Specificity and kinetic effects of nitrophenol analogues that activate myosin subfragment 1

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2,4-Dinitrophenol (DNP) activates the myosin ATPase of mammalian skeletal muscle in the presence of Ca^{2+} or Mg^{2+} , and inhibits it when the bivalent cations are replaced by K^{+} and EDTA. Activation of Mg^{2+} -ATPase is abolished by the presence of unregulated actin. 3-Nitrophenol (3-NP) is also an activator, whereas other analogues (2-nitrophenol, 2-NP, and 4-nitrophenol, 4-NP) are much less effective. Concentrations required for their half-maximal effects ($K_{0.5}$) range from 2 to 15 mM for 3-NP and DNP in the presence of different cations, and the sequence for the analogues is $3\text{-NP} \leq \text{DNP} \ll 2\text{-NP} \approx 4\text{-NP}$, which is apparently unrelated to either hydrophobicity or pK. DNP and 3-NP have almost identical effects on the ATPase activity of chymotryptic subfragment 1 as they do on myosin, which is an indication that their target is the globular head region rather than the tail, or the 18 kDa (regulatory) light chain.

Analysis of the ATP concentration dependence for subfragment-1 ATPase in the presence of Ca^{2+} or Mg^{2+} shows that DNP activates only at high substrate concentrations, becoming increasingly effective with ATP concentrations in the physiological range. At low substrate concentrations, DNP inhibits hydrolysis by increasing the apparent K_m for ATP at the catalytic site. In the presence of Mg^{2+} , it mimics the effect of actin, which increases the K_m and accelerates the release of products following hydrolysis. At high substrate concentrations, activation by DNP appears to involve a kinetic component with low affinity for ATP that can increase the overall reaction rate by a factor of 2- to 9-fold, depending on the bivalent cation. This low-affinity component is either induced by the drug (in the presence of Mg^{2+}) or shifted by the drug to a lower ATP concentration range (in the presence of Ca^{2+}).

INTRODUCTION

2,4-Dinitrophenol (DNP) is a weakly acidic, hydrophobic compound that has been extensively characterized as an uncoupler of oxidative phosphorylation in mitochondria. At low concentrations ($\approx 10^{-5}$ M), it binds to the mitochondrial inner membrane, where it acts as a protonophore [1]. At higher concentrations, it directly affects the activity of a number of membrane-bound ATPases, including the FoF1 ATPase of mitochondria [2], the Mg^{2+} -ATPase of erythrocytes [3] and the glutathione transport ATPase of erythrocytes [4]. Analogues of DNP have been found to activate or inhibit the Ca^{2+} , Mg^{2+} -ATPase of sarcoplasmic-reticulum membranes, depending on the occupancy of high- and low-affinity sites for Ca^{2+} and ATP [5,6].

DNP has also been shown to affect myosin, actomyosin and *in vitro* models of muscle contraction. Early reports described its ability to inhibit ATP hydrolysis and superprecipitation in myofibrils and actomyosin, and to reduce unloaded shortening in glycerinated muscle fibres [7–10]. However, it also proved to be a potent activator of both the Ca^{2+} - and Mg^{2+} -activated ATPase activities of isolated myosin [8,11–13]. The mechanism of activation is still not known, and it has not been examined systematically in the light of current knowledge of the structure and catalytic cycle of the enzyme. The early studies showed that activation by DNP was reduced or eliminated when experiments were carried out at low temperatures, in the absence of bivalent cations, at a low concentration of ATP (10^{-4} M), or in the presence of other nucleotides [8,11–14]. It was also reduced by actin, principally at low ionic strength under conditions where actin and myosin would form cross-bridges ([10,15]; see also

[8]). A superficial resemblance between the effects of DNP and of thiol reagents led to experiments that essentially ruled out the possibility of DNP reacting with a thiol group on myosin [16,17]. While these early experiments point to a possible effect of DNP on the catalytic site, they also suggest that the action of DNP is susceptible to changes in conformation that alter the rate-limiting step of the reaction. This raises the possibility that DNP could be useful as a probe for the conformational state of myosin during catalysis.

The proteolytic fragment S1 (myosin subfragment 1) is less complex than myosin and has been widely used for studies of the reaction mechanism. This fragment is derived from a single myosin heavy chain and retains the catalytic and actin-binding sites, as well as one or two of the three myosin light chains [18]. It has the advantage of being soluble at low ionic strength, and thus provides a homogeneous preparation for kinetic studies. In this report we show that the effect of DNP on S1 in the presence of different cations is similar to its effect on intact myosin, and we use S1 to characterize the ATP concentration dependence of DNP activation in the presence of Ca^{2+} and Mg^{2+} . The specificity and structural basis for the action of the drug on myosin are explored using a series of analogues that differ in pK and hydrophobicity.

EXPERIMENTAL

Reagents

Nitrophenols, PMSF, ATP (Grade I), ADP, α -chymotrypsin (type 1-S) and the dialysis membranes were from Sigma. The

Abbreviations used: DNP, 2,4-dinitrophenol; 2-NP, 2-nitrophenol; 3-NP, 3-nitrophenol; 4-NP, 4-nitrophenol; S1, myosin subfragment 1.

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Figure 1 Nitrophenol effects on myosin ATPase activity in the presence of Mg^{2+}

Concentration dependence for DNP (**A**) and 3-NP (**B**). Assays contained 3 mM ATP (total concentration), 0.1 mg/ml myosin, 118 mM KCl, 50 mM Hepes, pH 7.0, at 30 °C, and: (○) no added $MgCl_2$; (●) 2 mM $MgCl_2$; or (▽) 5 mM $MgCl_2$. Reactions were started by addition of 3 mM ATP and stopped by the addition of 0.1 M HCl containing activated charcoal. Higher concentrations of both nitrophenols were tested in other preparations, and no further activation was obtained (results not shown). (**C**) Comparison of nitrophenols in the presence of 5 mM $MgCl_2$: (○) DNP, (▼) 4-NP, (▽) 2-NP, (●) 3-NP. Results are $\bar{x} \pm S.E.$ ($n = 3$).

DE-52 anion-exchange resin was from Whatman. All other reagents used were of analytical grade.

$[^{32}P]P_i$ was from Instituto de Pesquisas Energéticas e Nucleares (São Paulo, Brazil). $[\gamma\text{-}^{32}P]ATP$ was purchased from New England Nuclear, or synthesized as described by Walseth and Johnson [19], as modified by Maia et al. [20].

Protein purification

Myosin and actin were prepared from rabbit longissimus dorsi muscle according to Bremel and Weber [21] and Pardee and Spudich [22] respectively. Purity was checked by SDS/PAGE [23]. The purified myosin was stored at -20°C in a solution consisting of 600 mM KCl, 50 mM Hepes (pH 7.0) and 50% (v/v) glycerol. Before use, the protein was diluted 12-fold in ice-cold water, centrifuged and resuspended to a concentration of 2 mg/ml in 600 mM KCl and 50 mM Hepes (pH 7.0).

S1 was prepared from purified myosin by digesting it with chymotrypsin for 10 min at room temperature [18]. PMSF was added to stop the reaction and the suspension was centrifuged to remove the rod region. The supernatant was loaded on to a Whatman DE-52 column (3.5 cm $\times$ 20 cm) equilibrated with 50 mM imidazole buffer (pH 7.0) and 1 mM dithiothreitol, and eluted with a linear NaCl gradient [0–200 mM NaCl/1 mM dithiothreitol/50 mM imidazole (pH 7.0)]. Fractions containing the cleaved heavy chain (110 kDa) together with light chain 1 (A1) or light chain 3 (A2) were pooled and concentrated nearly 10-fold by ultrafiltration under nitrogen with an Amicon YM30 membrane, and then dialysed against 50 mM KCl containing 50 mM Hepes (pH 7.0). Before use, the S1 was centrifuged for 21 min at $350\,000 g_{av}$ to eliminate aggregates. The remaining supernatant was then stored at 0°C and used within 3 days.

Myosin and actin concentrations were estimated by the biuret method [24] and S1 by its molar absorption coefficient, $A_{280}^{1\%} = 7.5$.

ATPase activity

The assay media used to measure the different activities of myosin and S1 contained 0.1 mg/ml protein, 3 mM ATP, 50 mM Hepes (pH 7.0) and: 0–600 mM KCl with 5 mM EDTA for K^+ ATPase activity; 70 mM KCl with 0–5 mM $CaCl_2$ for Ca^{2+} ATPase activity; 70 or 100 mM KCl with 0–5 mM $MgCl_2$ for Mg^{2+} ATPase activity; or 70 mM KCl, 5 mM $MgCl_2$ and 0.3 mg/ml actin for actin-activated ATPase activity. Control experiments showed that the effect of DNP on the S1 Mg^{2+} ATPase was the same in the presence of 50 mM or 100 mM KCl. In the myosin ATPase assays, 18 mM KCl was added with the protein. For experiments in the presence of $[\gamma\text{-}^{32}P]ATP$ (Figure 6), the concentration of S1 varied from 5.5 to 110 $\mu\text{g/ml}$, and BSA (40 $\mu\text{g/ml}$) was added [25,26].

Hydrolysis of ATP was measured at 30°C after a 2-min preincubation in the presence or absence of the nitrophenol, and was linear with time under all conditions tested. The reactions were started by the addition of unlabelled ATP or $[\gamma\text{-}^{32}P]ATP$ and quenched with an equal volume of activated charcoal suspended in 0.1 M HCl [27]. Reaction times were adjusted to ensure that no more than 10% of the substrate was hydrolysed. After centrifugation, the P_i remaining in the supernatant was assayed colorimetrically [28], or an aliquot containing $[^{32}P]P_i$ was applied to filter paper, dried and counted for radioactivity in a liquid-scintillation counter.

Analysis of the data

The kinetic parameters (K_m and V_m) for the hydrolysis of ATP by S1 were obtained by non-linear regression using the computer programs Enzfitter (Elsevier-Biosoft) or Sigmaplot 5.0 (Jandel Scientific). Data were fitted either to a simple Michaelis–Menten equation or to a model that, in analogy to plasma-membrane Ca^{2+} -ATPase [29], considers that a single class of substrate binding site can undergo interconversion between a high-affinity conformation that catalyses hydrolysis and a low-affinity non-

catalytic conformation that has a regulatory role, described by the equation:

$$v = \frac{V_{m1}}{1 + \frac{K_{m1}}{[S]} + \frac{[S]}{K_{m2}}} + \frac{V_{m2}}{\left(1 + \frac{K_{m2}}{[S]} + \frac{[S]}{K_{m1}}\right)} \quad (1)$$

where V_{m1} and V_{m2} are the calculated maximal hydrolysis rates and K_{m1} and K_{m2} are the apparent affinities of the enzyme for ATP in each conformation of the binding site.

RESULTS

Activation of myosin ATPase by different nitrophenols

In the presence of Mg^{2+} or Ca^{2+} and a high concentration of ATP (3 mM), DNP markedly accelerates ATP hydrolysis. Its effect on the Mg^{2+} -ATPase activity of myosin is shown in Figure 1(A).

As is well known, Mg^{2+} alone is a potent inhibitor of hydrolysis by purified myosin [30,31]. In agreement with previous reports [14], increasing concentrations of DNP activate Mg^{2+} -ATPase, with a $K_{0.5} \approx 15$ mM. With 32 mM DNP, activity is restored to the level observed in the absence of Mg^{2+} (Figure 1A). We now show that the analogue 3-nitrophenol (3-NP) has an effect that is similar to DNP, although it requires a lower concentration ($K_{0.5} \approx 5$ mM) and is maximally effective at 16 mM (Figure 1B). However, neither 2-nitrophenol (2-NP) nor 4-nitrophenol (4-NP) stimulates hydrolysis significantly (Figure 1C). Table 1 shows that DNP has a pronounced inhibitory effect when actin is present. In previous reports where actin was shown to attenuate activation of Mg^{2+} -ATPase by DNP, different forms of regulated actin were used [8,10,15]. The experiment in Table 1 indicates that this effect can be obtained with actin alone, in the absence of regulatory proteins.

DNP also potentiates the ATPase activity of myosin in the presence of 5–10 mM Ca^{2+} [11–13]. Figure 2(A) shows that this potentiation depends on the concentration of $CaCl_2$ added to the medium. With a saturating concentration of $CaCl_2$ (2–5 mM), activation is maximal (approx. 2.2-fold) with 16 mM DNP (Figure 2A). Activation by the analogue 3-NP is less pronounced, and biphasic (Figure 2B), whereas 2-NP has no effect whatever and 4-NP is slightly inhibitory (Figure 2C). 1,3-Dihydrobenzene also has no effect (results not shown).

Myosin catalyses its highest rate of ATP hydrolysis in the presence of K^+ , when bivalent cations are excluded from the medium [31]. The effect of DNP has been examined under these conditions using EDTA and actomyosin. In this case, DNP inhibited hydrolysis by approx. 30% [9], but these experiments are difficult to interpret because of the presence of actin, an inhibitor under these conditions [32]. Here we show that DNP has a pronounced inhibitory effect on K^+ -EDTA ATPase of isolated myosin, with a half-maximal effect at 2–4 mM (Figure

Figure 2 Nitrophenol effects on myosin ATPase activity in the presence of Ca^{2+} .

Concentration dependence for activation by DNP (A) and 3-NP (B) with: (○) no added $CaCl_2$, (■) 1 mM $CaCl_2$, (●) 2 mM $CaCl_2$, or (▽) 5 mM $CaCl_2$. Assays contained 3 mM ATP, 0.1 mg/ml myosin, 88 mM KCl and 50 mM Hepes, pH 7.0, at 30 °C. Reactions were started by the addition of ATP and stopped by the addition of 0.1 M HCl containing activated charcoal. (C) Comparison of nitrophenols in the presence of 5 mM $CaCl_2$: (○) DNP, (▽) 2-NP, (●) 3-NP. The results are $\bar{x} \pm S.E.$ ($n = 3$).

Table 1 Effect of DNP on the actin-activated activity of myosin

Assays contained 3 mM ATP, 0.1 mg/ml myosin, 88 mM KCl, 5 mM $MgCl_2$ and 50 mM Hepes (pH 7.0), at 30 °C. Reactions were started by adding ATP and stopped after 4 min by the addition of 1 M HCl containing activated charcoal. Values are given as means $\pm$ S.E. ($n = 3$).

Actin (mg/ml) DNP concentration (mM) ...	Myosin activity (nmol of P _i /min per mg)		
	0	8	16
0	20 $\pm$ 5	48 $\pm$ 3	76 $\pm$ 7
0.3	349 $\pm$ 52	233 $\pm$ 13	182 $\pm$ 43

3A) and an identical pattern of inhibition over a wide range of K^+ concentrations (Figure 3A, inset). A similar profile is observed with 3-NP, except that this analogue is slightly more potent ($K_{0.5} \approx 1$ –2 mM). Figure 3(B) shows the effects of the four nitrophenols in the presence of 0.6 M KCl. 4-NP and 2-NP are less effective than either 3-NP or DNP.

Figure 3 Inhibition of K⁺EDTA ATPase activity by DNP (A) and comparison of nitrophenols (B)

(A) Inhibition of K⁺EDTA ATPase activity by DNP in the presence of: (○) 18 mM KCl, (●) 68 mM KCl, (▽) 118 mM KCl, (▼) 218 mM KCl, (□) 418 mM KCl, or (■) 618 mM KCl. Assays contained 3 mM ATP, 0.1 mg/ml myosin, 1 mM EDTA and 50 mM Hepes, pH 7.0, at 30 °C. Inset shows data normalized to maximal activity without drug at each KCl concentration. (B) Comparison between nitrophenols in the presence of 618 mM KCl: (●) 3-NP, (○) DNP, (▼) 4-NP, (▽) 2-NP. The results are $\bar{x} \pm \text{S.E.}$ ($n = 3$).

Table 2 Activation of myosin and S1 Mg²⁺ATPase activities by DNP

Assays contained 3 mM ATP, 0.1 mg/ml myosin or S1, 118 or 70 mM KCl, 5 mM MgCl₂, 50 mM Hepes (pH 7.0) and either 0 or 16 mM DNP, at 30 °C. Values are given as means $\pm$ S.E. ($n = 3$).

	Activity (nmol of P _i /min per mg)		Activation (%)
	Control	DNP	
Myosin	18 $\pm$ 4	101 $\pm$ 11	588 $\pm$ 60
S1	73 $\pm$ 5	335 $\pm$ 36	478 $\pm$ 50

Figure 4 Comparison of nitrophenol effects on myosin (A), and S1 (B) and (C), activities in the presence of different concentrations of Ca²⁺

Assays contained 3 mM ATP, 0.1 mg/ml protein, 70 mM KCl and 50 mM Hepes, pH 7.0, and the CaCl₂ concentrations indicated on the abscissae, at 30 °C. Concentrations of DNP (A) and (B), or 3-NP (C) were: (○) zero, (■) 4 mM, (●) 8 mM, (▽) 16 mM or (▼) 32 mM. The results are $\bar{x} \pm \text{S.E.}$ ($n = 3$).

Myosin versus S1

In a previous report, Yamada et al. [15] showed that DNP activates the Mg²⁺ATPase activity of heavy meromyosin, a proteolytic fragment of myosin that retains both heads and major portions of all three light chains, but lacks most of the rod. The next experiments were carried out in order to determine whether the effects of DNP under different ionic conditions could be

Figure 5 Comparison of DNP and 3-NP (inset) effects on K^+ EDTA ATPase activities of myosin and S1

Assays contained 1 mM EDTA, 50 mM Hepes, pH 7.0, 3 mM ATP, 618 mM KCl and 0.1 mg/ml of either myosin (●) or S1 (○). Without the drug, the activity (nmol of P_i /min · mg) varied in different preparations from 1300 to 1600 for myosin and from 2100 to 3200 for S1. The results are $\bar{x} \pm S.E.$ [$n = 3$; $n = 2$ (inset)].

observed with the proteolytic fragment S1, which has only one head and lacks both the filamentous portion and the regulatory light chain LC_2 [18]. Table 2 shows that DNP stimulates Mg^{2+} ATPase of chymotryptic S1 to the same extent as it does with intact myosin. 3-NP has a similar effect (results not shown).

Potential of Ca^{2+} ATPase by different concentrations of DNP is also similar for myosin and S1 (Figures 4A and 4B). With S1, as with myosin, activation of Ca^{2+} ATPase by 3-NP is attenuated at concentrations above 4 mM, and may not occur at all unless a high concentration of Ca^{2+} is present (Figure 4C). Finally, in both preparations K^+ EDTA ATPase activity is inhibited by both drugs (Figure 5). 3-NP inhibits both preparations equally (Figure 5, inset), whereas DNP is somewhat more effective with myosin than with S1 (Figure 5). Nevertheless, it is clear that both nitrophenols exert most of their action on the globular head domain of the myosin molecule, regardless of which cation is present, and that the regulatory light chain is not required. In the presence of Ca^{2+} , the effect of 3-NP is biphasic, and thus appears to be more complex than that of DNP. Accordingly, DNP was used in experiments to determine the effect on kinetic parameters of chymotryptic S1 in the presence of Ca^{2+} and Mg^{2+} .

ATP concentration dependence

Figure 6(A) shows the ATP concentration dependence for the steady-state hydrolysis catalysed by S1 in the presence of Mg^{2+} . The low K_m (0.08 μ M) is consistent with previous studies in which K_m values of $\leq 0.1 \mu$ M were obtained [26,33–35]. As described elsewhere for S1 [25,36]; but see [33,34], the steady-state rate under control conditions follows a hyperbolic dependence on substrate concentration. Activation by DNP is observed only at ATP concentrations above approx. 0.5 μ M;

Figure 6 ATP concentration dependence for DNP activation of S1 ATPase in the presence of Mg^{2+} (A) and Ca^{2+} (B)

Assays contained 5.5–110 μ g/ml S1, the concentrations of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ indicated on the abscissae, 70 mM KCl, 5 mM $MgCl_2$ (A) or 5 mM $CaCl_2$ (B), 50 mM Hepes, pH 7.0, 40 μ g/ml BSA and either (○) zero or (●) 16 mM DNP, at 30 °C. Reactions were started with ATP and stopped after different intervals with 0.1 M HCl containing activated charcoal. Free cation concentrations range from 5 to 0.74 mM Mg^{2+} in (A) and 5 to 1 mM Ca^{2+} in (B); $MgATP^{2-}$ and $CaATP^{2-}$ concentrations are essentially identical with the ATP concentrations used. Inset to (A) shows the region from 0.05 to 1 μ M ATP on a linear scale. Activity in the absence of drug in (A) was fitted by a simple Michaelis–Menten model; all other curves in (A) and (B) were fitted using eqn. (1) (see the Experimental section). Similar results were obtained using three other preparations (see Tables 3 and 4 for averages).

below this value, DNP inhibits the ATPase (Figure 6A, inset). In Table 3, a comparison of the kinetic parameters for ATP concentrations below approx. 100 μ M shows a 4-fold increase in V_{m1} and an 18-fold increase in K_{m1} when 16 mM DNP is present. This response is distinctly different from that observed for the mitochondrial Mg^{2+} ATPase, where DNP decreased the apparent K_m with only a small increase in V_{max} , and its maximal effect occurred with a much lower concentration of the drug [2].

Table 3 Kinetic parameters of S1 Mg²⁺ATPase activity

Conditions were those described for Figure 6(A), with 11–110 $\mu\text{g/ml}$ S1. Values are averages $\pm$ S.E. of those obtained by non-linear regression analysis of the individual data sets using a simple Michaelis–Menten equation for 0 DNP and eqn. (1) (see the Experimental section) for 8 and 16 mM DNP ($n = 4$).

DNP (mM)	K_{m1} (μM)	V_{m1} (nmol of P_i /min per mg)	K_{m2} (mM)	V_{m2} (nmol of P_i /min per mg)
0	0.083 ± 0.009	48.6 ± 4.6	—	—
8	0.75 ± 0.10	154 ± 32	1.33 ± 0.15	312 ± 95
16	1.51 ± 0.18	186 ± 39	0.92 ± 0.16	435 ± 134

Table 4 Kinetic parameters of S1 Ca²⁺ATPase activity

Conditions were those described for Figure 6(B), with 5.5 $\mu\text{g/ml}$ S1. K_m and V_m values are averages $\pm$ S.E. obtained from individual data sets using eqn. (1) (see the Experimental section) ($n = 4$).

DNP (mM)	K_{m1} (μM)	V_{m1} (nmol of P_i /min per mg)	K_{m2} (mM)	V_{m2} (nmol of P_i /min per mg)
0	1.50 ± 0.3	1319 ± 67	3.9 ± 0.7	2741 ± 127
16	8.80 ± 3.2	1847 ± 275	0.75 ± 0.4	4151 ± 288

With ATP concentrations in the millimolar range, including the concentration used for the experiments in Figure 2 and Table 2, DNP promotes a second rise in the S1 ATPase activity (Figure 6A). This effect appears to be nearly saturated at 8 mM DNP (K_{m2} , V_{m2} , Table 3), and at the highest ATP concentration used (5 mM), it increases the activation to a factor of 6- to 9-fold in comparison with the control. These data were fit using eqn. (1) (see the Experimental section), which is described below.

In the presence of Ca²⁺, as observed previously [26,33,34], the affinity for ATP is lower than in Mg²⁺ (Figure 6B). Nevertheless, the effect of DNP at ATP concentrations up to 500 μM is qualitatively similar: it inhibits at low concentrations of ATP, followed by activation at higher concentrations. In control experiments (results not shown), the same activation profile was observed when the free Ca²⁺ concentration was kept constant (5 mM) over the entire range of ATP concentrations.

In contrast with Mg²⁺ATPase under control conditions, a second increase in the Ca²⁺ATPase activity occurs at high ATP concentrations (Figure 6B). Note that this increase becomes evident only when $[\text{ATP}] \geq 1$ mM. These data cannot be fitted by the simple Michaelis–Menten formulation for a single catalytic site, but they are consistent with more complex models: either two independent catalytic sites with different affinities but similar V_{max} values, or a model with a single site that exists in interconvertible, high- and low-affinity conformations. Both models yield two K_m values and two V_m values, and cannot be distinguished statistically. The second model has been used to analyse data obtained for a number of P-type ATPases, including the Ca²⁺,Mg²⁺ATPase of the sarcoplasmic reticulum [6,37–39]. The curves in Figure 6(B) and the fitted values for the kinetic parameters shown in Table 4 were obtained using the second model [see eqn. (1) in the Experimental section]. DNP acts as a competitive inhibitor at low ATP concentrations, where it increases K_{m1} by a factor of approx. 6, without significantly altering V_{m1} . In contrast, at higher ATP concentrations it has a striking effect as an activator, diminishing K_{m2} and increasing V_{m2} (Table 4). In the presence of Mg²⁺ and contaminating Ca²⁺

concentrations, the second K_m appears to be induced by the drug, since it is not normally apparent in the range of ATP concentrations tested. Experiments were also performed at high concentrations of ATP and Ca²⁺ using triphenylphosphine, a hydrophobic compound that strongly activates the Ca²⁺,Mg²⁺ATPase of sarcoplasmic reticulum [6]. No activation was observed.

DISCUSSION

The results described in this report show that DNP exerts its effects on myosin primarily through the globular head domain, with little or no influence from the filamentous rod portion of the molecule. Since the S1 preparation used was a mixture of S1(A1) and S1(A2), containing different proportions of light chains 1 and 3, a role for these light chains cannot be entirely ruled out. However, it is clear that neither rod nor light chain 2 is required for DNP's effects in the presence of Ca²⁺ or Mg²⁺ (Table 2 and Figure 4).

Specificity

In an effort to identify structural prerequisites for DNP effects, three analogues were chosen. 3-NP proved to be effective at a slightly lower concentration than DNP, and it exhibited maximum effects that were similar to or less than those of DNP. The other mononitrophenols were less effective, or inactive. The experiments with different cations in Figures 1–3 show a consistent sequence among the four nitrophenols in terms of $K_{0.5}$, so that 3-NP $\leq$ DNP $\leq$ 2-NP $\approx$ 4-NP. In a comparison among different hydrophobic compounds that affect the forward and reverse reactions of Ca²⁺,Mg²⁺ATPase from sarcoplasmic reticulum, Petretski et al. [5] showed that the more hydrophobic compounds were effective at lower concentrations. Thus $K_{0.5}$ values for the effects of 3-NP and 4-NP in their experiments were nearly identical, and much higher than for triphenylphosphine or diphenylamine, which are a great deal less soluble in water and more soluble in benzene than either of the mononitrophenols. The authors concluded that access to a susceptible region of the pump was determined by hydrophobicity of the compound, and postulated that the magnitude of the response may be determined by steric or structural factors [5].

On the basis of our limited selection of analogues, access to the myosin ATPase does not appear to be determined by the hydrophobicity of the compound. An index of hydrophobicity is provided by the solubilities of DNP and 2-, 3- and 4-NP in water, which are of the order of 10^{-4} , 10^{-2} , 10^{-1} and 10^{-1} mol/litre respectively [40]. Neither the $K_{0.5}$ value nor the maximal effect observed in our experiments varies in the same sequence as the solubility.

Recently, using S1 and skinned fibres, Wang et al. [41] analysed a new series of non-nucleotide ATP analogues from the 2-[(4-azido-2-nitrophenyl)amino]ethyl triphosphate (NANTP) family, where a substituted phenyl ring is attached through an amino group to ethyl or propyl triphosphate. The authors did not report relative binding affinities, but all of the analogues, including the 2-[(2-nitrophenyl)amino] and 2-[(2,4-dinitrophenyl)amino] derivatives, were hydrolysed by S1 in the presence of Mg²⁺, and hydrolysis was accelerated by actin. It is interesting to note that only analogues with a nitro group in the 2-position relative to the amino group were able to generate active shortening. The authors speculated that this property might reflect a requirement of the adenine ring, and that formation of an intramolecular hydrogen bond between the amino and nitro groups in these analogues would allow them to mimic this structure. Based on its crystal structure, DNP also forms a ring

in this way, via hydrogen bonding between the 2-nitro and the hydroxy group [42]. However, presumably 3-NP would not and, in any event, the marked contrast between the effects of DNP and either 2-NP or 4-NP appears to rule out any specific requirement for a nitro group at the *ortho* or *para* position of the ring in relation to the hydroxy group. Nucleophilic attack by one of these nitro groups seems unlikely for the same reason.

The pK values of the four analogues are 4.09 (DNP), 7.16 (4-NP), 7.23 (2-NP) and 8.35 (3-NP) [40]. Since the two most effective compounds lie at the extremes of this series, it is also apparent that ionization of the hydroxy group cannot account for the sequence of effects on the ATPase. However, it may be that polarization of the ring by withdrawal of π electrons is an important factor, with the combination of the two nitro groups at positions 2 and 4 having an effect equivalent in this regard to a single nitro group at position 3.

In a previous report, NMR data were presented to show that DNP enhances the asymmetry of Mg^{2+} co-ordination with the two terminal phosphoryl groups of ATP in aqueous solution, with no enzyme present [43]. Since 4 mM DNP caused a 30% increase in the spontaneous hydrolysis of MgATP (8 mM MgCl_2 with 8 mM ATP) at pH 7.4 and 60 °C, the authors proposed that the asymmetry destabilizes the O–P $_{\gamma}$ bond. It is not possible to say whether such an effect contributes to the increase in hydrolysis rates at the catalytic site of myosin, but there was no discernible effect of DNP on non-enzymic hydrolysis under our conditions.

Cation requirements

A striking feature of the DNP effect on myosin and S1 is the way it changes in the presence of different cations. The half-maximal effect occurs at a lower concentration in the presence of Ca^{2+} (Figure 2) than Mg^{2+} (Figure 1); and in the absence of either cation, when the enzyme is activated by K^+ , DNP inhibits rather than activates the enzyme (Figure 3). There is ample evidence that the binding of different cations changes the conformation of myosin. One example is the susceptibility to cleavage by chymotrypsin, which yields S1 in the presence of EDTA and heavy meromyosin in the presence of Mg^{2+} [18]. The data of Figure 2(A) suggest that the affinity for DNP as an activator may increase as the concentration of Ca^{2+} in the medium increases, as if saturation of Ca^{2+} (or CaATP^{2-}) binding sites causes a conformational change that affects DNP binding. This is not observed when K^+ is the activating cation (Figure 3A, inset). This difference may be related to the observation that the rate-limiting step with K^+EDTA is different from that in the presence of Ca^{2+} or Mg^{2+} [33]. The bivalent cations stabilize the $\text{M} \cdot \text{ADP} \cdot \text{P}_i$ transition state [44], leading to a decrease in the rate of dissociation of P_i from the $\text{M} \cdot \text{ADP} \cdot \text{P}_i$ complex [45].

As an activator, DNP has its most dramatic effect in the presence of Mg^{2+} (Figure 1; Tables 2 and 3), which is also the most interesting condition from a physiological point of view. Under these conditions, it has a dual effect that is remarkably similar to that reported for actin, which decreases the apparent affinity for MgATP at the catalytic site and greatly accelerates turnover [46]. The effect of actin on turnover, which predominates at physiological concentrations of ATP, is due to an enhanced rate of release of products (steps 3 and 4 in the following condensed scheme) rather than to an increase in the rate of cleavage (step 2) [44,46]:

DNP may have a similar effect when actin is absent. This hypothesis is supported by data obtained with heavy meromyosin: DNP increased the rate of P_i release to the medium (step 3), but did not alter the size of the P_i 'burst', which reflects accumulation of the complex $\text{M} \cdot \text{ADP} \cdot \text{P}_i$ [15]. It also depressed the equilibrium binding of ADP to myosin [47], suggesting an effect on step 4 as well. Activation of step 3 and/or step 4 could explain why DNP does not accelerate K^+EDTA ATPase activity. In this case the rate-limiting step is probably ATP cleavage (step 2) [33], and so any effect of DNP on later steps would not be detected. In fact, its pronounced ability to inhibit K^+EDTA ATPase activity probably means that DNP either directly or indirectly stabilizes the O–P $_{\gamma}$ bond within the catalytic site, rather than destabilizing it as reported for MgATP [43].

ATP concentration dependence

The data presented in Figure 6 (as well as in all of the earlier reports; see references in the Introduction) provide an explanation for acceleration of the Ca^{2+} and Mg^{2+} ATPase activities by DNP shown in Figures 2 and 3: DNP promotes activation of the ATPase by high ATP concentrations, in the physiological range. In the presence of Mg^{2+} , activation by high substrate concentrations only becomes evident when the drug is added (Figure 6A). In the presence of Ca^{2+} , activation by substrate occurs even in the absence of the drug, and in the presence of DNP it shifts to a lower concentration range (Figure 6B and K_{m2} in Table 4). DNP appears to increase the affinity of a low-affinity site for ATP that is capable of accelerating turnover. A similar effect has been reported for the Ca^{2+} , Mg^{2+} ATPase isolated from sarcoplasmic-reticulum membranes, in the presence of 3-NP and millimolar concentrations of Ca^{2+} [6]. The data obtained with DNP in the presence of high ATP concentrations contrast with the effect at the high-affinity site, where DNP appears to compete with ATP (K_{m1} in Tables 3 and 4).

In a number of previous reports in which myosin or heavy meromyosin was used, a biphasic effect of increasing ATP concentrations was observed [34,36,37]. The rise in activity at higher substrate concentrations has been attributed to artifacts such as aggregation of myosin filaments, reversed by high ATP [25], and contamination by a second, low-affinity ATPase [35,48,49], as well as to processes of possible physiological significance such as interaction between the two heads [36,50] and activation of turnover by a second, low-affinity regulatory site for ATP [34,51]. Later, the idea of a second site for ATP lost ground, since convincing physical evidence for a second ATP binding site on S1 was not found. In the present experiments (see also [34]), a single-headed myosin species was used, protein aggregates were removed by centrifugation, and albumin was present in the assay media. Contamination by a second ATPase is unlikely, since the S1 was purified by anion-exchange chromatography and, in addition, only one K_m is observed in the presence of Mg^{2+} and contaminating Ca^{2+} . These considerations have led us to interpret our data in terms of a regulatory site. This would not necessarily require a second ATP binding site on S1, which proceeds sequentially during each cycle through a series of conformations having different affinities for ligands [46]. The same is true for the P-type ATPases, where the increase in activity at high ATP concentrations has been attributed to a conformational change caused by ATP binding to a low-affinity conformation of the catalytic site, rather than to ATP hydrolysis at a second catalytic site [37,38].

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