BBA 45995

EFFECTS OF SUBSTRATE AND SUBSTRATE ANALOGUES ON THE TRINITROPHENYLATION OF MYOSIN

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(Received March 31st, 1970)

SUMMARY

The effects of ATP, ATP analogues, Mg^{2+} and actin on the trinitrophenylation of myosin and on the enzymic properties of trinitrophenylated samples were studied.

1. Trinitrophenylation of myosin was inhibited by the presence of ATP and its analogues during the treatment in the order ADP > ATP > pyrophosphate > AMP.

2. The alteration of the enzymic properties due to trinitrophenylation of myosin was prevented by the presence of ATP or ADP and somewhat less by that of pyrophosphate and AMP during trinitrophenylation, but only if Mg^{2+} was also present.

3. Neither the degree of trinitrophenylation nor the enzymic properties of the trinitrophenylated myosin were influenced by the presence of actin during the treatment of myosin.

INTRODUCTION

The trinitrophenylation of myosin by 2,4,6-trinitrobenzenesulphonate (TBS) has already been extensively studied[1-6]. It was observed that two ε-amino groups per $4.2 \cdot 10^5$ g of myosin are rapidly trinitrophenylated by TBS[1] and that trinitrophenylation is markedly retarded by the presence of ATP or phyrophosphate[2]. The enzymic properties of myosin ATPase (ATP phosphohydrolase, EC 3.6.1.3) were found to be essentially altered on trinitrophenylation[1, 3, 4, 6]. It was observed in our previous studies[6] that the change of the enzymic properties due to trinitrophenylation was appreciably less remarkable if ATP was present during the treatment of myosin with TBS, even if the same number of lysyl residues was trinitrophenylated as in the absence of the substrate. This observation suggested that ATP specifically prevents the trinitrophenylation of the lysyl residues which are involved in the ATPase activity of myosin and it seemed of interest to investigate also the effects of the ATP analogues, actin and different ionic conditions on the trinitrophenylation of these lysyl residues.

MATERIALS AND METHODS

Myosin was prepared essentially as described by PORTZEHL *et al.*[7]. The precipitation process was repeated 5 times to remove the last traces of myokinase.

Abbreviation: TBS, 2,4,6-trinitrobenzene sulphonate.

The last precipitate was dissolved in 0.5 M KCl + 0.02 M borate buffer (pH 7.4) and centrifuged at 105000 × *g* for 1 h. Fresh myosin (not older than 4 days) was used throughout. Actin was prepared as described in a previous paper[6].

Reagent grade chemicals were used throughout. TBS was a product of Sigma Chemical Co. The other products were from Reanal (Budapest).

The trinitrophenylation of myosin was carried out as described in the previous paper[6]. The number of the trinitrophenyllysine groups was evaluated by the method of OKUYAMA AND SATAKE[8] from the change in absorbance at 346 mμ ($\varepsilon = 1.45 \cdot 10^4$).

The ATPase activity was measured in a solution containing 1 mg of myosin per ml, 4 mM ATP, 0.6 M KCl, 25 mM Tris–maleate buffer (pH 7.4) and either 5 mM EDTA or 2 mM $MgCl_2$. The measurements were carried out at 20° on samples of 2 ml. Incubation was terminated by addition of 2 ml of 10 % trichloroacetic acid. P_i was measured by the method of FISKE AND SUBBAROW[9]. The ATPase activity was evaluated as μmoles P_i per mg of myosin per min.

Protein content was measured by the biuret method of GORNALL *et al.*[10].

RESULTS

The dependence of the trinitrophenylation of myosin on TBS concentration, as studied both in the presence and in the absence of ATP or its analogues, is shown in Fig. 1. In the presence of ATP or its analogues the trinitrophenylation decreases as compared with the samples treated in their absence. The order of inhibition is ADP > ATP > pyrophosphate > AMP. The data on ATP and pyrophosphate are in agreement with those obtained by TONOMURA *et al.*[2].

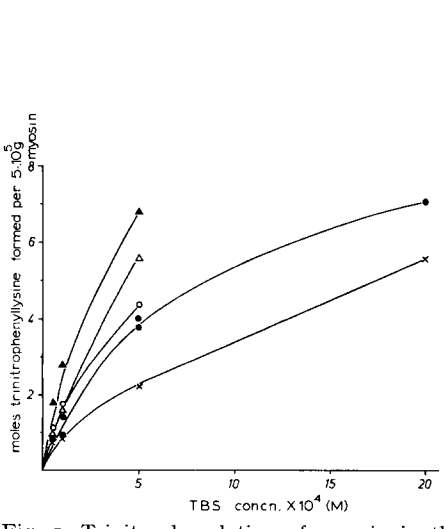

Fig. 1. Trinitrophenylation of myosin in the presence and absence of ATP or its analogues. Trinitrophenylation was carried out in the presence of 0.5 M KCl, 2 mM $MgCl_2$. Other constituents: ●, 8 mM ATP; ×, 8 mM ADP; ○, 8 mM pyrophosphate; △, 8 mM AMP; ▲, none.

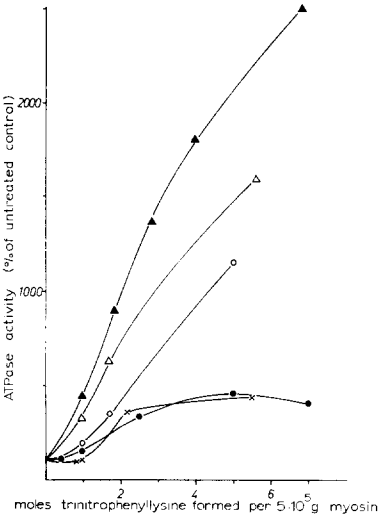

Fig. 2. The Mg^{2+}-mediated ATPase of myosin trinitrophenylated in the presence and absence of ATP or its analogues. ATPase activity measured in the presence of 2 mM $MgCl_2$. For conditions of trinitrophenylation and definitions of symbols see Fig. 1. Control (100%) activity: 0.0056 μmoles P_i per mg of myosin per min.

The ATPase activity of myosin trinitrophenylated under different conditions was measured in the presence of Mg^{2+} (Fig. 2) or EDTA (Fig. 3). The Mg^{2+}-mediated ATPase markedly increases as a function of trinitrophenylation. This increase becomes much less pronounced if ATP or ADP are present, and it is reduced but to a smaller extent also by the presence of pyrophosphate and AMP during the treatment.

The ATPase activity in the presence of EDTA, *i.e.* the K^+-activated ATPase[11], considerably decreases with increasing trinitrophenylation. The decrease is inhibited markedly if ATP or ADP and slightly if AMP or pyrophosphate is present during the treatment.

All the trinitrophenylation experiments mentioned so far were carried out in the presence of Mg^{2+} in order to inhibit the liberation of P_i from ATP during the treatment. It therefore seemed necessary to study specifically the effect of Mg^{2+} on the trinitrophenylation of myosin. Some of the myosin samples were trinitrophenylated in the presence of EDTA and ATP (EDTA was added to remove all Mg^{2+} contaminations). Since ATP is rapidly transformed by myosin to ADP in the presence of EDTA and K^+, the latter was replaced in these experiments by Na^+ to prevent the rapid breakdown of ATP during the trinitrophenylation[12]. Control runs were done to see if there was any difference in the degree of trinitrophenylation or in the enzymic properties of the trinitrophenylated myosin caused by the substitution of Na^+ for K^+ in the treatment. The dependence of the trinitrophenylation on the TBS concentration in both the presence and the absence of ATP studied under different ionic conditions is shown in Fig. 4. The experimental curves show that trinitrophenylation

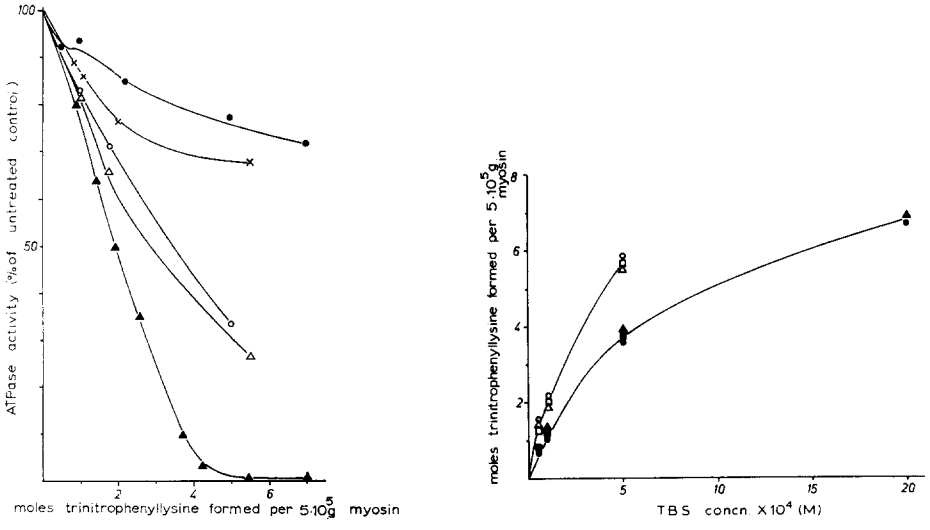

Fig. 3. The K^+-activated ATPase of myosin trinitrophenylated in the presence or absence of ATP or its analogues. ATPase activity measured in the presence of 5 mM EDTA. For conditions of trinitrophenylation and definitions of symbols see Fig. 1. Control (100%) activity: 1.06 μmoles P_i per mg of myosin per min.

Fig. 4. Trinitrophenylation of myosin in the presence and absence of ATP under different ionic conditions. Trinitrophenylation was carried out in the presence (●, ▲, ■) or in the absence (○, △, □) of ATP and in the presence of 0.5 M KCl and 2 mM $MgCl_2$ (○, ●); 0.5 M NaCl and 2 mM $MgCl_2$ (△, ▲) and 0.5 NaCl and 5 mM EDTA (□, ■).

is affected by the presence of ATP only, while it is entirely insensitive to the absence or presence of Mg^{2+} as well as to the species of K^+ or Na^+ in the test solution.

The effects of the presence of ATP and of different ionic conditions during the treatment on the enzymic properties of trinitrophenylated myosin are shown in Figs. 5 and 6. The Mg^{2+}-mediated ATPase markedly increases as a function of trinitrophenylation independently of the ionic conditions if ATP is absent during trinitrophenylation (Fig. 5). This increase is greatly prevented if Mg^{2+} and ATP are both present during the treatment. On the other hand, Mg^{2+}-mediated ATPase increases to almost the same extent as in the absence of ATP, if ATP and EDTA are present during the treatment. The activity curves do not vary with the species of monovalent cation present during trinitrophenylation.

A similar effect, but in the opposite sense, was observed in the measured values of the K^+-activated ATPase of the trinitrophenylated myosins (Fig. 6). The most marked decrease in the enzymic activity is observed in the samples trinitrophenylated in the absence of ATP; the decrease is less remarkable in myosin treated in the presence of both ATP and EDTA, and there is only a slight decrease when ATP and Mg^{2+} are present during the trinitrophenylation. There is no difference between the shapes of the activity curves in relation to whether K^+ or Na^+ is present during trinitrophenylation.

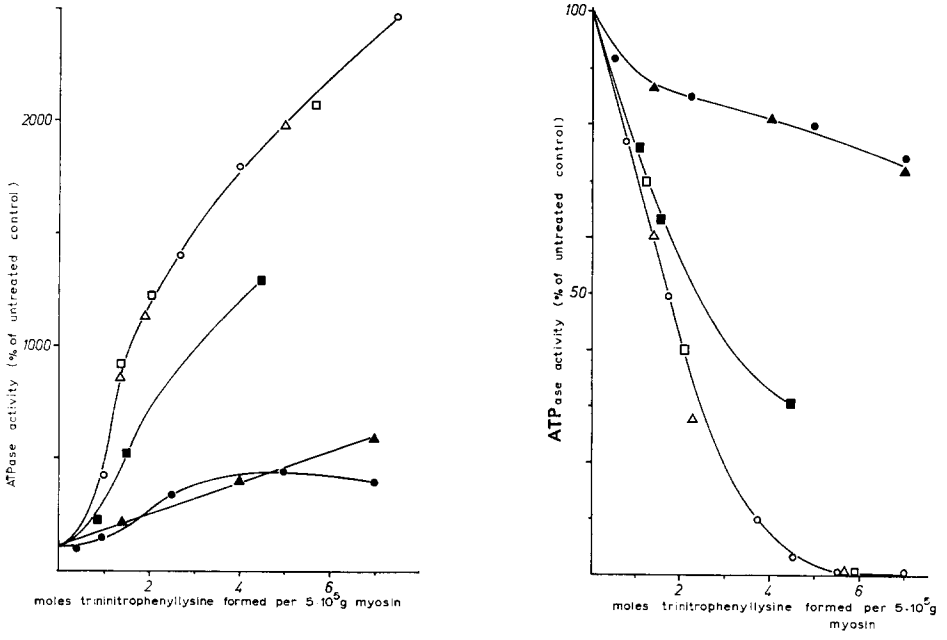

Fig. 5. The Mg^{2+}-mediated ATPase of myosins trinitrophenylated in the presence and absence of ATP under different ionic conditions. ATPase activity measured in the presence of 2 mM $MgCl_2$. For conditions of trinitrophenylation and definitions of symbols see Fig. 4. Control (100%) activity: 0.0052 μmoles P_i per mg of myosin per min.

Fig. 6. The K^+-activated ATPase of myosin trinitrophenylated in the presence and absence of ATP under different ionic conditions. ATPase activity measured in the presence of 5 mM EDTA. For conditions of trinitrophenylation and definitions of symbols see Fig. 4. Control (100%) activity: 1.11 μmoles P_i per mg of myosin per min.

The effect of the presence of actin on the trinitrophenylation and enzymic parameters of myosin was also studied. As is apparent from Table I, neither of these parameters was found to be sensitive to the presence of actin during the treatment.

TABLE I

THE DEPENDENCE OF TRINITROPHENYLATION AND ATPASE ACTIVITY OF MYOSIN ON THE PRESENCE OF ACTIN DURING TRINITROPHENYLATION

Conditions of trinitrophenylation: 10 mg of myosin per ml, 2.5 mg of actin per ml if present, 50 mM KCl, 25 mM Tris–maleate buffer (pH 7.4). At the end of the reaction after the removal of the excess of TBS actin was removed by the method of SZENT-GYÖRGYI[20] with three precipitations by dilution and resolution in 0.5 M KI + 6 mM $Na_2S_2O_3$. After the last KI treatment myosin was precipitated by dilution and dissolved in 0.5 M KCl. The precipitation was repeated once more to remove KI.

Treatment	*Moles of trinitrophenyllysine formed per* $5 \cdot 10^5$ *g of myosin*	*ATPase activity (μmoles* P_i *per mg myosin/min) measured in the presence of*	
		2 mM $MgCl_2$	*5 mM EDTA*
—	—	0.0042	0.76
0.1 mM TBS	2.42	0.022	0.175
0.5 mM TBS	5.93	0.089	0.069
□ + actin present	—	0.0046	0.75
0.1 mM TBS + actin present	2.15	0.024	0.165
0.5 mM TBS + actin present	6.14	0.092	0.061

DISCUSSION

The present experimental data clearly indicate that the presence of ATP has a double effect on the trinitrophenylation of myosin. First, it inhibits trinitrophenylation in general, independently of the ionic conditions of the test solution. Second, in the presence of Mg^{2+} it prevents specifically the trinitrophenylation of the lysyl residues having some role in the enzymic activity. The first effect, observed also by TONOMURA *et al.*[2], can be due to the fact that the binding of ATP to the positively charged lysyl residues prevents the trinitrophenylation of these groups[13]. The second effect can be explained by a conformational change induced by the combined effect of Mg^{2+} and ATP which abolishes the specifically high affinity toward TBS of those lysyl residues which are somehow involved in the ATPase activity and are localized in or near the active centre of myosin. This Mg^{2+}-dependent ATP-induced conformational change of the active site supports the view that different mechanisms are responsible for the splitting of ATP by myosin in the presence and in the absence of divalent cations[14, 15]. The conformation change due to the simultaneous presence of ATP and Mg^{2+} has also been observed by several investigators in the form of a change in the ultraviolet absorption spectrum of heavy meromyosin upon the addition of substrate or substrate analogues[16–18].

The effect of ATP in the presence of Mg^{2+} different from that in the absence of Mg^{2+} in the reaction of myosin with fluorodinitrobenzene was observed by BÁRÁNY *et al.*[19], who found that the incorporation of the dinitrophenyl residues into the myosin molecule increased in the presence of free ATP and decreased if MgAPT was present.

In the present experiments the number of incorporated trinitrophenyl groups was found to be the same when either ATP or MgATP was present. The interesting feature of the present observation is that other types of lysyl residues reacted in the presence rather than in the absence of Mg^{2+} and ATP, since the simultaneous presence of the two prevented specifically the trinitrophenylation of the lysyl residues playing some role in the ATPase activity of myosin. In the experiments of BÁRÁNY *et al.*[19] actin was found to inhibit markedly the dinitrophenylation of myosin, while in our experiments the presence of actin did not alter in any way the trinitrophenylation of myosin or the enzymic behaviour of the product. The difference between our results and those of BÁRÁNY *et al.* is probably due to the different nature of the reagents. Fluorodinitrobenzene was found to react primarily with tyrosyl and cisteyl residues of myosin in their experiments[19], while TBS reacts under the conditions used in our experiments specifically with the lysyl residues.

The effect of ADP is similar to that of ATP on both the trinitrophenylation and the enzymic properties of the trinitrophenylated myosin with the inhibitive effect on the trinitrophenylation being even more enhanced. The effects of AMP and pyrophosphate show a similar trend but are considerably less apparent than those of the other two materials.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. N. A. Biró for his kind interest and helpful suggestions and to Mrs J. Monori-Kovács for her help in the preparation of the paper.

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