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Sulphate is a competitive inhibitor of the binding of nucleotide to myosin

A comparison with phosphate

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By the use of rapid reaction methods (rapid flow quench and stopped flow) it has been shown that sulphate is a competitive inhibitor of the binding of ε-ATP and ATP to myosin. At low ionic strengths, the K_i was in the micromolar range. Under several conditions used sulphate was more effective than phosphate. Neither anion was very effective in inhibiting the binding of ε-ATP to actomyosin.

Myosin; Sulfate inhibition; Reaction kinetics; Cryoenzymology

1. INTRODUCTION

Ammonium sulphate is a commonly used reagent in the purification and concentration of enzymes. In particular, its use has become standard practice with myosin and its subfragments. The sulphate ion is often introduced in enzyme assay systems, e.g. as $MgSO_4$ with ATP handling enzymes, as a buffer component or in linked assay systems (where enzyme suspensions in ammonium sulphate are added).

We wish to report that SO_4^{2-} is a competitive inhibitor of the binding of nucleotide to myosin with a K_i of about 20 μM in buffers of low ionic strengths. It is more effective than P_i [1]. Strikingly, sulphate had much less effect on the binding to actomyosin: here K_i = 1.5 mM under the same conditions. In addition to caution regarding the effect of SO_4^{2-}, we suggest that by its use information can be gained about the active sites of myosin and actomyosin.

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Abbreviations: S1, subfragment 1 of myosin; ε-ATP, 1-N^6-etheno-ATP

It is generally agreed that myosin hydrolyses ATP according to the Bagshaw-Trentham [2] scheme:

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M\cdot ATP \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} M^*\cdot ATP \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}}$$

$$M^{**}\cdot ADP\cdot P_i \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} M^*\cdot ADP\cdot P_i \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}}$$

$$M^*\cdot ADP \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} M\cdot ADP \underset{K_1}{\rightleftharpoons} M + ADP$$

Scheme 1

where M is subfragment 1 (S1) of myosin. Asterisks indicate different protein conformations.

Here we studied the effects of sulphate and P_i on the first two steps of scheme 1 using as substrate ATP (rapid flow quench method) or the fluorescent analogue ε-ATP (fluorescence stopped flow).

2. MATERIALS AND METHODS

2.1. *Proteins and reagents*

The purification of myosin and S1 has been described in [3], and that of actin and ε-ATP (Sigma) respectively in [4,5]. [γ-^{32}P]ATP was obtained from Amersham International. Sulphate and P_i were used as the potassium salts.

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2.2. *Rapid reaction experiments*

The binding of ATP to S1 was followed using the unlabelled ATP chase technique. In a rapid flow quench apparatus [6], S1 plus [γ-^{32}P]ATP reaction mixtures are quenched in a large excess of non-radioactive ATP and finally in acid and the $^{32}P_i$ determined. Since k_{-2} is close to zero and $k_2 \gg k_4$ [6,7], by this method one follows specifically the two-step binding of ATP (scheme 1) with the kinetics:

$$k = k_2[ATP]/(K_1 + [ATP]) \quad (1)$$

By measuring k at different [ATP], K_1 and k_2 are obtained.

The binding of ϵ-ATP to S1 was monitored in a fluorescence stopped-flow apparatus as in [5]. The excitation and emission wavelengths were 290 and 340 nm, respectively. With water as solvent, acrylamide was included for amplification of the signal [8]. Upon mixing S1 with ϵ-ATP two fluorescence transients are obtained [9]: the fast one is due to tight binding of the nucleotide (i.e. formation of M*·ϵ-ATP) and the slow one probably to a partially active S1 [5]. Here, we exploited the kinetics of the fast component; its kinetics (k) varied hyperbolically with [ϵ-ATP] and K_1 and k_2 were obtained from eqn 1. The dissociation of acto-S1 was followed by light scattering using the same stopped-flow apparatus with excitation and emission wavelengths at 340 nm.

3. RESULTS AND DISCUSSION

3.1. *Sulphate is a competitive inhibitor of binding of ϵ-ATP to S1*

The kinetics of nucleotide (ATP or ϵ-ATP) binding to S1 were reduced by sulphate. Bagshaw and Trentham [1] showed that P_i inhibits competitively the binding of ATP to S1 and if we assume that sulphate does likewise, following their treatment, we can write:

$$\frac{1}{k_0} = \frac{K_1 + [S]}{k_2[S]} + \frac{K_1[I]}{k_2 K_i[S]} \quad (2)$$

where S denotes the substrate (ATP or ϵ-ATP), I the inhibitor (sulphate), K_i its inhibition constant and k_0 the constant obtained from the kinetics of nucleotide binding in the presence of sulphate. Thus, if [I] = 0, then $k_0 = k$ (eqn 1).

The binding of ϵ-ATP to S1 was studied by fluorescence stopped-flow in the presence of

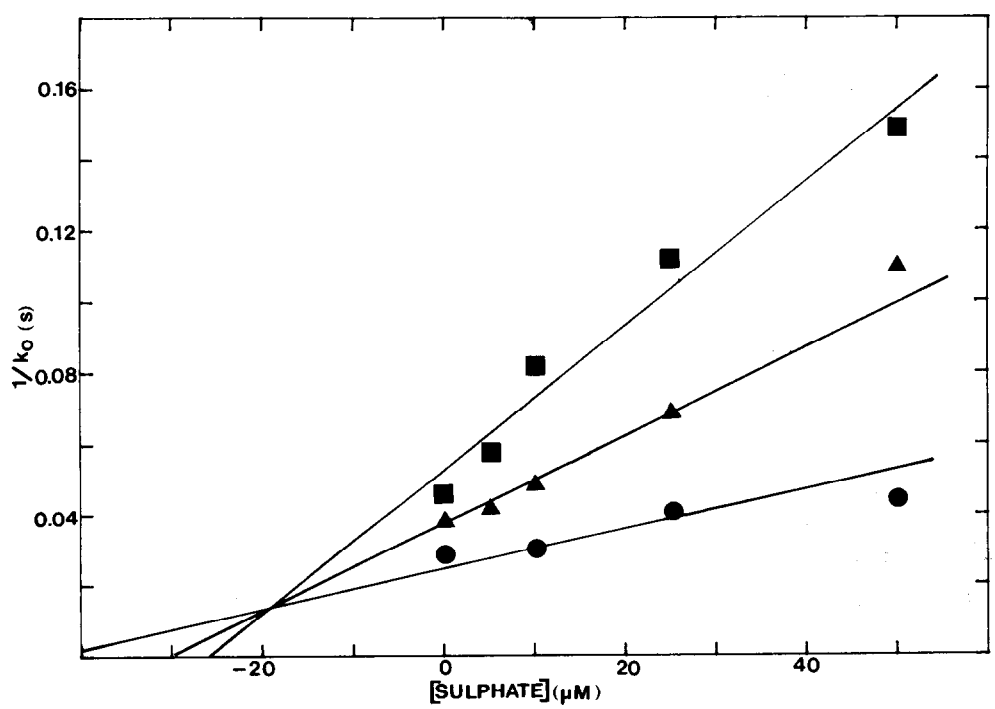

Fig.1. Effect of sulphate on the rate of binding of ϵ-ATP to S1 at 15°C. Data were obtained from fluorescence stopped-flow experiments and treated using eqn 2 with [ϵ-ATP] = 15 μM (■—■), 25 μM (▲—▲) or 50 μM (●—●). The ϵ-ATP/S1 ratio was kept constant at 7.5. Conditions: 50 mM Tris acetate, pH 8, 5 mM KCl, 2 mM Mg acetate in 40% ethylene glycol.

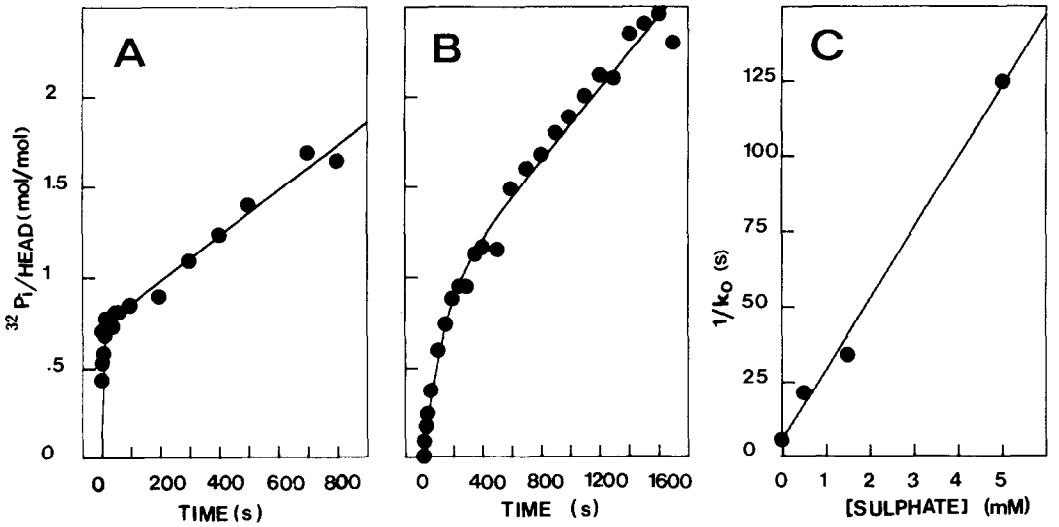

Fig.2. Effect of sulphate on the kinetics of ATP binding to S1 followed via the ATP chase method at −7.5°C under the conditions given in fig.1. Reaction mixtures (2.7 μM S1 plus 10 μM [γ-^{32}P]ATP) contained no sulphate (A), k = 0.15 (± 0.02) s^{-1}, k_{cat} = 1.2 (± 0.2) × 10^{-3} s^{-1} or 5 mM sulphate (B), k_0 = 0.008 (± 0.001) s^{-1}, k_{cat} = 1 (± 0.1) × 10^{-3} s^{-1}. (C) Dependence of $1/k_0$ on [sulphate]. A K_i of 17 μM was obtained using eqn 2 with K_1 = 1 μM and k_2 = 0.17 s^{-1} [3].

sulphate. k_0 was obtained from the fast transient component (see above). When k_0^{-1} is plotted vs [sulphate] at a series of fixed [ε-ATP] in 40% ethylene glycol at 15°C (fig.1), the lines obtained could be fitted to eqn 2 with k_2 = 70 s^{-1}, K_1 = 40 μM [5] and K_i adjusted to 20 μM. The lines converged at a point above the inhibitor axis with coordinates ($-K_i$,$1/k_2$).

Thus, our results confirm that similarly to P_i [1], sulphate inhibits competitively the binding of nucleotide to S1: its effect is to increase the apparent value of K_1 but k_2 remains unchanged.

The competitive effect of sulphate is in accord with certain non-kinetic observations, e.g. the finding that it reduces the efficiency of trapping of nucleotides by cross-linking of SH1 and SH2 of S1 [10]. Further, with S1 containing a fluorescent label (at SH1), $MgSO_4$ caused the same fluorescence quenching as ATP, ADP or PP_i [11]. Taken together, the kinetic and non-kinetic data strongly suggest that sulphate binds to the ATPase site of S1.

3.2. *Effect of sulphate and P_i on the binding of ATP to S1 under cryoenzymic conditions*

The effects of the two anions were studied by the unlabelled ATP chase technique at −7.5°C where the kinetics are easier to follow [3]. Typical progress curves at −7.5°C for the binding of ATP to S1, with or without sulphate, are given in fig.2A,B. As with ε-ATP, the binding process with ATP was retarded by the presence of sulphate. The dependence of the kinetics of the binding process upon [sulphate] is shown in fig.2C. This gives a K_i of 17 μM which is very similar to that found with ε-ATP (20 μM, table 1). Under the same conditions, the K_i for P_i is 100 μM and 125 μM with ε-ATP as substrate (table 1). We note that these data were obtained through the use of quite different methods: fluorescence stopped flow with ε-ATP and a chemical sampling method with ATP.

Whereas the kinetics of nucleotide binding to S1 were sensitive to sulphate, the steady-state rate of ATP hydrolysis was much less so. Thus, at the concentrations used above, sulphate had little effect on this parameter. However, at 10 mM, sulphate reduced the steady-state rate by about 30% at −7.5°C. Since in this case, a transient burst phase was not discerned via the ATP chase method, we suggest that at high [sulphate] k_0 becomes rate-limiting (in the absence of sulphate this is k_6 under the same conditions [3]). There was little effect of 50 mM sulphate on the K^+-EDTA and the Ca^{2+}-ATPase activities of S1.

Table 1

Effect of experimental conditions on the binding of sulphate and P_i to S1

Nucleotide	Experimental conditions				Kinetic constants				
	Temperature (°C)	Ethylene glycol (%)	[KCl] (mM)	pH	K_1 (μM)	k_2 (s^{-1})	$K_i(SO_4^{2-})$ (μM)	$K_i(P_i)$ (μM)	$K_i(SO_4^{2-})/K_i(P_i)$
ϵ-ATP	15	0	100	8	–	–	1600 ± 200	2900 ± 300[a]	1.8 ± 0.4
ϵ-ATP	15	0	5	8	–	–	100 ± 20	150 ± 30	1.5 ± 0.6
ϵ-ATP	15	40	5	8	40 ± 20	70 ± 10	20 ± 2	170 ± 20	8.5 ± 3
ϵ-ATP	−7.5	40	5	8	67 ± 17	10 ± 4	20 ± 5	125 ± 25	6.2 ± 3
ATP	−7.5	40	5	8	1 ± 0.3	0.17 ± 0.05	17 ± 5	100 ± 20	5.9 ± 3

[a] Bagshaw and Trentham [1] determined a value of 1.5 mM under similar conditions

K_i values were obtained from fluorescence stopped-flow (ϵ-ATP) or ATP chase (ATP) experiments. K_1 and k_2 for ϵ-ATP are from [5] and for ATP from [3].

3.3. *Effect of experimental conditions on binding of sulphate and phosphate with ϵ-ATP as substrate*

The K_i values for the two anions were determined under different conditions. By carrying out experiments at $[S] \ll K_1$ it was unnecessary to determine the individual constants K_1 and k_2 under each condition: only their ratio is required because here eqn 2 reduces to:

$$1/k_0 = (K_1/k_2 \cdot [\epsilon\text{-ATP}])(1 + [I]/K_i) \quad (3)$$

and K_i is obtained from the slope of a plot $1/k_0$ vs [I]. The results are listed in table 1. Since myosin has positively charged residues at its ATPase site [12], one might expect its interaction with sulphate to be purely ionic which would explain the effect of ionic strength in increasing the K_i. Further, it would explain the relative insensitivity of K_i to temperature: ionic interactions are governed principally by the product of dielectric constant and temperature [13] which varies little with temperature in hydro-organic solvents [14].

A number of ATP handling enzymes have 'glycine-rich loops' which are believed to play a special role in the binding of the phosphoryl group of the substrate [15–18]. The heavy chain of rabbit muscle myosin has two glycine-rich loops, one of which is conserved in certain other myosins and it has been suggested that it forms part of the ATPase site ([12,18], but see [19]). With adenylate kinase it is known that sulphate binds specifically to a glycine loop at the active site, thus explaining its inhibitory effect [18]. With myosin, we suggest that sulphate also binds to an active-site loop, thus preventing the nucleotide from interacting.

3.4. *The effect of sulphate and P_i on the interaction of ϵ-ATP with acto-S1*

The kinetics of ϵ-ATP binding to acto S1 in 40% ethylene glycol were much less affected by sulphate and P_i than were those of S1. For example, at 15°C in 40% ethylene glycol, the K_i for sulphate increases dramatically from about 20 μM with S1 to 1.5 mM with acto-S1 and from 150 μM to 1.75 mM for P_i. This confirms that actin induces changes in the ATPase site of myosin [20].

We also studied the effect of both anions on the kinetics of dissociation of acto-S1 by ϵ-ATP (by light scattering). The inhibitory effects of the two anions were very similar: under the same conditions as before, the K_i values for sulphate were 1.5 mM in binding and 0.6 mM in dissociation. With P_i the K_i values were identical for both processes (1.5 mM).

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