Effect of 2,3-Butanedione Monoxime on Myosin and Myofibrillar ATPases. An Example of an Uncompetitive Inhibitor†

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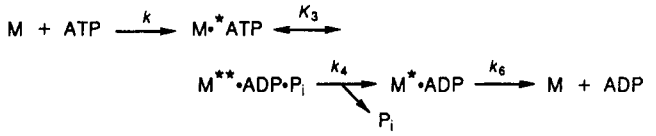
ABSTRACT: 2,3-Butanedione monoxime (BDM) reversibly inhibits force production in muscle. At least part of its action appears to be directly on the contractile apparatus. To understand better its mechanism of action, we studied the effect of BDM on the steps of myosin subfragment 1 Mg^{2+}-ATPase in 0.1 M potassium acetate, pH 7.4. Because of the rapidity of certain processes, we experimented at 4 °C and our main technique was the rapid flow quench method. By varying the experimental conditions (relative concentrations of reagents, time scale, quenching agent), it was possible to study selectively the different steps of the S1 Mg^{2+}-ATPase:



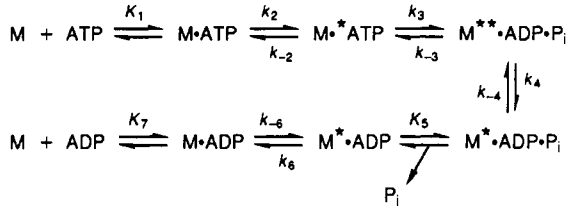
At saturation (20 mM), BDM had two major effects on the ATPase. First, it increased the equilibrium constant of the cleavage step (K_3) from 2 to >10. Second, it slowed the kinetics of the release of P_i by an order of magnitude (k_4; from 0.054 to 0.004 s^{-1}). By contrast, the kinetics of the binding of ATP (k) and the release of ADP (k_6) were little affected by BDM. Thus, the oxime appears to interact specifically with M**•ADP•P_i, and it is a rare example of an uncompetitive inhibitor. Its effect is to reduce the steady-state concentration of the "strong" actin binding state M*•ADP and to increase that of the "weak" binding state, M**•ADP•P_i. The effect of BDM on the initial ATPase of Ca^{2+} activated myofibrils was very similar to that on S1 ATPase. Thus, with myofibrils too BDM seems to exert its main effect subsequent to the initial binding and cleavage steps. We discuss our results with reference to the effect of BDM on the contractile properties of muscle fibers.

Muscle contraction involves the sliding of interdigitating myosin and actin filaments. This sliding process is driven by a cyclic interaction of the myosin heads (cross-bridges) with actin (thin filament) coupled to Mg^{2+}-ATP hydrolysis. The ATPase sites are on the cross-bridges, and it is thought that the contraction process is modulated by the various intermediates on the ATPase pathway, given by the Bagshaw–Trentham scheme (Trentham et al., 1976) (see Scheme I).

A major problem in understanding muscle contraction is to correlate the chemical steps of actomyosin ATPase with tension development. A way to aid toward this correlation is to seek ligands that interact specifically with particular intermediates of the ATPase and then to determine their effects on muscle contraction. If they inhibit the ATPase, such ligands are uncompetitive inhibitors; there are few examples of this way of perturbing a system.

2,3-Butanedione monoxime (BDM)[1] may be useful in correlating the chemistry of myosin ATPase with muscle contraction. Several reports show that BDM reversibly depresses twitch and tetanic contraction in muscle [e.g., Lang and Paul (1990) and Yagi et al. (1992)]. In whole muscle or mechanically skinned fibers, BDM may act at several points. It inhibits the Ca^{2+} release from sarcoplasmic reticulum (Fryer et al., 1988; Horiuti et al., 1988). Lang and Paul (1991) showed that BDM inhibits the Ca^{2+} channel current in single cells of guinea-pig taenia caeci smooth muscle. However, in chemically skinned fibers (in which the sarcoplasmic reticulum has been removed), BDM may inhibit the contractile apparatus by specifically suppressing cross-bridge attachment (Mulieri & Alpert, 1984; Horiuti et al., 1988; Lenart et al., 1989). Indeed, Higuchi and Takemori (1989) suggest that the suppression of contraction of muscle fibers by BDM is due to its direct and reversible action on the myosin heads.

Scheme I [a]



[a] M represents myosin and the asterisks indicate different conformations of the myosin.

Higuchi and Takemori (1989) studied the effect of BDM on myosin Mg^{2+}-ATPase. They showed that the ATPase decreased as the BDM concentration was increased. At 10 mM BDM the remaining activity was about 25%. No further loss of activity occurred at 20 mM BDM. Their P_i burst

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BDM, 2,3-butanedione monoxime; S1, myosin subfragment 1; Tris, tris(hydroxymethyl)-aminomethane; Ap5A, P^1,P^5-bis(adenosine-5′) pentaphosphate.

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Scheme II

$$M + ATP \xrightarrow{k} M^{*}ATP \xleftarrow{K_3}$$

$$M^{**}\cdot ADP\cdot P_i \xrightarrow{k_4} M^{*}\cdot ADP + P_i \xrightarrow{k_6} M + ADP$$

measurements suggested that the equilibrium constant for the chemical step, K_3 (Scheme I), is not affected by BDM. They suggest that BDM stabilizes $M^{**}\cdot ADP\cdot P_i$ and that it decreases the ATPase of myosin by slowing down the rate-limiting release of P_i (k_4). In accordance with this suggestion, Oetliker et al. (1991) showed that BDM had little effect on the K^+–EDTA ATPase activity of myosin where the kinetics of the P_i release are not rate limiting and there is no P_i burst.

We describe here the effect of BDM on the kinetics of the steps of myosin Mg^{2+}-ATPase (Scheme I). Under our conditions, $K_1 \gg [ATP]$, and the two first steps can be reduced to one with an apparent constant $k = k_2[ATP]/K_1$. In the time range used in most of our experiments, step 3 is fast and can be considered to be a rapid equilibrium. Finally, the concentrations of P_i and ADP are much lower than the equilibrium constants K_5 and K_7. By taking these approximations into account, we reduce Scheme I to the working Scheme II. In Scheme II, the kinetics of the P_i release are described by k_4 (apparent kinetics $k_o = k_4K_3/(1 + K_3)$). The kinetics of the ADP release are described by k_6.

Because of the rapidity of certain of the processes of Scheme II, we carried out our experiments at 4 °C. The buffer used had an ionic composition mimicking that obtaining in vivo (Houadjeto et al., 1992). We show that the only constants that are changed significantly by BDM are K_3 and k_4.

MATERIALS AND METHODS

Proteins and Reagents. Myosin and its subfragment (S1) were prepared from rabbit skeletal muscle (Weeds & Taylor, 1975) and myofibrils from rabbit psoas muscle as in Houadjeto et al. (1992). Protein concentrations were determined as in Houadjeto et al. (1992). Myosin head ATPase site concentrations were determined from cold ATP chase experiments (Barman & Travers, 1985). [γ-^{32}P]ATP was from Amersham International, and BDM was from Fluka Chemie, Switzerland. Solutions containing BDM were made up within 1 h of the experiment and kept on ice.

Experimental Conditions. Experiments were carried out at 4 °C. The buffer was 0.1 M potassium acetate, 5 mM KCl, 2 mM magnesium acetate, and 50 mM Tris adjusted to pH 7.4 with acetic acid. These were the conditions that we used in our studies on the myofibrillar ATPases (Houadjeto et al., 1991, 1992).

In certain experiments BDM was included in the reaction mixtures. Identical results were obtained whether the S1 or ATP or both were incubated with BDM before the experiments. In most of the experiments reported below, the BDM was included with the ATP rather than with the S1.

Quenched Flow Experiments. The apparatus used has been described (Barman & Travers, 1985).

Two types of experiment were carried out. In *P_i burst experiments* S1 + [γ-^{32}P]ATP reaction mixtures are quenched in acid (22% trichloroacetic acid plus 1 mM KH_2PO_4) and the [^{32}P]P_i determined by the method of Reimann and Umfleet (1978). These experiments were carried out under multi-turnover (e.g., Figures 1 and 4) or single-turnover conditions (e.g., Figure 5). In the former, one obtains information on the kinetics of the cleavage step (step 3 of Scheme II) and the steady-state rate. In single-turnover experiments one obtains information on the cleavage and the release of the P_i steps (steps 3 and 4).

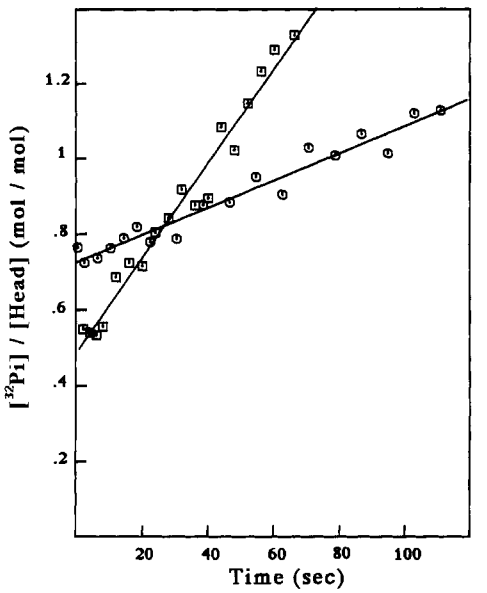


FIGURE 1: Steady-state time courses at 4 °C for S1 ATPase in the absence (□) or presence (○) of 20 mM BDM. The reaction mixtures (2 μM S1 + 10 μM [γ-^{32}P]ATP) were quenched in acid at the times indicated and the [^{32}P]P_i was determined.

In *cold ATP chase experiments*, S1 + [γ-^{32}P]ATP reaction mixtures are aged for time t and quenched in a large molar excess of unlabeled ATP. The quenched reaction mixtures are incubated at 20–25 °C and after 2 min are stopped by the addition of acid and the [^{32}P]P_i is determined. By plotting [^{32}P]P_i versus time one obtains a transient burst phase of amplitude equal to the ATPase site concentration of the S1 and kinetics $k = k_2[ATP]/K_1 + [ATP]$. For a discussion of the cold ATP chase method, see Barman and Travers (1985).

The data obtained from the flow-quench experiments are presented in the form [^{32}P]P_i/[Head] (mol/mol) versus time (e.g., Figure 1). [Head] represents S1 molarity (i.e., it is a protein concentration) whether with S1 itself or with myofibrils. Although the active site (ATPase) concentrations of the preparations were obtained by the ATP chase method, we prefer this way of presenting the data instead of normalizing to active site concentrations. Consequently, in the text, amplitudes are experimentally obtained and are with reference to S1 molarities.

When the effect of BDM upon some kinetic parameter of S1 ATPase is determined, the experiments with and without the oxime were carried out using the same preparation of S1 and on the same day. We give further details on the treatment of data in Houadjeto et al. (1992).

Fluorescence Stopped-Flow Experiments. These were carried out using the apparatus described by Tesi et al. (1988). The reaction mixtures were excited by light at 300 nm, and the emitted light at 340 nm was analyzed.

RESULTS

Effect of BDM on the Overall Mg^{2+}-ATPase of S1. The effect of 20 mM BDM on S1 Mg^{2+}-ATPase is shown in Figure 1. The experiments were carried out with the same preparation of S1 which titrated 0.71 mol of active site/mol of S1.

There are two noteworthy features of these experiments. First, BDM decreased the k_{cat} from 0.018 s^{-1} to 0.004 s^{-1}, i.e., by a factor of 4.5. Second, it increased the amplitude of the

Table I: Effect of BDM upon Certain Kinetic Constants of Myosin ATPase at 4 °C[a]

constant	without BDM	with 20 mM BDM
k_2/K_1 (μM^{-1} s^{-1})	1.8 (±0.2)	1.2 (±0.1)
$k_3 + k_{-3}$ (s^{-1})	16 (±2)	30 (±3)
K_3	2.0 (±0.2)	≥10
k_o (s^{-1})	0.036 (±0.004)	0.0034 (±0.0004)
k_4 (s^{-1})	0.054 (±0.008)	0.0040 (±0.0005)
k_6 (s^{-1})	0.036 (±0.005)	0.044 (±0.004)
k_{cat} (s^{-1})	0.018 (±0.002)	0.0040 (±0.0004)

[a] The buffer was 0.1 M potassium acetate, 5 mM KCl, 2 mM magnesium acetate, and 50 mM Tris–acetate, pH 7.4.

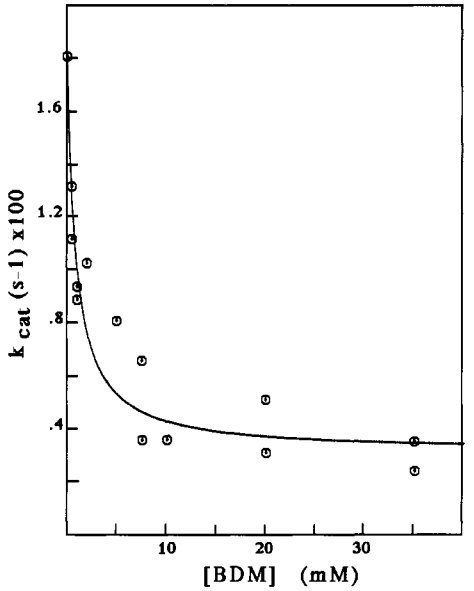


FIGURE 2: Dependence of k_{cat} upon the BDM concentration. The reaction mixtures (2 μM S1 + 10 μM [γ-^{32}P]ATP + BDM as indicated) were incubated as shown in Figure 1 and quenched in acid and the [^{32}P]P_i was determined.

initial P_i burst phase from 0.49 to 0.73 mol of P_i/mol of S1. This strongly suggests that BDM increases the equilibrium constant of the cleavage step. Estimates for this constant, K_3, are given in Table I.

The dependence of k_{cat} upon the BDM concentration is given in Figure 2. The k_{cat} decreased with the BDM concentration until it reached a plateau of about 25% remaining activity, in agreement with Higuchi and Takemori (1989). Interestingly, their experiments were carried out at 4.3 mM ATP compared with our 10 μM—it seems, therefore, that the concentration of ATP does not affect the inhibition by BDM. Except where otherwise stated, our experiments were carried out at 20 mM BDM, i.e., well on the 25% plateau. At concentrations less than 20 mM, the k_{cat} versus [BDM] plot appears to be hyperbolic, giving an apparent K_d for BDM of about 1 mM (Figure 2).

BDM Has a Small Effect on the Initial Binding of ATP. We then studied the effect of BDM on certain of the steps of S1 ATPase, starting with the initial binding steps (steps 1 and 2, Scheme I) which we investigated by the cold ATP chase method.

Typical ATP chase experiments with and without 20 mM BDM are illustrated in Figure 3. They were carried out at the same concentrations of S1 and ATP and the initial binding kinetics appear to be very similar: with each there was a rapid rise of tightly bound ATP followed by the steady-state rate. Experiments were also carried out at 0.57 μM ATP; the results obtained from both sets of experiments are given in Table II.

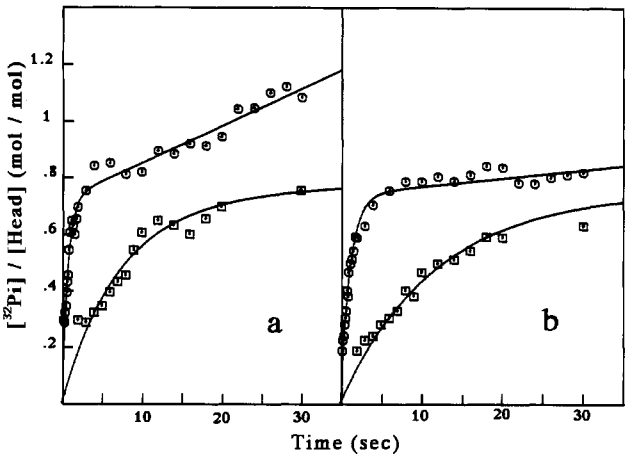


FIGURE 3: Binding of ATP to S1 measured by the cold ATP chase method in the absence (a) or presence (b) of 20 mM BDM. The reaction mixtures (0.22 μM S1 + 0.75 μM [γ-^{32}P]ATP) were quenched in 10 mM ATP, and the [^{32}P]P_i was determined after a final acid quench. For (○), the time scale is as indicated; for (□) it is divided by 10. The curves were fitted to exponentials followed by linear phases.

Table II: Effect of BDM upon the ATP Binding Kinetics to S1 at 4 °C[a]

[ATP] (μM)	k (s^{-1})	k_2/K_1 (μM^{-1} s^{-1})	amplitude of rapid rise (mol of [^{32}P]Pi/mol of S1)
	without BDM		
0.57	1.0 (±0.1)	1.75 (±0.15)	0.76 (±0.03)
0.75	1.4 (±0.2)	1.85 (±0.15)	0.72 (±0.02)
	with 20 mM BDM		
0.57	0.72 (±0.05)	1.25 (±0.10)	0.74 (±0.03)
0.75	0.87 (±0.05)	1.15 (±0.10)	0.73 (±0.02)

[a] The data are from cold ATP chase experiments. k refers to the kinetics of the tight binding of ATP. At [ATP] ≪ K_1 (as here), $k = k_2$[ATP]/K_1. For further details see legend to Figure 3.

Under our experimental conditions, K_1 and k_2 (Scheme I) are large and cannot be obtained by our equipment; since $k_{-2} \ll k$, the kinetics give directly the second-order binding constant for ATP, k_2/K_1, (Houadjeto et al., 1992). From Table II, this constant decreases by about 30% in the presence of 20 mM BDM. This is a small change, and we conclude that the effect of BDM on the initial binding of ATP is small. It is noteworthy that the transient burst amplitudes in the chase experiments were not affected by BDM; thus the oxime does not seem to affect the tight binding of ATP to S1.

Effect of BDM upon the Cleavage and Release of P_i Steps. The effect of BDM upon the transient kinetics of the P_i burst phase ($k_3 + k_{-3}$) is illustrated in Figure 4. Each time course consists of a transient lag phase (presumably a manifestation of the initial binding process, K_1 and k_2) which was followed by a rapid rise of kinetics $k_3 + k_{-3}$. On the time scale used, the subsequent steady-state rates could not be discerned. BDM had the effect of increasing somewhat $k_3 + k_{-3}$: from 22 s^{-1} in its absence to 30 s^{-1} in its presence. In agreement with the steady-state experiments (Figure 1), the amplitude of the P_i burst increased with BDM.

On the time scale of seconds, step 3 (Scheme I) becomes a rapid equilibrium defined by K_3. From the P_i burst experiments illustrated in Figures 1 and 4, it appears that BDM increases K_3. A more sensitive way to determine a change in K_3 is to carry out P_i burst experiments under single-turnover conditions. Such experiments are precise since all the [γ-^{32}P]ATP is turned over, provided that [ATPase site]

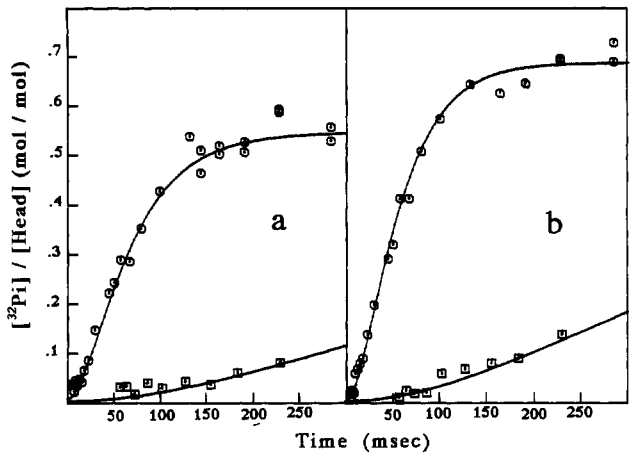


FIGURE 4: Transient kinetic time course of a P_i burst without (a) or with (b) 20 mM BDM. The reaction mixtures (7.8 μM S1 + 38.7 μM [γ-^{32}P]ATP) were quenched in acid and the [^{32}P]P_i was determined. For (O), the time scale is as indicated; for (□) it is divided by 10. The curves were simulated using Scheme II and the constant in Table I.

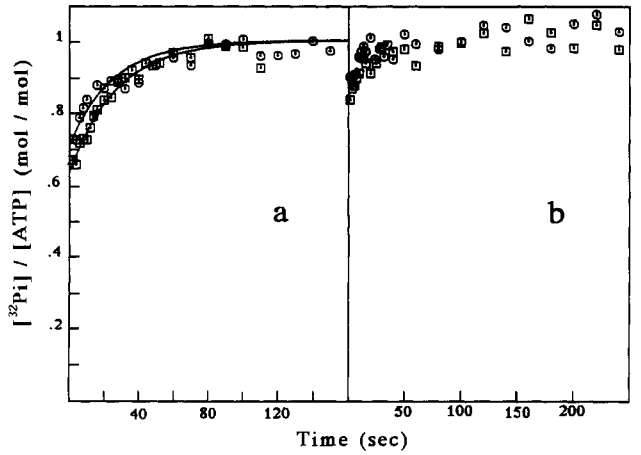


FIGURE 5: Single-turnover time courses of the hydrolysis of ATP by S1 in the absence (a) or presence (b) of 20 mM BDM at 4 °C. The reaction mixtures (4 μM S1 + 0.27 μM [γ-^{32}P]ATP) were quenched in acid, and the [^{32}P]P_i was determined. The results of two experiments are given in each figure (O, □). In (a), the curves were fitted to single exponentials.

$\gg$ [ATP], and they can give estimates for K_3 and k_4 that are independent of the ATPase site concentration. Bagshaw and Trentham (1973) have shown that in single-turnover experiments with S1 on the $1/k_{cat}$ time scale the progress curve consist of a rapid rise of amplitude $K_3/(1 + K_3)$ followed by a slow exponential of $k_o = k_4K_3/(1 + K_3)$ leading to the complete hydrolysis of the ATP.

Two typical single-turnover experiments in the absence of BDM are given in Figure 5a: there was a rapid rise (kinetics not obtained in the time range used) of amplitude 0.67 mol of P_i/mol of ATP and a slow exponential of 0.036 s^{-1}. Bagshaw and Trentham (1974) obtained $k_o = 0.036$ s^{-1} at 5 °C in a buffer of ionic strength and pH similar to ours. These experiments confirm, first, the value for K_3 found under multiturnover conditions (Figure 1). Second, from the relationship $k_{cat} = k_ok_6/(k_o + k_6)$, they allowed us to calculate k_6 (Table I).

In the presence of BDM (Figure 5b) the rapid rise was large (0.85–0.9 mol of P_i/mol of ATP) which confirms that the oxime increases K_3. Because of this large rise, it was difficult to obtain a value for the final kinetics leading to the complete hydrolysis of the ATP. This final rise, although very small, was reproducible. It is probably not due to free S1 as increasing the BDM concentration to 50 mM did not reduce it. Nor does it seem to be due to the tail end of the kinetics of formation of M**·ADP·P_i (i.e., corresponding to the rapid rise) as it was not reduced by increasing the concentration in S1. The final rise could be due to a relatively fast release of P_i or to the presence of secondary ATPase sites (Tesi et al., 1989). In conclusion, we were unable to obtain k_o in the presence of BDM by the single-turnover method. We addressed this problem by carrying out two types of experiment: fluorescence stopped-flow (k_o obtained directly) and ADP displacement using the flow–quench method (k_6 obtained directly and k_o from the relationship $k_{cat} = k_ok_6/(k_o + k_6)$).

Fluorescence Stopped-Flow Experiments. When ATP interacts with S1, certain tryptophan residues are perturbed which gives rise to a fluorescence enhancement due mainly to the formation of M**·ADP·P_i (Biosca et al., 1984, and references cited therein). Thus, when ATP is mixed with S1 in a stopped-flow apparatus, there is a rapid increase in fluorescence (kinetics a function of k and $k_3 + k_3$), a plateau, and when the ATP has been hydrolyzed there is a slow decrease in fluorescence due to the decomposition of M**·ADP·P_i. The kinetics of the slow decrease is that of the P_i release with the apparent rate constant $k_o = k_4K_3/(1 + K_3)$ (Bagshaw & Trentham, 1973).

Typically, our reaction mixtures were 4 μM S1 + 4 μM ATP with the conditions under Experimental Conditions. Upon mixing, there was a rapid increase in fluorescence of k_{obs} = 2 s^{-1}. With 20 mM BDM, $k_{obs} = 1.6$ s^{-1} (curves not shown). This similarity confirms that the oxime has little effect on the ATP binding kinetics. Unfortunately, the kinetics of the following slow decrease in fluorescence were confounded by a slow drift which made estimates of k_o difficult. This drift is presumably due to denaturation of the S1 as the reaction mixtures were subjected to UV irradiation for minutes [e.g., Bagshaw and Trentham (1974)]. Nevertheless, it appeared that 20 mM BDM reduced k_o by at least a factor of 5.

Kinetics of the Release of P_i (k_o) and ADP (k_6) from ADP Displacement Experiments. The overall k_{cat} is determined by the two slowest steps on the S1 pathway, the sequential release of the products P_i and ADP, and is given by $k_ok_6/(k_o + k_6)$ (Bagshaw & Trentham, 1974). In the absence of BDM, the results of the flow–quench experiments gave values for k_{cat} and k_o from which k_6 was calculated (Table I). The estimates for k_o obtained from the fluorescence stopped-flow experiments were in broad agreement with the k_o in Table I. In the presence of BDM, we could not obtain an estimate of k_o from the flow–quench experiments, and there was some uncertainty as to the estimate obtained by the stopped-flow method. We next carried out ADP displacement experiments using the flow–quench method from which k_6 is obtained directly and k_o by calculation (Bagshaw & Trentham, 1974). In these, S1 is incubated with ADP before the addition of the [γ-^{32}P]ATP. It was important to include the inhibitor Ap5A (Lienhard & Secemski, 1973) as myokinase activity was present even in purified S1.

The rationale of these experiments is that the radioactive ATP is mixed with M*·ADP (Scheme I) rather than S1 and, therefore, that the ATP can only bind after the ADP has come off, via k_6. The ATP concentration chosen was such that the overall kinetics of the formation of the measured intermediate M**·ADP·P_i (at 20 μM ATP about 10 s^{-1}) are much faster than k_6 (0.036 s^{-1}, see above).

A typical ADP displacement experiment is shown in Figure 6a in which S1 + ADP was mixed with ATP. There was a

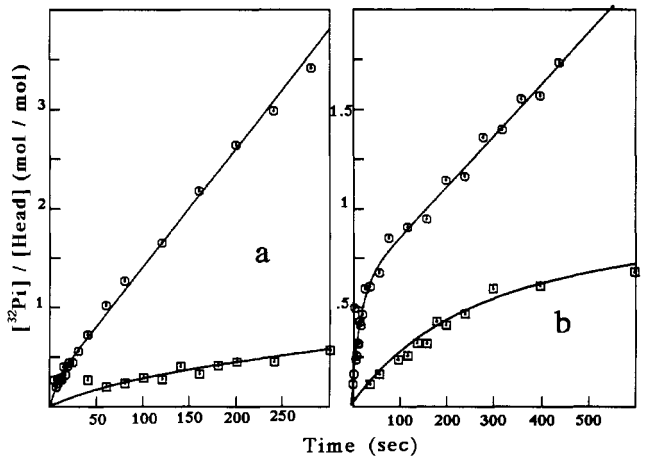
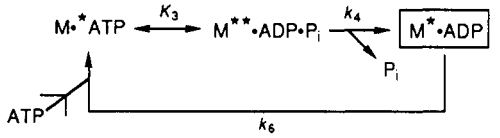


FIGURE 6: Displacement of ADP from S1 by ATP in the absence (a) or presence (b) of 20 mM BDM. S1 (4 μM) + 20 μM ADP was mixed with 40 μM [γ-^{32}P]ATP with or without 20 mM BDM. The concentration of Ap5A was 25 μM. The reaction mixtures were quenched in acid, and the [^{32}P]P_i was determined. For (○), the time scale is as indicated; for (□) it is divided by 10. The curves were fitted to exponentials followed by linear phases.

Scheme III



small transient burst phase followed by a steady-state rate similar to that without ADP. It is noteworthy that in this experiment the transient burst phase fitted well to a curve going through the origin. The concentrations of S1 and ADP were 2 μM and 10 μM, respectively. When the ADP concentration was reduced to 5 μM a similar curve was obtained (data not shown). This shows that ADP binds tightly to S1 (K_d < 1 μM) under our experimental conditions.

As shown by Bagshaw and Trentham (1974), this type of experiment can be interpreted in terms of Scheme III, a simplification of Scheme II on a time range where binding steps are fast and the cleavage step is a rapid equilibrium. In Scheme III, M*•ADP is the starting complex. Upon the addition of [γ-^{32}P]ATP, the progress curve consists of a P_i burst defined by kinetics $\lambda = k_o + k_6$ and amplitude $k_6\alpha/\lambda - k_ok_6/\lambda^2$ where $\alpha = K_3/(1 + K_3)$. The P_i burst is followed by a steady-state rate of ATP hydrolysis which, when corrected for active site concentration of the S1 used (here 0.74 mol of site/mol of S1), gives k_{cat}.

The P_i burst size in Figure 6a was small, and its kinetics were difficult to obtain with precision. This is explained by the small difference between k_6 and k_{cat} with S1 alone. Nevertheless, the data points fitted well to a curve traced using the values for k_o, k_6, and K_3 obtained from P_i burst experiments in the absence of ADP (Figures 1, 4, and 5). Thus, with *P_i burst kinetics*, we obtained 0.087 (±0.036) s^{-1}, and calculated 0.070 (±0.016) s^{-1}; and with P_i burst amplitude, we obtained 0.23 (±0.04) and calculated 0.20 (±0.05) mol of P_i/mol of S1.

BDM reduces significantly k_{cat} (Figure 1), and its effect upon the ADP displacement kinetics is illustrated in Figure 6b. There was a significant increase in the burst amplitude (0.60 ± 0.03 mol of P_i/mol of S1). Its kinetics (0.045 ± 0.005 s^{-1}) and those of the following steady-state rate (0.0025 ± 0.0001 s^{-1}) were lower than in the experiment without BDM. From the characteristics of this P_i burst (size, kinetics) and

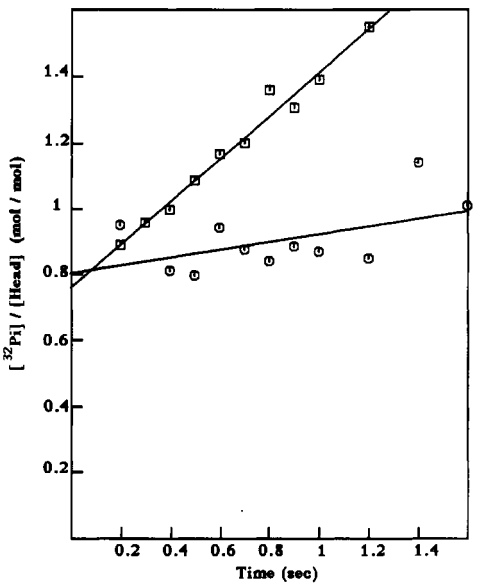


FIGURE 7: Steady-state time courses at 4 °C for Ca^{2+}-activated myofibrillar ATPase in the absence (□) or presence (○) of 20 mM BDM. The reaction mixtures (3 μM in myosin heads + 30 μM [γ-^{32}P]ATP) were quenched in acid at the times indicated, and the [^{32}P]P_i was determined. The buffer contained 0.1 mM $CaCl_2$.

the following steady-state rate and using the expressions for these parameters given above, we were now able to obtain estimates for k_o and k_6 which are given in Table I. The P_i burst amplitude in this experiment was large, which confirms a large K_3.

From the ADP displacement experiments, therefore, it appears that k_o is reduced significantly by BDM, in agreement with the fluorescence stopped-flow experiments. Thus, the small end rise seen in the P_i burst experiments under single-turnover conditions (Figure 5b) is too fast to be due to the release of P_i. On the other hand, the kinetics of the release of ADP (k_6) are not changed significantly by BDM.

Effect of BDM on the Ca^{2+}-Activated Myofibrillar ATPase. When ATP is added to activated myofibrils, they overcontract rapidly and lose their structures and it is difficult to obtain their Mg^{2+}-ATPase activity. However, by working at 4 °C and sampling in the hundreds of milliseconds time range, one can obtain the activity before overcontraction occurs (Houadjeto et al., 1991).

The effect of 20 mM BDM on the ATPase activity of contracting myofibrils is shown in Figure 7. It is noteworthy that whereas BDM did not seem to affect the amplitude of the initial burst phase, it decreased the following steady-state rate of ATP hydrolysis by a factor of about 6: without BDM, steady-state rate = 0.65 s^{-1}, with BDM 0.11 s^{-1}. With myofibrils, P_i burst amplitudes are larger than with S1 (Houadjeto et al., 1992) so any further increase by BDM would be difficult to discern.

DISCUSSION

Our aim in this work was to determine which intermediates on the myosin ATPase reaction pathway (Schemes I and II) interact with the oxime BDM. As a probe for this interaction, we determined the kinetic properties of several of the intermediates in the presence and absence of BDM. In particular, we wished to test the suggestion of Higuchi and Takemori (1989) that BDM interacts specifically with the key intermediate M**•ADP•P_i and that in doing so it decreases the rate of the P_i release. We analyze our results in terms of this hypothesis.

From our results, it appears that BDM has two major effects on S1 ATPase. First, it increases the equilibrium constant of the cleavage step, K_3. This is in disagreement with Higuchi and Takemori (1989), who concluded that the oxime does not affect this constant. Second, the rate of the release of P_i was decreased by an order of magnitude by BDM. This is in agreement with the prediction of Higuchi and Takemori (1989). These two effects would be simultaneously explained if BDM interacts with and in some way stabilizes the key intermediate M**·ADP·P_i. If it interacts with nucleotide-free S1, it does so without affecting significantly the binding of ATP. Further, as BDM does not affect the kinetics of the release of ADP, it does not appear to interact with M*·ADP either. To sum up, BDM appears to be an uncompetitive inhibitor of S1 ATPase. This type of inhibition is very rare [e.g., Dixon and Webb (1964) and Gutfreund (1975)].

One aim of muscle research is to interpret physiological phenomena with reference to the biochemical pathway for myosin and actomyosin ATPases [e.g., Taylor (1979)]. So, does the rather specific effect of BDM upon S1 ATPase tell us how it lowers force and stiffness in skinned fibers?

It is noteworthy that BDM affects similarly the ATPases of the organized myofibrils and dispersed S1 ATP systems (Figures 1 and 7). Thus, it appears that with myofibrils too BDM exerts its effect subsequent to the initial binding and cleavage steps. Further, the effect suggests that as with S1 the P_i burst with myofibrils is due to the accumulation of M**·ADP·P_i rather than to free P_i (Scheme I).

To return to our question: If force and stiffness are related to the degree of cross-bridge attachment in the fiber, we can come to certain tentative conclusions. With S1 ATPase, BDM reduces the rate of formation of the key intermediate M*·ADP (k_4 is decreased by a factor of more than 10; Table I) without significantly affecting its rate of decomposition (via k_6). The effect of BDM, then, is to decrease the number of bridges in the "strong" actin binding state M*·ADP and to increase those in the "weak" state, M**·ADP·P_i. Measurements by complementary fluorometric techniques (M. Geeves, personal communication) support our conclusions regarding the myosin ATPase while suggesting additionally that the binding of BDM may reduce the affinity of certain cross-bridge species for actin. It has already been suggested that BDM inhibits the contractile apparatus by suppressing cross-bridge attachment [e.g., Mulieri and Alpert (1984)]. It could be that the ratio k_4 to k_6 is important in determining the physiological state of the fiber.

In conclusion, we show that BDM interacts rather specifically with a key intermediate on S1 and probably myofibrillar ATPases: M**·ADP·P_i. This specificity shows that BDM could be a useful tool in the correlation of the chemical steps of myosin ATPase with the physiological parameters in the fiber.

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Registry No. BDM, 57-71-6; ATPase, 9000-83-3; ATP, 56-65-5; ADP, 58-64-0; P_i, 14265-44-2.