THE REACTION OF MYOSIN WITH A BROMOALKYL ANALOG OF ADENOSINE TRIPHOSPHATE

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1. Introduction

ATP affinity analogs are of special usefulness in the study of the binding and hydrolysis of ATP by myosin ATPase during the contraction cycle.

A number of these ATP analogs have been synthesized and their reaction with myosin described [1–3]. It was reported [4] that the synthesis of a brominated adenosine-5′ ester of maleic acid and pointed out its potential use as an active-site-directed inactivator of certain adenosine nucleotide dependent enzymes, attacking the phosphate binding site.

In this report we describe an improved synthesis of a similar derivative, 2′–3′ isopropylidene adenosine 5′ dibromohydrogen succinate, and its reaction with rabbit skeletal myosin and its chymotryptic subfragment-1.

2. Experimental

2.1. *Materials and methods*

ADP, ATP and AMP-PNP were obtained from Boehringer and Soehne, Mannheim, and 2′–3′ isopropylidene adenosine from Aldrich. Rabbit myosin was prepared according to [5], subfragment-1 was prepared from myosin by digestion [6,7] with chymotrypsin (Worthington) pretreated with tosyl-L-Lysine chloromethyl ketone [8]; it was purified on Sephadex G-200 and stored at 4°C in 0.2 M Tris–HCl buffer (pH 7.5). Actin was prepared as described [9] and purified according to [10]. Protein concentrations were estimated by extinction using $E_{280}^{1\%}$ 5.5 cm^{-1} for myosin [11]; 7.5 cm^{-1} for subfragment-1 [7] and 11.0 cm^{-1} for actin [12].

ATPase activities were determined at 25°C in 50 mM Tris–HCl (pH 7.5) in the presence of 2.5 mM ATP. Conditions for K^+EDTA assays were: 1 M KCl, 5 mM EDTA, for calcium ATPase: 250 mM KCl, 5 mM $CaCl_2$; for magnesium-dependent ATPase: 250 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA. P_i liberated was measured colorimetrically by the procedure [13].

Assays in presence of actin were performed in a pH-stat with a medium containing 6 mM KCl, 50 mM Tris–HCl (pH 8), 1.5 mM $MgCl_2$, 1 mM ATP, and 14 μM actin for 0.5 μM subfragment-1.

The inactivation reactions were performed at room temperature in 0.2 M Tris–HCl (pH 7.5) 0.1 M KCl, with myosin 1.45 mg/ml or subfragment-1 0.730 mg/ml. The inactivator was added in 0.2 M Tris–HCl buffer (pH 7.5).

Association and dissociation reactions of actin-myosin were studied by the turbidity method [14].

2.2. *Synthesis*

2′–3′ Isopropylidene adenosine 5′ hydrogen mealeate was prepared from recrystallized maleic anhydride and 2′–3′ isopropylidene adenosine as described [4].

The product (500 mg) eluted from Amberlite CG 50 I (H^+ form) was freed from traces of 2′–3′ isopropylidene adenosine and maleic acid by filtration on Sephadex LH-20 (5 × 90 cm) equilibrated and eluted with distilled water 100 ml/h.

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2′–3′ Isopropylidene adenosine 5′-hydrogen maleate appeared after 1 l in vol. 140 ml. It was removed by lyophilisation (yield 36%) and it was pure by cellulose thin-layer chromatography (solvent, ethanol : 1 M ammonium acetate (pH 6.5) 7:3, v/v).

$C_{17}H_{19}N_5O_7$ (mol. wt 405.3)			
Calculated	50.3 C	4.6 H	17.2 N
Found	49.2 C	4.8 H	16.9 N

NMR analysis measured in D_2O provided resonance peaks consistent with the assigned structure.

Freshly prepared 2′–3′ isopropylidene adenosine 5′-hydrogen maleate (0.5 mM) was dissolved in 6 ml 4.8 M KBr solution (pH 6.5). Bromine in 4.8 M KBr was added (1.6 mM) and the solution was allowed to stand for 12 h in the dark at room temperature. The mixture was adjusted to pH 4.9 with 0.25 N NaOH and excess bromide eliminated under nitrogen.

The decolorized solution was applied on a Sephadex LH-20 column (fig.1). Five ultraviolet absorbing products were obtained and their purities were checked by thin-layer chromatography. They were characterized by NMR and elementary analysis; I was 2′–3′ isopropylidene adenosine 5′-hydrogen maleate, II and III were 2′–3′ isopropylidene adenosine 5′ (mono bromo hydrogen maleate), IV was 2′–3′ isopropylidene adenosine 5′ (dibromo hydrogen succinate) and V was a 8-bromo derivative of II.

IV was further purified on a Sephadex LH-20 column and obtained in 31% yield.

$C_{17}H_{19}N_5Br_2O_7$ (mol. wt 565.6)				
Calculated	36.1 C	3.3 H	12.3 N	28.3 Br
Found	35.1 C	3.2 H	12.1 N	26.9 Br

NMR analysis of IV showed that it lacked ethylenic hydrogens:

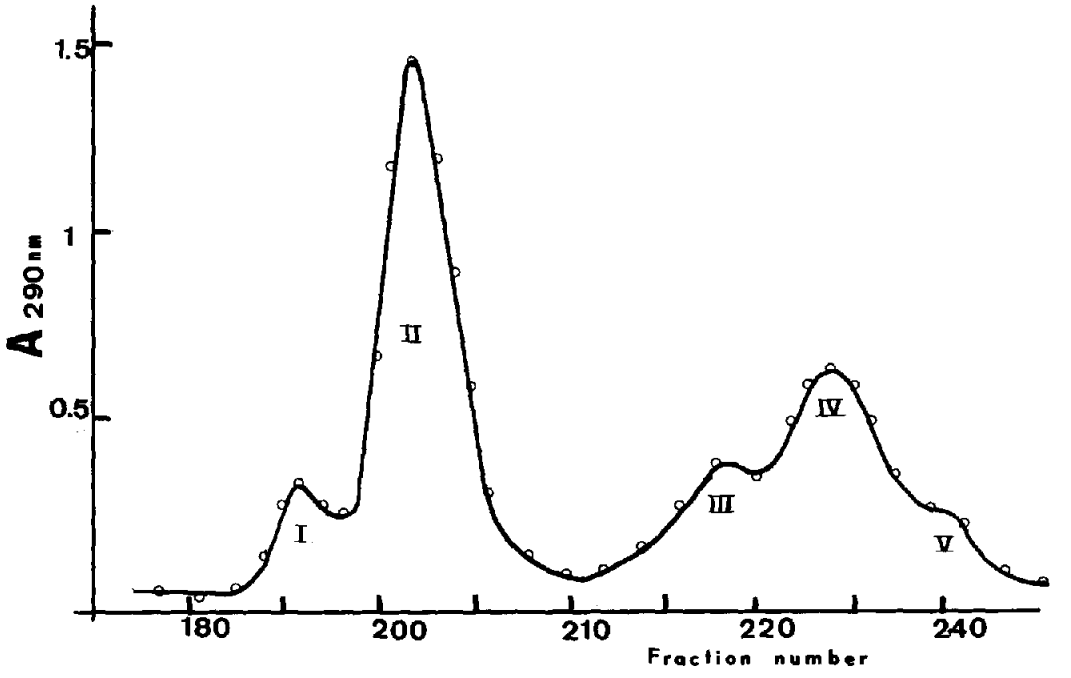
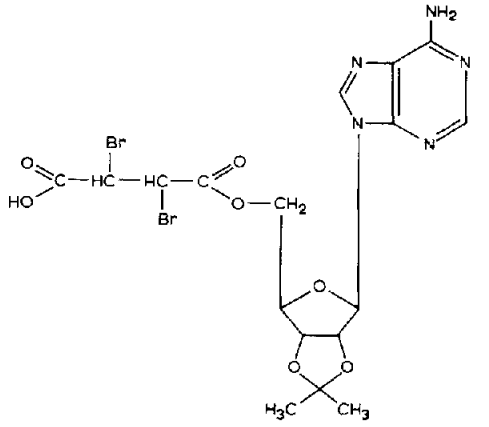


Fig.1. Separation of brominated derivatives of 2′–3′ isopropylidene adenosine 5′-maleate on Sephadex LH-20: the column (5 × 90 cm) is loaded with 13 ml reaction mixture and eluted with distilled water 60 ml/h. The fractions (7 ml) are monitored by $A_{290\ nm}$.



The reactions of this compound with myosin and subfragment 1 were investigated.

3. Results and discussion

As 2′–3′ Isopropylidene ATP was shown to be a good substrate for myosin [15], we used 2′–3′ isopropylidene adenosine 5′-hydrogen maleate for the bromination step.

In our hands bromination of the double bond in aqueous solution under the conditions used in [15] did not give successful results. However, when bromination was performed in saturated KBr [16] and the reaction mixture fractionated on Sephadex LH-20, five different derivatives were identified. In preliminary experiments product IV was found to be the most powerful inhibitor of the K^+EDTA ATPase activity of myosin and it was selected for further investigations.

In the presence of IV, myosin loses its K^+EDTA ATPase activity (fig.2) with first order kinetics.

A plot of the observed rate constant versus concentration of IV showed saturation kinetics (fig.3A and B) and gave k_{max} 1.25 min^{-1} for myosin and 0.38 min^{-1} for subfragment-1.

In addition, the plot deviated from a hyperbolic curve, having a sigmoidal character. Thus a Lineweaver-Burk plot showed positive cooperativity. Treatment of the kinetic data according to [17] gave n 1.85, K_i 34 mM for myosin and n 1.78, K_i 28 mM for subfragment-1. The presence of ADP, pyrophosphate or the ATP analog AMP–PNP not only did not protect but markedly accelerated the rate of

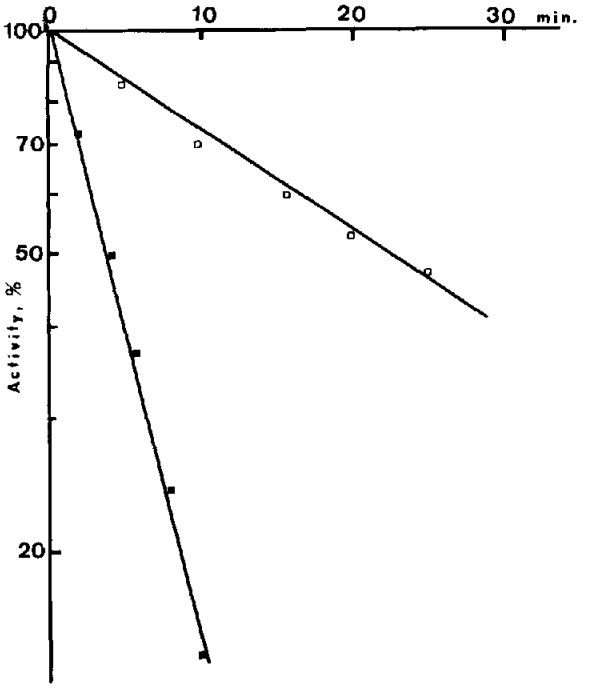


Fig.2. Inactivation of myosin K^+EDTA ATPase in absence and presence of AMP–PNP with 5×10^{-3} M of IV: the semi logarithmic plots are determined with (■) and without (□) AMP–PNP (2×10^{-5} M).

inactivation (fig.2). As shown in fig.3, the inactivation in presence of AMP–PNP also exhibited saturation kinetics with k_{max} 1 min^{-1}, K_i 11 mM, n 1.92 for myosin and k_{max} 0.35 min^{-1}, K_i 6.5 mM, n 0.94 for subfragment-1. The Mg^{2+}- and Ca^{2+}-dependent ATPase activities were not modified by treatment of myosin or subfragment-1 with IV either in the absence or the presence of AMP–PNP. In contrast, the Mg^{2+}–ATPase activity of acto-subfragment-1 was progressively inhibited in the same manner as found for K^+EDTA ATPase (fig.4). The results of turbidity measurements (table 1) indicated modified myosin, was able to interact with actin to the same extent as the native enzyme but that the actin-modified myosin complex could not be dissociated by Mg^{2+}–ATP.

The results indicate that IV specifically inactives the K^+EDTA stimulated ATPase of myosin and subfragment-1 and behaves as a site specific reagent. The sigmoidal character of the saturation curve suggests the presence of two dependent sites per myosin head.

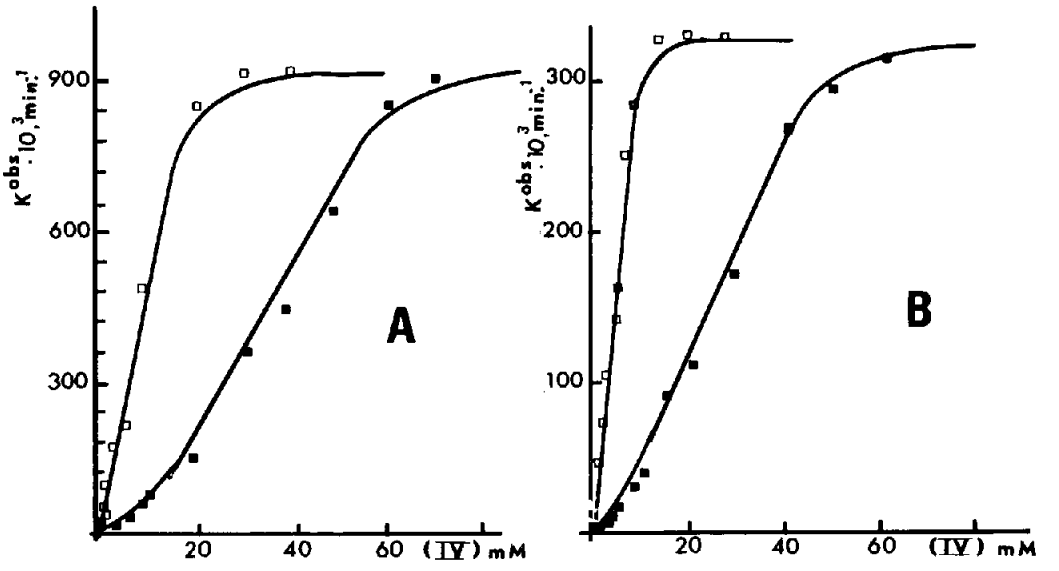
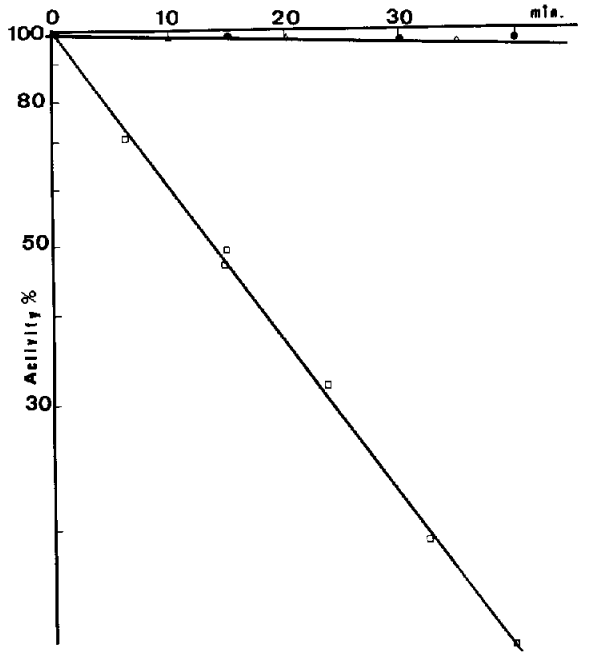


Fig.3. Effect of concentration of IV on the rate of inactivation of myosin and subfragment-1 K^+EDTA ATPase: (A) Myosin treated in absence (■) and in presence (□) of 2×10^{-5} M AMP–PNP. (B) Subfragment-1 treated in absence (■) and in presence (□) of 2×10^{-5} M AMP-PNP.



Furthermore the effect of AMP–PNP on the inactivation rate suggests the interdependence of two different nucleotide binding sites. This last effect is similar to that reported [18,19] for the disulfide analog of ATP, 6,6′-dithiobis (inosinylimidodiphosphate). However, in contrast to the latter derivative, IV does not affect the Ca^{2+}– or Mg^{2+}–ATPase activities of myosin and owing to its chemical structure, it probably reacts with different amino acid side chains. Further, it does not inhibit actin binding as does the disulphide analog [20]; instead it alters the Mg^{2+}–ATP site so that dissociation of actomyosin cannot occur. This is consistent with the observed inactivation of the actomyosin Mg^{2+}–ATPase, since it is now well established that ATP cleavage takes place after dissociation of the actomyosin complex by the substrate [21]. Experiments are underway to determine the stoichiometry of the reaction and to identify the modified site.

Fig.4. Inhibition of actin-activated Mg^{2+}–ATPase activity of subfragment-1 by 6×10^{-3} M of IV: (△) Ca^{2+}–ATPase, (●). Mg^{2+}–ATPase, (□) Mg^{2+}–ATPase in presence of actin. Reaction conditions were as described in the Materials and methods section.

Table 1
Turbidimetric measurement of actomyosin association-dissociation before and after treatment by IV

	In absence of actin	In presence of actin	
		Without Mg^{2+}ATP	With Mg^{2+}ATP
Native myosin	0.004	0.129	0.009
Modified myosin	0.002	0.112	0.092

Myosin, 1.4 mg, in 10 mM Tris–HCl, 100 mM KCl, 1 mM $MgCl_2$ (pH 8) 1 ml, were mixed with 0.480 mg actin in the same buffer. Turbidity was estimated by $A_{400\ nm}$ in the absence and presence of 50 mM $MgCl_2$ + 1.6 mM ATP

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