Dimethyl sulfoxide enhances the effects of P_i in myofibrils and inhibits the activity of rabbit skeletal muscle contractile proteins

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In the catalytic cycle of skeletal muscle, myosin alternates between strongly and weakly bound cross-bridges, with the latter contributing little to sustained tension. Here we describe the action of DMSO, an organic solvent that appears to increase the population of weakly bound cross-bridges that accumulate after the binding of ATP, but before P_i release. DMSO (5–30%, v/v) reversibly inhibits tension and ATP hydrolysis in vertebrate skeletal muscle myofibrils, and decreases the speed of unregulated F-actin in an *in vitro* motility assay with heavy meromyosin. In solution, controls for enzyme activity and intrinsic tryptophan fluorescence of myosin subfragment 1 (S1) in the presence of different cations indicate that structural changes attributable to DMSO are small and reversible, and do not involve unfolding. Since DMSO depresses S1 and acto-S1 MgATPase activities in

the same proportions, without altering acto-S1 affinity, the principal DMSO target apparently lies within the catalytic cycle rather than with actin–myosin binding. Inhibition by DMSO in myofibrils is the same in the presence or the absence of Ca^{2+} and regulatory proteins, in contrast with the effects of ethylene glycol, and the Ca^{2+} sensitivity of isometric tension is slightly decreased by DMSO. The apparent affinity for P_i is enhanced markedly by DMSO (and to a lesser extent by ethylene glycol) in skinned fibres, suggesting that DMSO stabilizes cross-bridges that have ADP· P_i or ATP bound to them.

Key words: cryoprotection, inorganic phosphate affinity, organic solvents, skinned muscle fibre, viscosity.

INTRODUCTION

A fundamental feature of chemomechanical coupling in muscle is the sequential formation of cross-bridges that are weakly bound and those that are strongly bound (for reviews, see [1,2]). Agents that increase the population or prolong the lifetime of one cross-bridge state over another potentially can provide access to intermediates that normally occur only as a small fraction of the myosin heads. 2,3-Butanedione monoxime and a number of ATP and P_i analogues have been exploited extensively with this objective [3–13]. Lowering the temperature has also provided valuable information, since the kinetics of some of the steps are affected more than others [14,15]. Ethylene glycol, which is used as an anti-freeze in most studies carried out at sub-zero temperatures, also modifies catalysis and cross-bridge kinetics in its own right, without changing structure or mechanism in any fundamental way [15–22]. It has been particularly valuable in providing access to the very fast reactions involving ATP binding, isomerization and actin dissociation at the beginning of the cycle [20,23–25]. However, ethylene glycol has more complex effects in preparations that contain regulated thin filaments [26–28]. Recently, Stehle et al. [28] presented evidence to show that ethylene glycol inhibits the formation of strong cross-bridges by shifting the thin-filament complex toward the off-position, and not by favouring myosin–nucleotide complexes that bind weakly to actin.

Dimethyl sulfoxide (DMSO) is a dipolar, aprotic solvent that, like ethylene glycol, is used widely as a cryoprotectant [29], and is also used to solubilize small organic molecules. It activates

some enzymes and inhibits others, in many cases reversibly [30]. We became interested in DMSO because, like ethylene glycol, it inhibits ATP hydrolysis by different energy-transducing enzymes, including the F_0F_1 -ATPase of mitochondria and the Ca^{2+} , Mg^{2+} -ATPase of the sarcoplasmic reticulum, and at the same time it activates the reverse reaction (for reviews, see [31,32]). These observations suggest that DMSO favours the binding of inorganic phosphate (P_i) or ADP over that of ATP, either by stabilizing the appropriate protein conformation or by altering the reactivity of products at the active site. A primary aim of the present study was to determine whether DMSO has a similar effect on myosin ATPase, and to evaluate the possible consequences for the distribution of cross-bridges among different intermediate states, as shown below:

In this simplified scheme, actin (A) and myosin (M) interact sequentially to form weakly and strongly bound cross-bridges, depending on the association of myosin with ATP and products. Only strongly bound bridges (AM, AM·ADP and part of the AM·ADP· P_i population) contribute significantly to motility and isometric tension. The P_i -bound forms grouped under AM·ADP· P_i include both weakly and strongly bound cross-bridges [1,2].

In neuromuscular preparations, DMSO has complex, multiple effects, since it alters membrane permeability and inhibits both pre- and post-synaptic events [33,34]. However, a low con-

Abbreviations used: DTT, dithiothreitol; f_u , fraction of uniformly moving filaments; HMM, heavy meromyosin; RhPh, rhodamine/phalloidin; S1, myosin subfragment 1; s_u , mean speed of uniformly moving filaments.

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centration of DMSO (1 %, v/v) depressed contraction in isolated skinned fibres of the frog, suggesting a direct effect on the contractile apparatus [35]. In preliminary experiments we found that DMSO, like ethylene glycol [27], also inhibits isometric tension in mammalian skinned fibres, but it can either activate or inhibit the hydrolysis of ATP by myosin in solution, depending on the cation present [36]. Here we have examined the effects of DMSO on the steady-state properties of myosin subfragment 1 (S1), acto-S1, myofibrils and skinned fibres, and in motility assays using acto-heavy meromyosin (HMM). For comparative purposes, selected experiments were repeated using ethylene glycol when comparable data were not already available in the literature. It is noteworthy that DMSO, unlike ethylene glycol, inhibits the MgATPase of myosin to the same extent regardless of whether or not actin or regulatory proteins are present. Both solvents, but especially DMSO, cause a marked increase in the apparent affinity for P_i in skinned fibres, and the data point to an increase in the population of weakly bound, non-force-generating cross-bridges.

Some of these data have appeared in abstract form [36,37].

EXPERIMENTAL

Proteins

Myofibrils, myosin, HMM and S1 were prepared from rabbit longissimus dorsi, skinned fibres were from rabbit psoas, and actin was from rabbit or from chicken pectoralis. Natural myofibrils were prepared from fresh or stored muscle as described previously [38], but using 5 mM $MgCl_2$ and a cocktail of protease inhibitors. For some experiments myofibrils were subsequently desensitized in low salt at pH 7.6 (to remove troponin and tropomyosin), as described by Schaub et al. [39]. Solutions used for the preparation and storage of myofibrils contained 0.2 mM PMSF, 0.3 mg/ml soybean trypsin inhibitor, 4 μ g/ml leupeptin and 1 mM dithiothreitol (DTT). Myofibrils were stored in 50 % (v/v) glycerol at $-20^\circ C$ until use, and then diluted in 6 vol. of 50 mM KCl in 50 mM Hepes (pH 7.0), centrifuged (755 g for 20 min) to remove glycerol, and resuspended in the same buffer. Protein concentrations were measured using the biuret reaction, and molar concentrations were calculated from molecular mass values of 520 kDa for myosin and 42 kDa for actin, and an estimate of 50 % (w/w) myosin in myofibrils. Myosin was prepared according to the method of Bremel and Weber [40], and stored at $-20^\circ C$ in 300 mM KCl, 25 mM imidazole (pH 7.0) and 0.5 mM DTT in 50 % (v/v) glycerol. HMM was obtained by chymotryptic digestion of myosin, as described by Kron et al. [41].

S1 was prepared from fresh myosin [42] using chymotrypsin and EDTA, thereby eliminating the regulatory light chain. After passage through an anion-exchange column, fractions containing S1(A1) and S1(A2) were pooled and concentrated to ~ 25 mg/ml by ultrafiltration under N_2 , followed by dialysis overnight (10 mM P_i , 100 mM KCl, 2 mM DTT, 20 mM imidazole, pH 7.0, and 0.2 mM EDTA). The protein solution was diluted to 50 % using glycerol and stored under liquid N_2 in small aliquots; before use it was dialysed overnight against 50 mM KCl in 50 mM Hepes (pH 7.0) and centrifuged for 22 min at 350 000 g to remove aggregates. Actin was prepared as described by Pardee and Spudich [43], and stored as the acetone powder. It was polymerized in small batches using MgATP and KCl, and kept at $0^\circ C$ for no more than 2 weeks. For motility assays, F-actin was fluorescently labelled with rhodamine/phalloidin (RhPh; Molecular Probes, Eugene, OR, U.S.A.), as described by Kron et al. [41]. S1 and HMM concentrations were determined spectrophotometrically, assuming absorption coefficients ($A_{280}^{1\%}$) of 7.5

Figure 1 Inhibition of ATP hydrolysis by native (NMF) and desensitized (DMF) myofibrils

Assays contained 50 mM Hepes, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, 1 mM CaEGTA (free $Ca^{2+} = 20 \mu M$) and the concentrations of DMSO (v/v) indicated on the abscissa. Native (●) or desensitized (○) myofibrils (0.3 mg/ml; approx. $1.2 \mu M$ heads) were preincubated in the reaction medium for 1 min at $30^\circ C$. The reaction was started with 3 mM ATP and quenched with acid after 2 min. 100% activity (per myosin head; mean $\pm$ S.E.M.; $n = 4$ or 5) was $7.5 \pm 2.4 s^{-1}$ for native myofibrils and $7.1 \pm 1.4 s^{-1}$ for desensitized myofibrils. The inset shows the same experiment in the absence of Ca^{2+} (1 mM EGTA); 100% activity was $1.0 \pm 0.3 s^{-1}$ (●) and $6.1 \pm 1.6 s^{-1}$ (○).

and $6.0 cm^{-1}$ respectively, and molecular masses of 110 and 350 kDa respectively. Actin concentration was measured using a biuret assay.

ATPase assays

Assays in the presence of Ca or EDTA contained 3–5 mM ATP, 50 mM Hepes (pH 7.0), the concentrations (v/v) of organic solvent indicated in the Figure/Table legends, and either 600 mM KCl with 5 mM EDTA (K^+ ATPase) or 70 mM KCl with 5 mM $CaCl_2$ (CaATPase). Reactions were started by adding ATP, after preincubating the protein with the other components for 2 min at $30^\circ C$, and arrested after 2–20 min. Single time-point assays and appropriate blanks were carried out in duplicate, based on control experiments for each protein showing that hydrolysis was linear with time, within experimental error. P_i release was measured colorimetrically, as described in [44]. The P_i standard curve was not altered by organic solvent. Assays for S1 and acto-S1 MgATPase activity were performed as described above, except that the medium contained 3–5 mM ATP, 20 mM imidazole, 1 mM EGTA, 7.5 mM $MgCl_2$ and 10–30 mM KCl, with or without actin. Values for K_m and V_{max} (k_{cat}) for actin were obtained by non-linear regression using the equation $v = k_{cat}/(1 + K_m/[actin])$, after subtracting the activity in the absence of actin. Conditions for the myofibrillar ATPase assays are given in the legend to Figure 1. Because of the long time scale used, the rates reported for Ca^{2+} -activated or desensitized myofibrils correspond to over-contracted fibrils, and are therefore underestimates of the true values [28].

Fluorescence measurements

Fluorescence measurements were carried out in an Hitachi F4500 spectrofluorimeter under the same conditions and with the same S1 concentration (0.05 mg/ml) as in the ATPase assays, except

that Hepes replaced imidazole and ATP was omitted. Buffer containing DMSO or ethylene glycol was warmed to room temperature (27 °C) and mixed with S1 from stock, in a 3 ml quartz cuvette. Excitation was at 280 nm and emission spectra were recorded from 300 to 400 nm, with entry and exit slits of 5 nm. Fluorescence intensity and centre of spectral mass were calculated using the software supplied by the manufacturer to obtain ΣI_λ , the area under each curve, where I_λ is the intensity at wavelength λ ; the average emission wavelength, or spectral centre of mass, is given by $\Sigma(\lambda \cdot I_\lambda)/\Sigma I_\lambda$. A red-shift in fluorescence emission indicates increased exposure of tryptophan residues that are normally less accessible to solvent, and is usually found to accompany protein unfolding [45]. Small blank values obtained with buffer plus solvent were subtracted from the area calculated for each curve. Controls in which S1 was incubated with 0 or 50% (v/v) DMSO for up to 30 min showed that fluorescence emission reached a steady value within the time required for mixing and temperature equilibration (~2 min).

Motility assays

All aspects of motility experiments were performed and analysed as described previously [46–48]. ATP-insensitive heads were removed from HMM by ultracentrifugation in the presence of unlabelled F-actin and MgATP [41]; the HMM concentration was subsequently determined using the Bradford assay [48a]. Flow cells were constructed and imaged as described previously using a saturating HMM density (250 $\mu\text{g/ml}$) [46,47]. Motility assays were conducted in actin buffer (25 mM KCl, 25 mM imidazole, 4 mM MgCl_2 , 1 mM EGTA and 1 mM DTT, pH 7.4) plus various concentrations (v/v) of DMSO. Immediately prior to the motility assay, 2 mM ATP, 16.7 mM glucose, 100 $\mu\text{g/ml}$ glucose oxidase, 18 $\mu\text{g/ml}$ catalase and an additional 40 mM DTT were added to minimize photo-bleaching and photo-oxidation [41,47]. The temperature was 30 °C.

RhPh-labelled F-actin speed distributions were obtained from video recordings by using hardware and Expert Vision software from Motion Analysis Systems (Santa Rosa, CA, U.S.A.) [46,47,49]. Data were digitized at 10 frames per s. The fraction of uniformly moving filaments (f_u) was obtained from the proportion of filament paths meeting the criterion for uniform motion [centroid unambiguously tracked along its path for at least 2 s, and the ratio of S.D. to mean speed (r_v) < 0.5]. The mean speed of uniformly moving filaments (s_u) was calculated as the unweighted mean of mean speeds from those filament paths meeting the criterion for uniform motion. When s_u was < 5 $\mu\text{m/s}$, the data were smoothed, a subset of the data was retained to yield an effective sampling rate of 2 frames per s, and the criterion for uniform motion was made more stringent (r_v < 0.3) [46,47]. To facilitate comparisons among data obtained on different days, s_u was normalized to that obtained in control conditions (actin motility buffer with no added DMSO).

Isometric tension

Psoas fibres were permeabilized, stored and mounted for isometric tension recording as described in Sorenson et al. [50]. Solutions were stirred vigorously in a 0.3 ml chamber and maintained at 15 °C. In a representative subset of the fibres used in these experiments, the diameter was $64 \pm 4 \mu\text{m}$ (mean $\pm$ S.E.M.; $n = 8$) and maximal isometric tension was $2.1 \pm 0.3 \text{ kg/cm}^2$. Fibres that lost more than 15% of their original tension were discarded. For experiments at 26 °C, fibres were first activated at pCa 4.4 ($-\log[\text{Ca}^{2+}] = 4.4$) at 15 °C and then exposed to pCa 4.4 at 26 °C until a new steady state was attained, followed by relaxation at 5–10 °C. Maximum tension was measured at an

ionic strength of 0.2 M with 4 mM MgATP^{2-} , 2 mM free Mg^{2+} and pCa 4.4, obtained by using 152 mM potassium propionate, 6.0 mM magnesium acetate, 4.4 mM $\text{K}_2\text{Na}_2\text{ATP}$, 5 mM CaEGTA and 20 mM imidazole propionate (pH 7.0). Solutions containing higher Ca^{2+} concentrations (to pCa 3.7) were obtained by adding small amounts of CaCl_2 to the pCa 4.4 activating solution. For pCa–tension curves, the magnesium acetate concentration varied from 6.0 to 6.4 mM, and part of the CaEGTA was replaced by K_2EGTA . Addition of organic solvent without diluting the ionic components was achieved by preparing solutions at twice the final concentrations given above and bringing them up to their final volume with water, DMSO or ethylene glycol. Solutions with different concentrations of P_i were obtained by mixing pCa 4.4 solutions containing 0 and 30 mM P_i ; in the latter, the potassium propionate concentration was decreased to 104 mM and that of magnesium acetate was increased to 8.1 mM. Ionic species were calculated as described [51].

Reagents

ATP, EGTA, EDTA, DTT, imidazole, propionic acid, magnesium acetate, protease inhibitors and enzymes for motility assays were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ethylene glycol and DMSO were from Sigma or from Vetec (Rio de Janeiro, Brazil), and were stored in the dark. Identical results were obtained with several different lots, and representative experiments were repeated in the presence of 2 mM DTT, with the same results. Solution viscosity and relative viscosity (normalized to the viscosity of water) were measured at 30 °C, as described in [52], using a No. 75 Cannon–Manning semi-micro viscometer (AH Thomas Scientific, Philadelphia, PA, U.S.A.). The anion-exchange resin (DE52) used to purify S1 was from Whatman. Water was distilled and deionized using Milli-Q resins (Millipore-Waters Corp., Bedford, MA, U.S.A.) or the equivalent (VanWaters and Rogers, South Plainfield, NJ, U.S.A.).

RESULTS

Myofibrils and skinned fibres

The MgATPase activity of native myofibrils was inhibited by DMSO, with a half-maximal effect at 5–10% (v/v) (Figure 1). [Note that all concentrations of organic solvents are given as % (v/v), unless indicated otherwise.] In order to evaluate the influence of regulatory proteins on this profile, experiments were also performed using desensitized myofibrils, which lack the troponin/tropomyosin regulatory system (see the Experimental section). In this preparation, Ca^{2+} sensitivity is lost, and activity is maximal in the presence of EGTA as well as in the presence of Ca^{2+} [39]. Accordingly, hydrolysis of ATP by the desensitized myofibrils (per myosin head) was the same in the presence and absence of Ca^{2+} (7.1 ± 1.4 and $6.1 \pm 1.6 \text{ s}^{-1}$ respectively; means $\pm$ S.E.M.), whereas the activity of the native, regulated myofibrils was greater with Ca^{2+} than with EGTA. We note that the rates for calcium-activated myofibrils are underestimated (see the Experimental section), and also that the rates for relaxed myofibrils are somewhat higher (i.e. less well regulated) than in other reports (e.g. [53]). Nevertheless, activity was reduced 7-fold in the absence of Ca^{2+} (from $7.5 \pm 2.4 \text{ s}^{-1}$ with Ca^{2+} to $1.0 \pm 0.3 \text{ s}^{-1}$ in EGTA). Figure 1 (main panel) shows that the profile of inhibition by DMSO was identical in native and desensitized preparations in the presence of Ca^{2+} , and very similar in the presence of EGTA (normalized data shown in inset to Figure 1). Under slightly different conditions, with no added

Figure 2 Isometric tension in rabbit psoas skinned fibres

Fibres were activated at pCa 4.4 in different concentrations of DMSO (% v/v), at 15 °C (●) or 26 °C (○). P, observed isometric tension; Po, maximal isometric tension. Recovery of control tension (no DMSO) after each sequence is shown (upper right). Data are means $\pm$ S.E.M. for six (●) and three (○) fibres.

Ca^{2+} , DMSO had a similar effect on unregulated actomyosin [54]. The similarity among the four curves shown in Figure 1 distinguishes the effects of DMSO from those of ethylene glycol, which is more effective as an inhibitor when Ca^{2+} , troponin and tropomyosin are present than when they are absent [26,28].

Maximal isometric tension (at pCa 4.4) in skinned fibres was also attenuated by the solvent. At 15 °C, inhibition was half-maximal at $\sim 10\%$ DMSO (1.3 M), and nearly complete at 20 % (Figure 2). With ethylene glycol (at 10 °C), higher concentrations were required: inhibition was half-maximal at 15 % (2.4 M) [27]. Inhibition of tension by DMSO occurred rapidly (< 5 s) and was readily reversible by washing (results not shown). At 12 % DMSO, the extent of inhibition was not changed when the Ca^{2+} concentration was increased by a factor of 3–5 (results not shown). For comparison with the measurements of ATP hydrolysis and motility (carried out at 30 °C), inhibition of tension by DMSO was examined in some fibres at a higher temperature, with similar results (Figure 2).

DMSO decreased the apparent sensitivity of skinned fibres to Ca^{2+} . Although this was not studied in detail, the mid-point of the pCa–tension curve (pCa_{50}) was shifted to the right, decreasing from a pCa of 5.97 ± 0.05 (mean $\pm$ S.E.M.; $n = 3$) in controls to pCa 5.84 ± 0.06 and 5.71 ± 0.04 ($P < 0.05$) as the DMSO concentration was raised from 0 % to 6 % and 12 % respectively. The Hill coefficient was unchanged. Maruyama et al. [27] reported a decrease of 0.4 pCa unit in 15 % ethylene glycol, a concentration at which the effect on maximal isometric tension was essentially identical with that seen with 10 % DMSO.

Motility of unregulated actin filaments

Using *in vitro* motility assays with unregulated thin filaments, we found that s_U was reduced by DMSO, with half-maximal inhibition at 7.5 % (1.06 M) (Figure 3), similar to its effect on myofibrillar ATPase activity. For comparison, as shown by the broken line in Figure 3, half-maximal inhibition of s_U by ethylene glycol required a much higher concentration (20 %, or 3.6 M) [46]. At these concentrations of DMSO (inset, Figure 3) or ethylene glycol [46], there was no significant change in f_U , the fraction of filaments moving uniformly. The data shown by the symbols in Figure 3 represent 25854 filament paths (972527 individual measurements) recorded from 19 flow cells. Inhibition

Figure 3 Inhibition of *in vitro* motility by DMSO

Assays used unregulated RhPh-labelled F-actin and a saturating density of rabbit skeletal muscle HMM at 30 °C. Values of f_U (inset) and s_U (main panel) of uniformly moving filaments were obtained for individual flow cells by automated motion analysis (Experimental section). Broken lines are fits to data obtained under the same conditions for ethylene glycol [52]. Each point (○) represents the value for a single flow cell; for s_U , values are means $\pm$ S.D. of 629–2379 filament paths. Control values (◆) were 0.80 ± 0.03 for f_U and $6.05 \pm 0.15 \mu\text{m/s}$ for s_U ($n = 3$ flow cells).

of motility by 30 % DMSO was demonstrated to be fully reversible by flushing control actin buffer (no added DMSO) into a flow cell after completion of data recording at 30 % DMSO (results not shown).

The data of Figures 1–3 make it clear that DMSO is a more effective inhibitor than ethylene glycol. Recently, evidence has been presented to show that increased viscosity and the accompanying diffusional limitations to cross-bridge motion can account for much of the inhibition of filament motility by ethylene glycol [46]. In order to ascertain whether differences in viscosity could explain the differences between the effects of ethylene glycol and DMSO in the experiments of Figure 3, we carried out viscosity measurements on water and on a 10 % (w/w) solution of each solvent at 30 °C. The relative viscosities were 1.38 (DMSO) and 1.32 (ethylene glycol), a difference of only 5 %, showing that the additional inhibitory activity of DMSO cannot be ascribed to differences in viscosity. Also, because inhibition by ethylene glycol was observed previously to be slightly greater than expected for an effect of solvent viscosity alone [46], the present results imply that inhibition by DMSO must involve a mechanism beyond increased viscosity.

ATP hydrolysis and reversibility

The catalytic activity of myosin and its proteolytic subfragments varies greatly depending on the cation present [13,56]. The steady-state kinetics of ATP hydrolysis in the presence of DMSO and different cations were investigated using the chymotryptic subfragment S1. Figure 4 shows that inhibition by 0–30 % DMSO requires the presence of a bivalent cation. With Mg^{2+} or Ca^{2+} , the activity decreased monotonically with increasing DMSO concentration. Previous studies have shown that the CaATPase of S1 is also inhibited by ethylene glycol [16]. However, unlike DMSO, ethylene glycol had no effect on MgATPase activity at concentrations up to 50 % [15–17].

Figure 4 ATP hydrolysis by S1 in the presence of Mg^{2+} (●), Ca^{2+} (○) or K^+EDTA (△)

S1 (0.45 μM) was preincubated for 2 min at 30 °C in the presence of different cations, with or without various concentrations of DMSO (% v/v). The reaction was started with ATP (3 mM) and quenched with acid after 5–20 min. Data are means $\pm$ S.E.M. for assays carried out in duplicate with three or four different preparations of S1.

Table 1 Reversal of the effects of DMSO on S1 ATPase activity

In experiment D, S1 (18 μM) was incubated for 2 min at 30 °C in the presence of 15% (v/v) DMSO and then diluted 20-fold into the appropriate reaction medium containing 3 mM ATP and no added DMSO, and samples were taken at 2, 4, 6, 8 and 10 min for P_i determinations. In the controls (experiments A–C), the concentrations of protein (0.45 μM) and DMSO (as shown) were the same during preincubation as in the assay media. Values shown are means $\pm$ S.E.M. ($n = 3$), normalized to the activity in experiment A (0% DMSO present), which ranged from 3.5 to 5.9 s^{-1} in K^+EDTA and from 1.7 to 6.4 s^{-1} in Ca^{2+} .

Experiment	DMSO present in assay (% v/v)	Activity (%)	
		K^+EDTA ATPase	Ca^{2+} ATPase
A	0	100	100
B	0.75	98 $\pm$ 6	99 $\pm$ 1
C	15	160 $\pm$ 17	42 $\pm$ 2
D	15, diluted to 0.75	102 $\pm$ 6	100 $\pm$ 8

The two solvents also differ in the absence of bivalent cations: the K^+EDTA ATPase exhibited a biphasic response to DMSO, characterized by activation (~ 1.5 -fold; $P < 0.05$) as the concentration was raised to 10%, followed by a decrease in activity at concentrations above 30% (Figure 4). Essentially identical results were obtained with myosin (not shown), where the biphasic response was observed at a low concentration of KCl (50 mM) as well as with 600 mM KCl. However, when we measured the K^+EDTA ATPase activity of S1 in the presence of 0–50% ethylene glycol, there was no effect up to a concentration of 30%, and then the activity decreased to 30% of the control value as the ethylene glycol concentration was raised to 50% (results not shown). Fast kinetic experiments have shown that the step that limits k_{cat} in the presence of EDTA precedes P_i release, thereby differing from the rate-limiting step for MgATP or CaATP [56]. Thus measurement of k_{cat} under these conditions has the potential for revealing perturbations that occur earlier in the cycle. Taken together, these data suggest that moderate concentrations of DMSO (up to 20–30%) accelerate ATP cleavage or a related isomerization, whereas ethylene glycol does not.

Figure 5 Intrinsic tryptophan fluorescence of S1 in the presence of DMSO (A) or ethylene glycol (B)

Enzyme (0.45 μM) was incubated for 2 min at ~ 27 °C in 0–50% (v/v) DMSO (A) or ethylene glycol (B), 50 mM Hepes, pH 7.0, and either 5 mM MgCl_2 plus 70 mM KCl (●) or 5 mM EDTA plus 600 mM KCl (▲). Data show areas of spectra obtained at 300–400 nm (excitation at 280 nm) after subtraction of the area recorded in the absence of S1. The inset shows examples of S1 fluorescence spectra in 0%, 30% and 50% organic solvent in the presence of MgCl_2 , with broken lines at the bottom representing buffer $\pm$ 50% solvent. Similar data with essentially the same centre of mass were obtained in media containing Ca or EDTA (results not shown). In (A), the example shows an increase in area with 30% DMSO; however, on average, after correcting for buffer plus solvent ($n = 3$; main panel), there was no significant increase with up to 40% DMSO.

An important control for studies with organic solvents is related to the question of reversibility. As noted above, tension recovers readily in Ca^{2+} -activated skinned fibres exposed to 20% DMSO (Figure 2), and motility recovers after exposure to 30% DMSO (Figure 3). The next experiments were designed to evaluate the possibility that DMSO might inhibit the catalytic activity of S1 in solution by causing deleterious structural changes. In the absence of nucleotide, S1 incubated with 15% DMSO and then diluted 20-fold regained its original activity (compare experiments B and D in Table 1). In addition, the intrinsic tryptophan fluorescence of S1 was measured in the presence of Ca^{2+} , Mg^{2+} or EDTA and 0–50% DMSO. Up to 30% DMSO, fluorescence intensity was essentially unchanged, or increased only slightly in proportion to the increase in solvent

Figure 6 Cation concentration dependence for the effects of DMSO on S1 CaATPase (A) and K⁺EDTA ATPase (B) activities

Assays were performed as described for Figure 4. Values are means $\pm$ S.E.M. ($n = 3$).

concentration (Figure 5A), with no change in the position of the curve (spectral centre of mass; see the Experimental section) (inset, Figure 5A). There was little difference when data were obtained using Ca²⁺ (results not shown) instead of Mg²⁺ (Figure 5A). The lack of a red-shift in the emission spectrum can be taken as evidence against any major alteration in the S1 global fold as a result of incubation with DMSO. Above 30% DMSO, larger changes in intensity occurred (Figure 5A), and the centre of mass exhibited only a small blue-shift (< 1 nm). For comparison, fluorescence was also measured after partial denaturation of the protein in 8 M urea in the presence of EDTA. In this case, the fluorescence intensity decreased to 33% of the original value and there was a small red-shift (2 nm) in centre of mass (results not shown). These two experiments indicate that DMSO ($\leq 30\%$) does not cause large structural perturbations of S1.

With ethylene glycol, fluorescence emission was constant over a wide range of concentrations (0–50%) (Figure 5B). Travers and Hillaire [16] reported a linear increase in the difference spectra (absorption change at 287 nm) over the same range, and concluded that the solvent produced purely local changes rather than a major structural perturbation.

Large differences in k_{cat} are observed in aqueous medium depending on whether Mg²⁺, Ca²⁺ or K⁺EDTA is present (see initial data points in Figure 4). These differences are associated with differences in cleavage of the different metal–substrate complexes [56]. It is well known that the binding of different cations also results in different conformational states of the protein (e.g. [42]), and it has been suggested that cation binding also modulates activity [55]. Changes in K_m with increasing

Table 2 Effect of DMSO on the steady-state parameters for acto–S1

The reaction mixtures contained 10 mM KCl, 20 mM imidazole chloride, 7.5 mM MgCl₂, 1 mM EGTA, 5 mM ATP, S1 (0.45 μ M), actin (1.2–11.9 μ M) and 0, 10 or 30% (v/v) DMSO, at 30 °C. Activity in the absence of actin was subtracted from each value to obtain the actin-activated activity. Data shown are means $\pm$ S.E.M. for n experiments, based on fitting each data set by non-linear regression using the equation $v = k_{\text{cat}}/(1 + [K_m]/[\text{actin}])$.

DMSO (% v/v)	K_m (actin) (μ M)	k_{cat} (s ⁻¹)	n
0	16.7 $\pm$ 4.8	6.6 $\pm$ 1.3	5
10	19.3 $\pm$ 6.7	5.7 $\pm$ 1.3	2
30	20.0 $\pm$ 4.8	3.1 $\pm$ 0.7	3

concentrations of KCl (0.1–1 M) are consistent with this idea [55,57]; thus the dependence of activity on the concentration of these cations is, in part, a reflection of the native protein structure. The increase in activity with increasing Ca²⁺ or K⁺ concentration is shown in Figure 6. The presence of 15% DMSO inhibited the CaATPase (Figure 6A) and stimulated the K⁺EDTA ATPase (Figure 6B), but did not alter the cation concentration profile in either case.

With a higher DMSO concentration (40%; inhibitory for the K⁺EDTA ATPase), the [K⁺]-dependence differed from that observed in the control (Figure 6B). This observation suggests that a moderate concentration of DMSO (10–20%) does not cause a major conformational change (unfolding or denaturation), but that higher concentrations ($\geq 30\%$) might do so. A similar conclusion can be drawn from two experiments in which the protein was preincubated for different periods (1–12 min) in the presence of DMSO before adding ATP. When 10% or 15% DMSO was present, the rate of P_i release following the addition of ATP was similar regardless of the preincubation period, and remained linear with time until > 30% of the substrate was hydrolysed. In 30% DMSO, on the other hand, inhibition increased progressively as the preincubation was extended beyond 2 min (results not shown).

Affinity for actin

The data presented so far indicate that DMSO ($\leq 30\%$) is an effective and reversible inhibitor of myosin catalytic activity, as well as of isometric tension and filament sliding. In preparations involving thin filaments, actin–myosin interactions provide another potential target, one that is of particular interest for chemo-mechanical coupling. The influence of DMSO on cross-bridge affinity was evaluated from the K_m^{-1} for actin, based on the increase in acto–S1 ATPase activity with increasing concentrations of F-actin [58]. The inhibition by DMSO was essentially identical at different actin concentrations, and the fitted curves revealed a decrease in k_{cat} , but no change in K_m , with 10% or 30% DMSO (Table 2). This result is consistent with the experiment shown in Figure 7(A), in which inhibition of S1 MgATPase by DMSO is seen to be proportionally the same in the absence and the presence of actin (inset, Figure 7A), despite the substantial activation of ATPase in the presence of actin (Figure 7A).

The experiment of Figure 7(A) was repeated using ethylene glycol, which inhibited the acto–S1 activity with a half-maximal effect at a concentration of 30–40% (Figure 7B). In contrast with the results with DMSO (Figure 7A), S1 MgATPase was unaltered by ethylene glycol (Figure 7B). The data shown for acto–S1 (Figure 7B) are consistent with earlier studies in which acto–S1 (ATPase) and fibres (contraction velocity) were used [16,27].

Figure 7 Inhibition of S1 MgATPase by DMSO (A) or ethylene glycol (B) in the presence or absence of actin

S1 (0.45 μ M) was preincubated with organic solvent in the absence (●) or presence (○) of F-actin (7.1 μ M), and the reaction was started with 5 mM ATP. Other components are given in the legend to Table 2. Data are means $\pm$ S.E.M. in (A), and are from a typical experiment in (B). The insets show averages and S.E.M.s (in some cases, less than the size of the symbol) obtained after normalizing data from each of three preparations to the activity without organic solvent in each data set.

Affinity for P_i

The affinity of the catalytic site for P_i is low, and difficult to demonstrate using S1 or acto-S1 in solution [59,60]. However, it can be estimated readily for comparative purposes by measuring isometric tension in skinned fibres, for which values of 3–6 mM have been reported [8,61]. Consistent with other reports, Figure 8(A) shows that, in the absence of DMSO, 30 mM P_i depressed maximal Ca^{2+} -activated tensions by approx. 55%, with a half-maximal effect ($K_{0.5}$) at $\sim$ 4 mM. This effect is interpreted as a partial reversal of the cross-bridge cycle, where the binding of P_i to AM $\cdot$ ADP leads to the formation of AM $\cdot$ ADP $\cdot$ P_i (see Introduction), followed by the dissociation of actin [62–66].

DMSO had a striking effect, not only adding to the inhibition by P_i , but accentuating it. Thus 5% DMSO and 2 mM P_i separately reduced tension by about the same amount (18% and 22% respectively), but together they caused 54% inhibition (Figure 8A). DMSO at 10% reduced tension to 47% of the control value in the absence of P_i , but blocked tension completely

Figure 8 Inhibition of isometric tension by DMSO and P_i in skinned fibres

Fibres were exposed sequentially to increasing concentrations of P_i at pCa 4.4 and 15 $^{\circ}$ C; ionic strength, Mg^{2+} and $MgATP^{2-}$ were kept constant (see the Experimental section). Each sequence started with a control tension (in the absence of DMSO and P_i), and ended the same way ('recovery'). In (A), data are means $\pm$ S.E.M. for 4–6 fibres in the presence of 0% (●), 5% (○) and 10% (△) DMSO, normalized to maximal control tension (P_o ; obtained in the absence of DMSO and P_i). In (B), each curve is normalized to its own value in the absence of P_i .

when P_i was raised to 30 mM. Ethylene glycol also enhanced the inhibition by P_i , with a concentration of 20% reducing the tension to zero in 30 mM P_i , compared with a decrease of only 65% ($P < 0.05$) in the absence of P_i (results not shown). Both solvents increased the apparent affinity for P_i ; with 10% DMSO, $K_{0.5}$ decreased to < 0.05 mM (Figure 8B). In 5% DMSO (Figure 8B) and 20% ethylene glycol (results not shown), $K_{0.5}$ was intermediate in value (1–2 mM).

It has been argued on theoretical grounds that force should decrease linearly with $\log[P_i]$, rather than showing saturation as expected from P_i binding according to the law of mass action [64,67]. This prediction was tested for the two curves in Figure 8(A) (0 and 5% DMSO), where measurable tensions were obtained over a wide range of P_i concentrations. When the control in Figure 8 was plotted against $\log[P_i]$ (1–30 mM), a linear plot ($r = 0.997$) with a negative slope was obtained (-27% per decade increase in $[P_i]$). With 5% DMSO, however, the plot of tension against $\log[P_i]$ was linear over only a small range, and reached a plateau at $\sim$ 15 mM P_i .

DISCUSSION

The data presented here indicate that DMSO has a remarkably specific effect on myosin: it inhibits the ATPase activity to the same extent regardless of whether actin, Ca^{2+} or regulatory proteins are present. Filament motility and isometric tension are

also depressed, with a DMSO concentration dependence similar to that observed for myofibrillar ATPase. This does not appear to be due to a decrease in actin–myosin interactions, since the K_m for actin is unaltered by the solvent, and inhibition of S1 is identical in the presence and the absence of actin. Up to a DMSO concentration of $\sim 30\%$, inhibition is readily reversible, and only minor structural changes can be detected by fluorescence spectroscopy on S1. Above 30% DMSO, structural effects become more pronounced, and reversibility is eventually compromised.

The action of DMSO resembles in some respects that of ethylene glycol, but the effects of the two solvents differ in ways that suggest that the primary target for DMSO lies within the catalytic cycle of S1, whereas ethylene glycol acts on the acto–S1 interface and on the regulatory proteins, as well as on S1. First, DMSO (unlike ethylene glycol) inhibits k_{cat} for S1 and acto–S1 to the same extent (Figure 7). Thus the effect of DMSO on acto–S1 can be attributed to its action on S1 alone. Secondly, in contrast with ethylene glycol [26,28], DMSO is just as effective in the presence of regulatory proteins and Ca^{2+} as in their absence (Figure 1). Finally, both solvents cause an increase in the viscosity of the medium, which would tend to reduce filament sliding speed [46,52]. However, Figure 3 shows that a 10% DMSO solution has a much greater effect on the motility of unregulated F-actin than 10% ethylene glycol, although the viscosities are nearly the same. Thus it is clear that increased solvent viscosity is not the primary mechanism of inhibition by DMSO.

DMSO has its most striking effect on the interaction with P_i . In fibres, DMSO produces a pronounced increase in the inhibition of steady-state tension by P_i (Figure 8); a qualitatively similar (but less pronounced) effect is observed with ethylene glycol. This appears to be associated with an increase in affinity for P_i , an increase that approaches 100-fold in the presence of 10% DMSO (Figure 8). Since we did not use an ATP-regenerating system, a major source of P_i contamination in fibre studies, we can assume that our solutions with no P_i added contained less than $0.1\text{ mM } P_i$, as measured by Potma et al. [66]. However, P_i accumulation due to fibre ATPase activity will raise this value, and an accurate assessment of P_i affinity, especially in the presence of DMSO, will require additional experiments and use of a P_i mop. In any case, it is clear that the enhanced inhibition by P_i within the fibre would contribute significantly to the inhibition of tension by DMSO, even when no P_i has been added to the bathing medium (as in Figure 2). We note that the increase in P_i dependence shown for DMSO and ethylene glycol contrasts markedly with data obtained recently using poly(ethylene glycol) 4000. This polymer of ethylene glycol increased isometric tension in skinned fibres in the presence of different concentrations of P_i , without affecting P_i affinity [68].

Ca^{2+} regulation

In a previous report, Kurebayashi and Ogawa [35] found that 1% DMSO caused the pCa–tension curve of frog skinned fibres to shift to higher Ca^{2+} concentrations. We also saw a decrease in Ca^{2+} sensitivity in rabbit skinned fibres in the presence of DMSO, which could be due to a decrease in the Ca^{2+} affinity of troponin C. Ethylene glycol also decreases Ca^{2+} sensitivity [27], although the shift is larger with ethylene glycol than with DMSO. We are not aware of any measurements of Ca^{2+} binding to troponin C in the presence of ethylene glycol, but it is noteworthy that this solvent causes a pronounced increase in Ca^{2+} affinity at all four sites of isolated, purified calmodulin, a closely related EF-hand protein [69]. Ca^{2+} binding to isolated troponin C from rabbit skeletal muscle was not affected by DMSO [35]. Thus it seems

unlikely that either solvent would cause a decrease in Ca^{2+} affinity through a direct effect on troponin C.

The P_i experiments of Figure 8 suggest another explanation for the apparent decrease in Ca^{2+} affinity in fibres. Different authors have shown that P_i shifts the pCa–tension curve to the right [65,70,71]. This shift has been attributed to a decrease in the lifetime of tension-generating cross-bridges [70], or, more specifically, to a change in the co-operative effect of strong cross-bridges on thin-filament activation. This effect is attributable to a redistribution of cross-bridges from $AM \cdot ADP$ to $AM \cdot ADP \cdot P_i$ states (see Introduction), some of which are weakly bound (see [65] for a discussion). Therefore lowered Ca^{2+} sensitivity would be expected to occur with any increase in P_i binding. If the concentration of P_i in the fibre interior rises to $0.1\text{--}0.5\text{ mM}$ during activation [66], the increase in P_i affinity caused by DMSO (Figure 8) or ethylene glycol would promote P_i binding and might thereby decrease the population of strong cross-bridges. This, in turn, would shift the pCa–tension curve towards higher Ca^{2+} concentrations, even without the addition of P_i to the bathing medium. Thus our data can be explained if DMSO acts solely on cross-bridges, and there is no need to suppose a change in Ca^{2+} binding to troponin C. This conclusion is consistent with the measurements of Kurebayashi and Ogawa [35] and with the observation that DMSO-evoked inhibition is similar regardless of whether actin, Ca^{2+} or regulatory proteins are present.

Inhibition of k_{cat}

The measurements of S1 ATPase activity in Figure 4 provide several clues to the basis for the inhibition of k_{cat} by DMSO. In principle, DMSO could inhibit the ATPase activity of S1 and acto–S1 by decreasing the forward rate of any step in the reaction cycle. However, with [ATP] in the millimolar range, the rates of binding to form $M \cdot ATP$ (see Introduction) and of the ensuing isomerization (to $M^* \cdot ATP$) are orders of magnitude higher than k_{cat} [24]. Furthermore, the K^+EDTA ATPase, which probably reflects the rate of the ATP cleavage step [56], is accelerated by the solvent (Figure 4). Therefore it seems likely that DMSO exerts its inhibitory effect by decreasing the forward rate of a step that occurs after ATP cleavage, or by enhancing reversal.

Both the MgATPase and CaATPase activities of myosin are limited by the rate of release of products [56]. In solution at 30°C , the release of ADP is much faster than the release of P_i [14], and would have to be lowered a great deal to affect k_{cat} . These considerations point to P_i release, or the isomerization that precedes it, as a likely target for the solvent. Nevertheless, we cannot exclude the possibility that DMSO shifts the equilibrium towards $(A)M \cdot ATP$ (see Introduction). We note that, in water, $AM \cdot ADP \cdot P_i$ states predominate in myofibrils and S1, whereas in acto–S1 at temperatures of $> 20^\circ\text{C}$ it is the ATP-bound states that prevail [53].

Cross-bridge intermediates

Decreased fibre force (Figure 2) and enhanced sensitivity to P_i (Figure 8) are consistent with the hypothesis that DMSO stabilizes weakly bound cross-bridges, presumably including P_i -bound states. Motility data (Figure 3) also support this idea. Evidence for an increase in the population of weakly bound bridges in the presence of DMSO comes from the observation that binding of F-actin to the HMM-coated surface persists at 30% DMSO, even though motility is completely (but reversibly) inhibited. If most of the cross-bridges were dissociated under these conditions, filaments would diffuse away from the surface,

as they do at low HMM density [72] or when weak interactions are decreased [73] in the absence of methylcellulose. Thus we infer that speed decreases in the motility flow cell for two primary reasons beyond any effect of altered solvent viscosity, which is small at the concentrations of DMSO used. First, as is found for analogues of strong-binding cross-bridges [74], cross-bridges that form stable complexes with P_i analogues can act as a load that opposes filament sliding [5,10], suggesting that similar behaviour could be expected with stabilization of the P_i -bound form by DMSO. Secondly, speed decreases because fewer cross-bridges contribute productively to filament sliding, analogous to the slowing that occurs at low HMM densities [72].

Other energy-transducing enzymes that have been studied in the presence of DMSO and ethylene glycol also exhibit pronounced increases in P_i affinity (see references in Introduction). This favours ATP synthesis, and appears to be related to changes in the structure or activity of water in the vicinity of the active site. Evidence that these solvents thereby promote the partitioning of P_i from the medium (hydrophilic) into the active site (hydrophobic) has been reviewed; changes in solvation of reactants and products also occur [31,32]. It may be that such effects contribute to the changes we see in actomyosin. For a 10% DMSO solution, the change in bulk water activity is small (4%) [75]. However, as a potent hydrogen acceptor, DMSO forms strong hydrogen bonds, and has been shown to displace water from the hydration shell surrounding hydroxy ions, increasing their activity [76]. According to George et al. [77], solvation of P_i can involve hundreds of kilojoules, depending on the ionic species that are present. Thus a small percentage change in solvation could easily involve the amount of energy associated with synthesis or hydrolysis of the terminal phosphate bond of ATP. Based on thermodynamic considerations, these observations have been incorporated into a proposal that the catalytic site of many ATPases and pyrophosphatases undergoes a hydrophilic-hydrophobic transition that is important for energy transduction [32]. There is evidence both for and against such a transition occurring at the active site of myosin [1,78–82], and the experiments reported here provide a direct comparison with data obtained from solvent perturbation of other energy-transducing enzymes.

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Rio de Janeiro, Financiadora de Estudos e Projetos, and Programa de Apoio ao Desenvolvimento Científico e Tecnológico (Brazil); and by grant HL 52558 from the National Institutes of Health (U.S.A.). A. C. M., G. M. C. A. and L. C. C. had fellowships from Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior, and L. C. S., A. R. and M. M. S. had fellowships from CNPq. We thank Dr Michael Regnier and Dr Leopoldo de Meis for critical comments on the manuscript, Dr A. M. Gordon for shared facilities and generous encouragement, S. O. Costa for the experiment with urea, Z. Luo for RhPh-labelled F-actin used in the motility assays, Josh Hawkins, Dr Bo Liang and Peggy Maker for assistance with motility assays and data analysis, and Dr A. Vievra for the use of his fluorescence spectrophotometer.

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Received 2 January 2001/18 May 2001; accepted 21 June 2001