K_0.K_I$ is relatively insensitive, K_{II} has a marked sensitivity to the nucleotide bound to S-1, ranging from greater than 100 with no nucleotide (Coates *et al.*, 1985) to <0.01 (Geeves *et al.*, 1986; Geeves & Jeffries, 1988) when the S-1 has ATP bound.

In this study, we demonstrate that BDM only affects the interaction between actin and S-1 in solution when ADP alone or both ADP and Pi are bound to S-1. Under these conditions. BDM stabilizes the A-state of the complex. This observation is supported by experiments in fibres where the transition between these two cross-bridge states can be perturbed by hydrostatic pressure.

Materials and methods

SOLUTION EXPERIMENTS

Proteins

Myosin subfragment-1 was prepared by chymotryptic digestion of rabbit myosin (Weeds & Taylor, 1975). F-actin was prepared by the method of Lehrer and Kerwar (1972) and pyrene labelled as described previously (Criddle *et al.*, 1985).

Stopped-flow

Rapid mixing experiments were carried out in a Hi-Tech Scientific stopped flow spectrophotometer SF-3L or SF-51. Pyrene fluorescence was excited at 365 nm and emission monitored through a KV393 glass filter. Protein fluorescence was excited at 290 nm and monitored through a WG345 glass filter. Signals from the photomultiplier were captured by an Infotech AD200 analog to digital converter using a Hewlett Packard 310 microcomputer or by a DAS16 analog to digital converter using a Vanilla 386 microcomputer. Data were analysed using a non-linear least squares routine (Edsall & Gutfreund, 1983) with software supplied by Hi-Tech Scientific. For all stopped flow experiments, at least five traces were averaged before fitting. In all experiments comparing the effects of BDM, paired experiments were carried out using protein from the same preparation with and without BDM.

Fluorescence titrations

Fluorescence titrations and some slow kinetic experiments were carried out on a Perkin Elmer LS-5B fluorescence spectrophotometer. Experiments comparing the effects of BDM were paired and used the same protein batches. Pyrene fluorescence was excited at 365 nm and emission monitored at 407 nm, protein fluorescence was excited at 295 nm and emission was monitored at 345 nm. Both fluorescence and emission used a 2.5 nm monochromator bandwidth. For kinetic experiments, data were collected via an RS232C interface to an Epson PC AX2 microcomputer using software supplied by Perkin-Elmer. For titrations, additions of S-1 were made automatically by a Harvard Instruments Pump-22 controlled by specially written software that also controlled the fluorometer, collected data, and corrected the fluorescence reading for dilution (McKillop & Geeves, 1993). Titrations were fitted by non-linear least squares to the following equation:

$$[A]_0.\alpha^2 - \alpha.([A]_0 + [M]_0 + K_d) + [M]_0 = 0 \quad (1)$$

where $[A]_0$ is the total concentration of pyrene-actin, $[M]_0$ is the total concentration of S-1 added, K_d is the dissociation constant and α is defined in terms of the fluorescence signal, F as:

$$\alpha = (F_0 - F)/(F_0 - F_\infty) \quad (2)$$

where F_0 and F_∞ are the fluorescence signals for zero and infinite S-1 concentrations respectively. With respect to *scheme II*, K_d is given by:

$$K_d = 1/(K_0.K_I.(1 + K_{II})) \quad (3)$$

K_{II} measurements

K_{II} was measured by a modification of the method described by Geeves and Jeffries (1988). The fluorescence of the pyrene group covalently attached to actin is quenched by approximately 70% when acto.S-1 goes from the A-state to the R-state (Criddle *et al.*, 1985, Geeves *et al.*, 1986). In the absence of nucleotide $K_{II} > 100$ and essentially all of S-1 is bound in the R-state (Coates *et al.*, 1985). In the work of Geeves and Jeffries (1988) addition of saturating ADP to acto.S-1, at high protein concentration, resulted in a change in pyrene fluorescence without dissociation of the two proteins. This increase in fluorescence was attributed to a decrease in the fraction of the acto.S-1 bound in the R-state and was used to estimate K_{II} according to the formula:

$$K_{II} = (F_A - F_L)/(F_L - F_S) \quad (4)$$

where F_A is the fluorescence the actin solution, F_S is the fluorescence after addition of S-1 and F_L is the fluorescence after addition of ADP or other ligand (all fluorescence values being corrected for dilution).

The experimental conditions that are of interest in this study are such that on addition of ADP some dissociation of actin and S-1 takes place. This problem is overcome by using the overall dissociation constant for the conditions used which was measured as described above. The fluorescence before addition of S-1 can be used to calculate the molar fluorescence of the A-state. When S-1 is added, the molar fluorescence of the R-state can be estimated using the approximation that all the actin is in the R-state. The dissociation constant in the presence of ligand gives the distribution of actin between dissociation actin and that in either the A-state or the R-state. This can be used with the fluorescence after ligand addition and the molar fluorescence of the A-state and the R-state to calculate K_{II} in the presence of ligand.

SKINNED MUSCLE FIBRE EXPERIMENTS

The full details of the apparatus, techniques and experimental protocols used for recording both steady-state and transient tension changes in single skinned rabbit psoas muscle fibres under hydrostatic pressure have been reported in detail previously (Geeves & Ranatunga, 1987; Fortune *et al.*, 1989, 1991). All experiments here used single skinned rabbit psoas muscle fibres. A segment (4 mm) of fibre was glued using nitrocellulose adhesive between the hook of the tension transducer (Akers AE801, Horten, Norway) and a second rigid hook fixed on the transducer assembly. The fibre segment was placed in relaxing solution for 5–10 min prior to activation via 3–5 min in preactivating solution. Steady force was recorded in the presence and absence of added BDM and/or inorganic phosphate over the range 0–20 mM.

The complex recovery of tension in maximally Ca-activated fibres following rapid pressure release has been described in detail by Fortune and colleagues (1991). Hydrostatic pressure was released from 10 MPa to 0.1 MPa within 1 ms and the previously reported three phase tension transient monitored in the presence and absence of 10 mM added BDM. Typically 3–4 such transients were recorded under each condition.

Details of the composition of experimental solutions are given elsewhere (Fortune *et al.*, 1989); they contained 50 mM imidazole as pH buffer (7.0 at 12°C) and their ionic strength was 200 mM. The desired amount of BDM was added to the control solution prior to the final adjustment of pH. Records of tension and pressure were stored as 4000 12-bit data points on a digital oscilloscope (Nicolet 3901). Data was transferred to hard disc and analysed as 400 data points on a Hewlett Packard 310 computer. Data was fitted using a non-linear least square fitting routine as described for the stop-flow data.

Results

SOLUTION STUDIES

The affinity of actin for S-1 in the presence of nucleotide

When S-1 binds to pyrene-actin, the pyrene fluorescence is quenched by 60–70% (Criddle *et al.*, 1985) and this can be used to monitor the formation of the acto.S-1 complex. Figure 1A shows fluorescence titrations when S-1 was added to pyrene-actin in the presence of ADP. The dissociation constant was estimated as 3.6 ± 0.46 μM from the least squares fit and this was increased to 5.4 ± 0.85 μM by the addition of 20 mM BDM. In the absence of nucleotide the dissociation constant was 0.3 ± 0.05 μM both in the presence and absence of BDM (data not shown). In the presence of 2 mM ADP and 90 mM phosphate, 20 mM BDM raised the dissociation constant of S-1 for actin from 7 ± 0.8 μM to 11 ± 1.5 μM (Fig. 1B). These results indicate that both phosphate and BDM weaken the interaction of actin and S-1 in the presence of ADP. BDM has little effect in the absence of ADP whereas Pi alone increases the dissociation constant to 1 μM (McKillop & Geeves, 1990).

Measurement of K_{II} in the presence of ADP

The weakening of the interaction between actin and S1 by BDM could be due to an effect on either the initial binding step (K_0K_1; *scheme* 2), the isomerisation (K_{II}) or a combination of these. This was examined by measuring the effect of BDM on K_{II}. K_{II} was measured as described in Materials and Methods. Addition of saturating ADP (2 mM) to acto.S-1 caused a greater relative rise in fluorescence in the presence of BDM than in its absence (137% and 72% respectively). This greater rise in the fluorescence was present even after correcting for the small dissociation of the proteins ($\approx 7\%$ and $\approx 10\%$ in the absence and presence of BDM respectively) and indicates that BDM causes a decrease in K_{II}. Thus K_{II} with ADP bound in the absence of BDM was 7.7 and was 2.8 in the presence of 20 mM BDM. A similar decrease was observed in two other paired experiments, though values of K_{II} varied by about twofold between protein batches.

Measurement of K_{II} in the presence of ADP and phosphate

K_{II} was also measured in the presence of both ADP and Pi. In this case the protocol was to add saturating ADP to the acto.S1 complex and then titrate in Pi. In so doing the ionic strength of the solution changes and so a control titration was performed using KCl in place of Pi. In the control titration the change in fluorescence intensity was approximately linear with KCl (a 0.7% increase in fluorescence for a millimolar rise in KCl concentration). The Pi titration was corrected for this linear change and K_{II} calculated from the remaining signal change. The correction was different in the presence of BDM (an increase of 0.4% per millimolar [KCl]). Figure 2 shows dependence of the calculated K_{II} on Pi concentration both in the presence and absence of BDM. At 20 mM phosphate the value of K_{II} corrected for ionic strength was 3.87 (uncorrected 2.60) with no BDM present and 0.48 (uncorrected 0.35) in the presence of 20 mM BDM. The variation in K_{II} with Pi was fitted to a hyperbola and gave $K_{0.5}$ (Pi concentration for half maximal effect) of 19 mM in the absence of BDM and 7 mM in the presence of BDM. These values and the estimated values of K_{II} at infinite Pi concentration were poorly defined, however, the three-

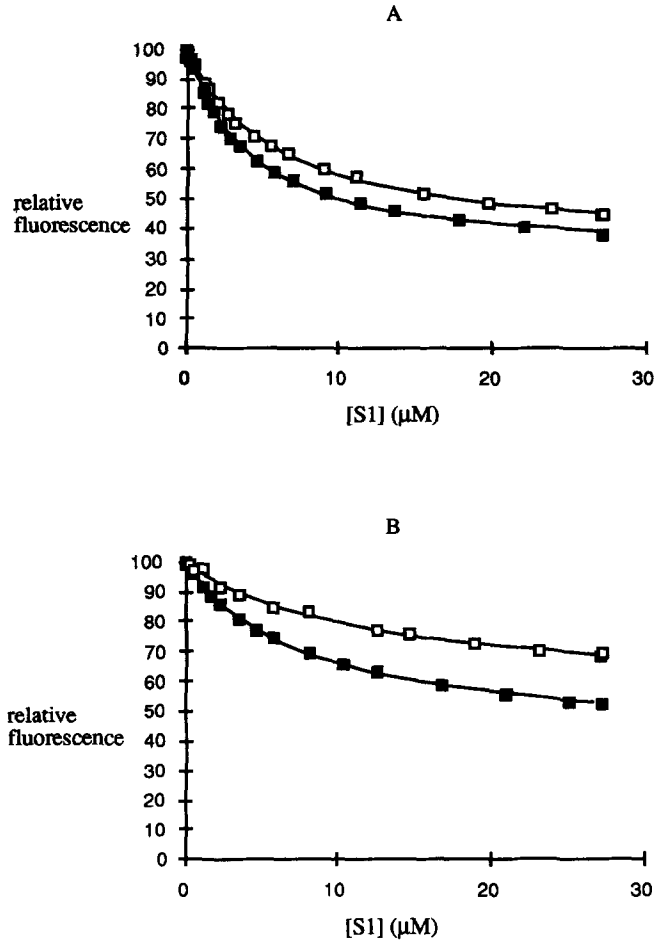

Fig. 1. Fluorescence titrations in the presence and absence of BDM. S-1 was added to 0.5 μM pyr-actin and the binding of S-1 to actin was monitored by the quench in pyrene fluorescence. For Fig. 1A, the buffer conditions were 20 mM MOPS, 140 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 2 mM ADP (pH 7.0) 20°C. The buffer conditions for Fig. 1B were 90 mM phosphate, 5 mM $MgCl_2$, 0.5 mM DTT, 2 mM ADP (pH 7.0) 20°C. In both cases, the closed squares are for the titrations with no BDM added while the open squares indicate the presence of 20 mM BDM. The data were fitted as described in the text and the fitted lines are shown. The dissociation constants fitted to each line are: Fig. 1A(no BDM) 3.6 μM; Fig. 1A (+20 mM BDM), 5.4 μM; Fig. 1B (no BDM), 7 μM; Fig. 1B (20 mM BDM), 11 μM.

fold larger drop in K_{II} in the presence of 20 mM phosphate clearly suggests that BDM either decreases $K_{0.5}$ or causes a larger decrease in K_{II} at saturating [Pi] or both. The decrease in K_{II} induced by BDM both in the absence and presence of Pi is sufficient to account for the increase in the dissociation constant measured in Fig. 1. Similar results were observed with proteins from two other protein preparations.

The rate of acto.S-1 association

The rate of association of actin and S-1 was measured in the presence of 2 mM ADP and 90 mM phosphate by rapidly mixing actin and S-1 in a stopped-flow fluorometer. As shown in Fig. 3 a plot of observed rate constant for this reaction as a function of S-1 concentration was a straight line and the second order rate constant ($K_0 k_{+1}$) for the association reaction obtained from the slope was 5.4 ($\pm$0.3) $\times 10^4$ $M^{-1}s^{-1}$ without BDM present and 4.1 ($\pm$0.3) $\times 10^4$ $M^{-1}s^{-1}$ in the presence of 20 mM BDM. In the presence of ADP alone and in the absence of nucleotide BDM induced no measurable change in the second order rate constant (data not shown). This is consistent with the proposition that $K_0.K_1$ is not changed by BDM.

The interaction of ADP and ATP with acto.S-1

The rate of ATP binding to acto.S-1 was examined by measuring the rate at which acto.S-1 dissociated when mixed with ATP in a stopped-flow fluorimeter. The variation of the observed rate with ATP concentration can be plotted and the slope gives the apparent second order binding constant for ATP binding to acto.S-1. We measured this to be 1.56 ($\pm$0.02) $\times 10^6$ $M^{-1}s^{-1}$ and in the presence of 20 mM BDM this was increased slightly to 1.84($\pm$0.06) $\times 10^6$ M^{-1} s^{-1} (data not shown). The binding constant for ADP to acto.S-1 can be measured using the

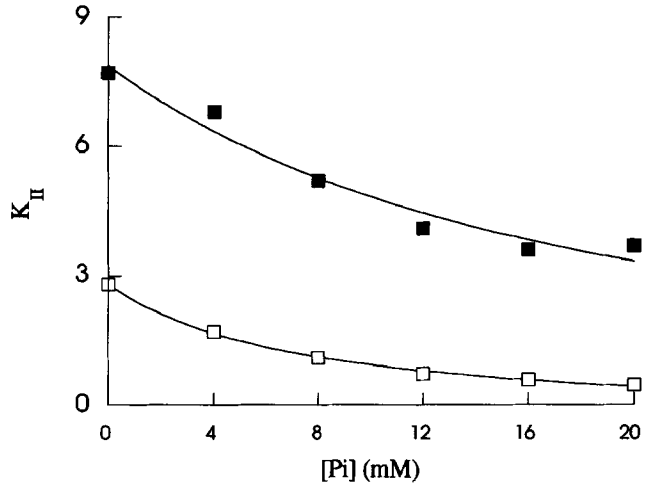

Fig. 2. The effect of BDM on the measurement of K_{II} in the presence of ADP and ADP with phosphate. The figure shows the effects of phosphate and BDM on K_{II}. The data were fitted to a rectangular hyperbola. The fit for the control data (closed squares) gave a half maximal effect for phosphate at 19 mM, those, in the presence of 20 mM BDM (open squares) gave a half maximal effect for phosphate at 7 mM. Initial buffer conditions were as in Fig. 1A, added phosphate was at pH 7.0.

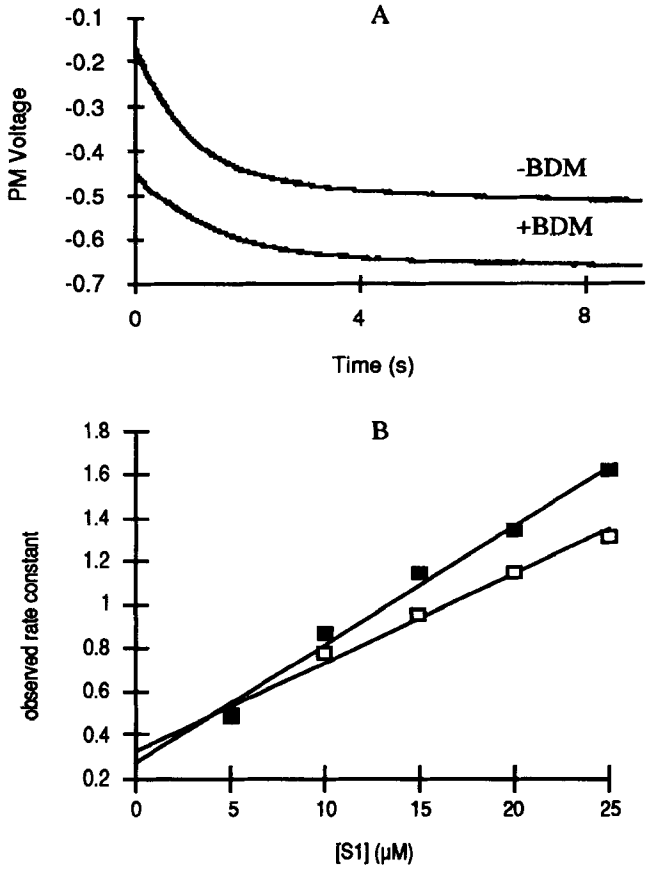

Fig. 3. The effect of BDM on the rate of S-1.ADP.Pi binding to pyr-actin. Figure 3A shows stopped flow traces obtained when 15 μM S-1 was rapidly mixed with 1 μM pyr-actin in the presence of 2 mM ADP and 90 mM phosphate. The upper trace shows the reaction without BDM while the lower trace shows the reaction in the presence of 20 mM BDM. The solid lines represent the least square fits to a single exponential plus drift. The observed rate constants are 1.04 s^{-1} without BDM and 0.71 s^{-1} in the presence of 20 mM BDM. Buffer conditions are as in Fig. 1B. Figure 2B shows the effect of S-1 concentration on these observed rate constants without BDM (solid squares) and in the presence of 20 mM BDM (open squares). Fitting a straight line to these plots gives K_0k_{+1} for the reaction. This is 5.4 ($\pm$0.3) $\times$ 10^4 M^{-1} s^{-1} without BDM and 4.1 ($\pm$0.3) $\times$ 10^4 M^{-1} s^{-1} with 20 mM BDM. The intercepts are 0.27 ($\pm$0.04) s^{-1} with no BDM and 0.32 ($\pm$0.09) s^{-1} in the presence of 20 mM BDM.

Table 1. Summary of the effects of BDM on myosin subfragment 1 in solution

	−BDM	*+BDM*
ATP binding to S1, K_1k_{+2}($M^{-1}s^{-1}$)	1.33 ($\pm$0.06) $\times 10^6$	0.89 ($\pm$0.03) $\times 10^6$
ATPase rate, $K_3k_{+4}/(1+K_3)$ (s^{-1})	0.038 $\pm$ 0.002	0.008 $\pm$ 0.001
ADP release rate, k_{+6} (s^{-1})	6.4 $\pm$ 0.04	8.6 $\pm$ 0.06
Affinity for actin (μM)		
S-1	0.3 $\pm$ 0.05	0.3 $\pm$ 0.05
S-1.ADP	3.6 $\pm$ 0.46	5.4 $\pm$ 0.85
S-1.ADP + 90 mM Pi	7 $\pm$ 0.8	11 $\pm$ 1.5
K_{II} for S-1.ADP	7.7	2.8
K_{II} for S-1.ADP + 20 mM Pi	3.7	0.48
Rate of actin binding, K_0k_{+1}($M^{-1}s^{-1}$)		
S-1.ADP	7.7 ($\pm$0.3) $\times 10^4$	8.0($\pm$0.4) $\times 10^4$
S-1.ADP + 90 mM Pi	5.4 ($\pm$0.3) $\times 10^4$	4.1 ($\pm$0.3) $\times 10^4$
ATP binding to acto.S-1 ($M^{-1}s^{-1}$)	1.56 ($\pm$0.03) $\times 10^6$	1.84 ($\pm$0.06) $\times 10^6$
Affinity of ADP for acto.S-1 (μM)	365 $\pm$ 36	336 $\pm$ 56

inhibition of the ATP-induced dissociation reaction by ADP. The dependence of the observed rate constant of the dissociation reaction on the ADP concentration can be described by:

$$k_{obs} = \frac{k_0}{1 + [ADP]/K_{ADP}} \quad (4)$$

where k_0 is the observed rate constant at zero ADP concentration and K_{ADP} is the dissociation constant for ADP (Siemankowski & White, 1984). The ADP dissociation constants with and without BDM were 365 ($\pm$36) μM and 336 ($\pm$56) μM respectively. This difference was not significant.

The rate of the ATP hydrolysis step on S1 ($k_3 + k_{-3}$)

The rate of the hydrolysis step ($k_{+3} + k_{-3}$; *scheme* 1) can be monitored by the slower of two fluorescence changes associated with ATP binding to acto.S-1 (Sleep & Taylor, 1976; Johnson & Taylor, 1978; Millar & Geeves, 1988). At 1°C and saturating ATP concentration the faster fluorescence change is rapid and is lost in the mixing time of the apparatus and the fluorescence change observed is the hydrolysis step. Millar and Geeves (1988) measured a rate for this step of 6 s^{-1} and we obtained a value of 6.4 ($\pm$0.04) s^{-1}. In the presence of 20 mM BDM, this rate was increased to 8.6 ($\pm$0.06) s^{-1}. Hermann and colleagues (1993) using quenched flow methods also found an increase in the rate of the hydrolysis step from 16 s^{-1} to 30 s^{-1} at 4°C (pH 7.4).

Single turnover of ATP by S-1

The rate of the isomerization that follows the hydrolysis of ATP is the limiting step in the S-1 ATPase at 20°C and can be measured by following the change in protein fluorescence during a single turnover when substociometric amounts of ATP are added to S-1 (Bagshaw & Trentham, 1974). The ATP is bound and hydrolyzed rapidly to give a high fluorescence state, M**.ADP.Pi. The fluorescence then decays at the rate $k_{+4}.K_3/(1+K_3)$ (*scheme* 1). When this experiment was performed in a fluorometer, 20 mM BDM reduced the observed rate of the fluorescence decay from 0.038 ($\pm$0.002) s^{-1} to 0.008 ($\pm$0.001) s^{-1}. This is similar to the result reported by Herrmann and colleagues (1993) who found rates in the presence and absence of BDM of 0.034 s^{-1} and 0.036 s^{-1} respectively using chemical quenching methods at 4°C. By measuring K_3 in the same experiment Herrmann and colleagues (1993) were able to show that 20 mM BDM increased K_3 from 2 to >10 at 4°C and therefore decreased the rate of the phosphate release step (k_{+4}; *scheme 1*) from 0.054 s^{-1} to 0.004 s^{-1}.

The rate of ADP dissociation from S-1

The rate of release of ADP was measured by mixing S-1.ADP with excess ATP. The observed rise in protein fluorescence is limited by the rate of ADP release (k_{+6}, *scheme 1*). This was 2.2 s^{-1} without BDM and 19 s^{-1} in the presence of 20 mM BDM. Herrmann and colleagues (1993) observed a small increase in this rate constant (from 0.036 s^{-1} to 0.044 s^{-1}) with addition of BDM at 4°C (pH 7.4).

SKINNED MUSCLE FIBRE STUDIES

Steady state measurements

The results presented so far point to the primary effects of BDM being on the interaction of Pi with acto.S-1 and S-1 with an additional effect on the A-to-R isomerization in acto.S-1. Pi binding to acto.S-1 is very weak in solution and is therefore difficult to quantitate precisely. Phosphate binds more readily to actomyosin during the ATPase cycle than when ADP and Pi are added at equilibrium (Sleep & Hutton, 1980). This effect has been well characterized in contracting skinned muscle fibres where the presence of millimolar phosphate (at constant ionic strength) inhibits the level of isometric force developed during active contraction; force being reduced by up to 60% at 20 mM Pi (Brandt *et al.*, 1982; Cooke & Pate, 1985; Pate & Cooke, 1989, Fortune *et al.*, 1989).

We examined the influence of BDM on the inhibition of isometric force by phosphate in a maximally activated skinned muscle fibre. Figure 4 shows the relationship between isometric force and phosphate concentration as [BDM] is varied from 0 to 20 mM. The data are shown analysed both as a binding curve and as a log[Pi] plot. Both analyses give a reasonable fit to the data. These results show the effects of Pi and BDM are additive and in combination can inhibit force by as much as 80%. Analysis in terms of a simple binding curve shows that the affinity of the fibre for Pi increases as [BDM] is increased. Conversely, if force is plotted as a function of [BDM] (not shown), the affinity of the fibre for BDM increases as [Pi] is increased. If the force is plotted as a function of log[Pi] (or log [BDM]), then a series of approximately parallel lines is produced which again demonstrates that the effects of Pi and BDM are additive. This result is consistent with the studies of acto.S-1 in solution and suggests that BDM stabilises the A-M.ADP.Pi complex (by stabilizing both the A-state and bound phosphate) and thereby inhibits both the ATPase rate and force generation in muscle fibres.

Pressure induced tension transients

Pressure induced tension transients from skinned muscle fibres can be used to examine the rate of phosphate binding to cycling crossbridges. It has previously been shown that increased hydrostatic pressure results in a reversible decrease in the maximally Ca-activated tension of a skinned muscle fibre (Fortune *et al.*, 1989). Following rapid release of pressure at 12°C (< 1 ms) a three phase tension response is seen: phase 1 is a further loss of tension in phase with pressure release, phase 2, a fast recovery (20–30 s^{-1}) and phase 3, a final slower recovery (2.5–3 s^{-1}). The marked phosphate dependence of the second phase is compatible with this step being closely coupled to the Pi release step and has led to the following model being proposed (Fortune *et al.*, 1991):

$$\text{A-M.D.Pi} \overset{K_{II}}{\rightleftharpoons} \text{A.M.D.Pi} \overset{K_{Pi}}{\rightleftharpoons} \text{A.M.D} + \text{Pi}$$

low force state high force states

Scheme III

where the transition from A–M.D.Pi to A.M.D.Pi is press;ure sensitaive and corresponds to step II of *scheme II*. K_{II} here is analogous to K_{II} in *scheme II* and K_{Pi} is the dissociation constant for phosphate release.

Figure 5 shows the effect of 10 mM BDM on the pressure induced tension transient for a single fibre. Data from five fibres showed that the apparent rate constant for phase 2 was significantly reduced ($p < 0.05$, paired Student's t-test) from 30.1 (±4.2) to 23.4 (±2.2), in the presence of 10 mM BDM. The effect on phase 3 was not significant. The rate of Pi binding to cycling crossbridges can also be examined using rapid photolysis of caged phosphate (Millar & Homsher, 1990). In preliminary studies the presence of BDM decreased the rate of tension fall following release of 5 mM Pi by 30%. (N. C. Millar & N. S. Fortune, unpublished observations). In both the caged Pi and the pressure jump studies the rate of observed relaxation has been attributed to:

$$\frac{k_{-II}[Pi]}{K_{Pi} + [Pi]} + k_{+II} \qquad (5)$$

These studies are compatible with BDM decreasing the rate of the transition by reducing k_{+II} or increasing K_{Pi}. As both solution and steady state fibre studies support an effect on K_{II} while not ruling out an increased phosphate

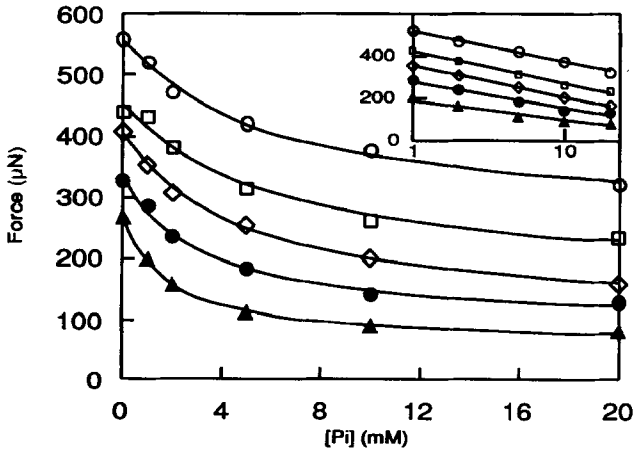

Fig. 4. The Pi and BDM concentration dependence of isometric force in a single rabbit psoas muscle fibre. The dependence of isometric force on phosphate concentration at five concentrations of BDM. Each data set is fitted to a hyperbola. BDM concentrations are represented by (○), (□), (◇), (●) and (▲) for 0, 2, 5, 10, 20 mM respectively. The fitted Ki values were 5.7 mM, 6.3 mM, 4.7 mM, 3.3 mM and 1.8 mM for 0, 2, 5, 10, 20 mM BDM respectively. The inset shows the same data plotted as a function of log[Pi] for each BDM concentration. When the force was plotted as a function of BDM concentration at each Pi concentration (not shown), the fitted Ki values were 6.1 mM, 9.0 mM, 9.1 mM, 6.9 mM, 4.7 mM and 4.4 mM for 0, 1, 2, 5, 10, 20 mM Pi respectively. Experiments were performed at ionic strength 0.2 M (pH 7.0) and 12°C.

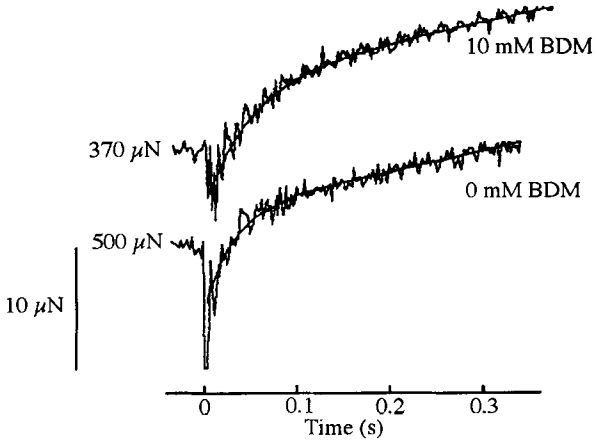

Fig. 5. Effect of BDM on pressure induced transients in single muscle fibres. This figure shows the pressure induced tension transient in a maximally Ca-activated muscle fibre in the presence and absence of 10 mM BDM. The transient is shown with best fit double exponential giving $1/\tau_2$ of 21 s^{-1} and 33 s^{-1} respectively in the presence and absence of BDM. For the slow phase $1/\tau_3$ is reduced from 2.4 s^{-1} to 2.1 s^{-1} by BDM. The fibre diameter was 75 μM and length 4 mm. The force per cross-sectional area was 131 kNm^{-2} in the control case and 68 kNm^{-2} in the presence of BDM. Buffer conditions were as for Fig. 4.

affinity, the area of overlap with the transient experiments is that a reduction in k_{+II} explains the transient data.

Discussion

The ability of BDM to alter the characteristics of actomyosin makes it useful as a tool for probing details of muscle function (Bagni *et al.*, 1992; McKillop & Geeves, 1993). Before the results of experiments on muscle fibres can be fully interpreted it is necessary to understand the effects of BDM on the interaction between myosin and nucleotide, Pi and actin. The solution studies presented here have demonstrated which events in the myosin and actomyosin ATPase cycle are sensitive to the presence of BDM. We have confirmed the results of Herrmann and colleagues (1993) that the principal effect of BDM on the S-1 ATPase is to reduce the rate of Pi release from S-1. These authors showed that this is due to stabilisation of the M**.ADP.Pi complex by both reducing the rate of the conformation change controlling Pi release (k_{+4}) and increasing the equilibrium constant of the ATP cleavage step (K_3).

We have found no evidence of any significant effect of up to 20 mM BDM on the interaction between actin and nucleotide free S-1 or on the binding of ATP or ADP to either S-1 or acto.S-1. Recent work by Zhao and Kawai (1993) has been interpreted as showing that BDM increases the affinity of nucleotide for actomyosin cross-bridges in contracting skinned rabbit psoas fibres. This observation is at odds with the results reported here and those of Herrmann and colleagues (1993) and Belknap and colleagues (1993). Although the observations of Zhao and Kawai (1993) are made using actomyosin which is assembled in a working muscle fibre it is difficult to see how a direct effect of BDM on ATP and ADP affinity would not be picked up in the solution studies presented here. The suggestion that this could be mediated through the thin filament proteins, troponin and tropomyosin (M. Kawai, personal communication), is not consistent with the observation that in solution the effect of BDM on the interaction of S-1 with reconstituted thin filaments can be explained by an effect on K_{II} alone; no direct effect is observed on thin filament regulation (McKillop & Geeves, 1993).

The results here suggest that if BDM does bind directly to S-1 then it does so without influencing either the actin or the nucleotide binding site. When either ADP alone or ADP and Pi are bound to S-1 then BDM does influence actin binding and we show that the affinity for actin is reduced K_{II}. According to the model of Geeves (1991) the isomerization from the A-state to the R-state of acto.S-1 results in weaker binding and accelerated release of both Pi and nucleotide from S-1. Thus inhibition of this isomerization by BDM will result in greater binding of Pi and ADP and inhibition of the ATPase. Thus, the presence of BDM stabilizes the A-state of actomyosin, the state in which actin is relatively weakly bound and nucleotide (and Pi) relatively strongly bound. In the absence of Pi, BDM reduces K_{II} less than 3-fold (ΔG increased from -4.99 to -2.52 kJ mol^{-1}) whereas in the presence of Pi, BDM reduces K_{II} by a factor of 8 (ΔG increased from -3.20 to $+1.78$ kJ/mol^{-1}). Whether the greater effect on K_{II} in the presence of 20 mM phosphate is due to an increase in phosphate affinity, the isomerization or both could not be accurately determined. An effect of BDM on K_{II} in the absence of bound nucleotide cannot be eliminated as K_{II} is much larger in the absence of nucleotide and our measurements may be insensitive to a small effect. K_0k_{+1} is not markedly affected by BDM and we made no estimate of the effect of BDM on k_{-1}, however, the changes observed in K_{II} are sufficient to account for the changes in overall affinity observed.

We detected no significant change in the affinity of ADP for acto.S-1 in the presence of BDM but the measurements only detect the ADP bound in rapid equilibrium with free ADP. An increase in occupancy of the A-M.D. state (expected from the decrease in K_{II}) in which the ADP is more tightly bound with a dissociation rate constant of $< 2\ s^{-1}$ (Geeves, 1989) would not be detected. In the absence of actin we observed a decrease in the rate at which ADP dissociates from S-1. The observed decrease was small but at lower temperatures Hermann and colleagues (1993) reported an increase in the rate of this process.

Pi binding to acto.S-1 is very weak and it is therefore difficult to achieve significant binding of Pi to acto.S-1 in solution. We cannot reliably estimate many of the rate or equilibrium constants for the species with Pi bound. However, some of these are accessible from the studies in skinned muscle fibres and are discussed below. The effect of BDM in the presence of ADP and phosphate is attributed to the A-to-R isomerization by extrapolation from the results with ADP only bound. The results are consistent with this, but all or some of the effects could be produced by an increase in phosphate affinity.

In summary the data presented here in the absence of actin are consistent with the primary effect of BDM being to stabilise the M**.ADP.P conformation of myosin. When actin is bound to myosin the primary effect is to shift the A-to-R equilibrium (K_{II}) towards the A-state. The R-state is postulated to be the force bearing or force generating state of the ATPase cycle. Thus BDM inhibition of the A-to-R transition will reduce the steady state concentration of force bearing states and so lead to a lower isometric force level in muscle fibres.

The results using skinned fibres show that BDM reduces the level of isometric force and that the effects of BDM and Pi are additive. In earlier studies, force reduction by phosphate was analysed as a simple binding curve with Pi behaving as in product inhibition and suggesting a binding constant of 3.5 mM at 20°C (Fortune *et al.*, 1989) and 3 mM at 10°C (Cooke & Pate, 1985). More recently Pate and Cooke (1989) suggested that, using thermodynamic arguments, force would be expected to have a linear dependence on the logarithm of phosphate concentration and the few studies which have managed to vary phosphate concentration by more than a factor of 20 support this view (Pate & Cooke, 1989; Millar & Homsher, 1992).

We have analysed our data both as hyperbolic inhibition curves, in which BDM and Pi both promote the affinity of each other, and as log plots. The data do not allow us to determine which is the more appropriate analysis but the comments of Dantzig and colleagues (1992) which suggest neither is completely satisfactory should be noted. However both analyses show the additive effect of the two treatments. The results are consistent with the model presented from solution studies in that both Pi and BDM when binding to acto.S-1 increase the energy change (and thus reduce the equilibrium constant) for the transition from the low force A-state to the high force R-state. The results from the pressure induced tension transients and the preliminary caged Pi experiments additionally demonstrate that BDM reduces the rate of the tension generating transition, compatible with the inhibition of the A to R transition in solution, probably by reducing k_{+II}. An effect on phosphate binding is not ruled out by this experiment though such an effect would involve weakening phosphate binding or would be small relative to the effect on K_{II}. This suggests that the effect on K_{II} is the major effect on the interaction of actin and myosin.

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Note added in proof

Since we submitted this paper, Y. Zhao and M. Kawai have published evidence that BDM reduces the equilibrium constant of the power stroke step in rabbit psoas muscle fibres (*Am. J. Physiol.* **266,** C437–47 (1994)). This is consistent with the main findings in our work.