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EFFECT OF TRINITROPHENYLATION ON MYOSIN ATPase

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SUMMARY

1. The enzymic and actin binding properties of myosins trinitrophenylated to different extents in the presence or absence of ATP have been studied.

2. The enzymic properties of myosin trinitrophenylated in the absence of ATP are different from those of myosin treated in the presence of ATP even on trinitrophenylating an equal number of lysyl residues. On trinitrophenylation in the absence of ATP the EDTA-(K^+-)activated ATPase and Ca^{2+}-activated ATPase decrease while the Mg^{2+}-activated ATPase considerably increases. In the presence of ATP the enzymic properties of myosin are much less affected by trinitrophenylation.

3. The actin binding capacity of trinitrophenylated myosin does not change, although its enzymic properties may be greatly altered, and even if its property to be activated by actin is completely lost.

INTRODUCTION

2,4,6-Trinitrobenzenesulfonate (TBS) specifically trinitrophenylates the NH_2 groups of proteins[1]. According to KUBO, TOKURA AND TONOMURA[2] two ε-NH_2 groups per $4.2 \cdot 10^5$ g myosin are rapidly and specifically trinitrophenylated by TBS. One of these ε-NH_2 groups is localized in a subfragment of myosin[3], subfragment-1, containing the active centre of myosin-ATPase (ATP phosphohydrolase, EC 3.6.1.3) and the actin binding site of myosin. The trinitrophenylation of the two lysyl residues greatly alters the characteristic properties of myosin ATPase[2,4,5]. In the presence of ATP or pyrophosphate, the trinitrophenylation of the rapidly reacting ε-NH_2 groups highly retarded[6]. It seemed of interest to find out whether the lysyl residues which are protected from trinitrophenylation by ATP have any role in the ATPase activity of myosin.

MATERIALS AND METHODS

Myosin was prepared essentially as described by PORTZEHL, SCHRAMM AND WEBER[8]. After the last precipitation it was dissolved in 0.5 M KCl + 0.02 M borate buffer (pH 7.4), and centrifuged with 105 000 $\times$ g in a Spinco preparative ultracentrifuge for 1 h.

Abbreviations: TBS, 2,4,6-trinitrobenzenesulfonate; TNP-, trinitrophenyl-.

Actin was extracted from acetone dry muscle powder prepared by the method of BÁRÁNY *et al.*[9]. The extraction was performed at 0° (ref. 10) and purified further by the method of MOMMAERTS[11].

Reagent grade chemicals were used throughout. TBS was a product of Sigma Chemical Co. The other products, including ATP, were from Reanal.

Myosin was trinitrophenylated at 20° for 10 min both in the presence and absence of ATP. The test solution contained 10 mg of protein per ml, 25 mM Tris–maleate buffer (pH 7.4), 0.5 M KCl to which TBS was added at different concentrations in each case. Whenever the effect of ATP was tested, 8 mM ATP and 2 mM $MgCl_2$ was added. The pH was kept at a constant value by performing the reaction in a Radiometer pH stat (TTT1/SBR2) and using 1.0 M KOH for neutralization.

Myosin was precipitated at the end of the reaction by dilution with 12 vol. of ice-cold distilled water and the pH was immediately adjusted to pH 6.2–6.4 in order to stop further trinitrophenylation. The suspension was centrifuged in a Spinco preparative ultracentrifuge with 10000 × *g* at 3° for 30 min. The precipitated myosin was resuspended in 100 vol. of ice-cold 40 mM KCl and was centrifuged again. Finally it was dissolved in 0.5 M KCl + 0.02 M borate buffer (pH 7.4) and left standing overnight, then centrifuged again with 10000 × *g* to obtain the clear supernatant used in the experiments. The number of trinitrophenyl-(TNP-)lysine groups formed was evaluated by the method of OKUYAMA AND SATAKE[1] from the absorbance change at 346 mμ ($\varepsilon = 1.45 \cdot 10^4$). Fresh myosin (not older than 4 days) was used throughout in the experiments.

The ATPase activity of myosin was measured in a solution containing, if not otherwise stated, 1 mg of myosin per ml, 4 mM ATP, 25 mM Tris–maleate buffer (pH 7.4) and, alternatively, 5 mM EDTA, or 5 mM $CaCl_2$, or 2 mM $MgCl_2$. The KCl concentration was varied from 0.1 to 0.6 M. 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[12]. The ATPase activity was evaluated as μmole P_i per mg myosin per min. The time of incubations was chosen so as to obtain a decomposition of the terminal phosphate of ATP to less than 25%.

The ATPase activity of the synthetic actomyosin was measured similarly in a solution containing 0.8 mg of myosin per ml, 0.132 mg of actin per ml, 2 mM $MgCl_2$, 12.5 mM Tris–maleate buffer (pH 7.4) and 2 mM ATP.

The actomyosin formation at high ionic strength was established from the measured ATP sensitivity. The measurements were carried out in an Ostwald viscometer, using the method of BÁRÁNY *et al.*[13] under the following conditions: 1 mg of myosin per ml, 0.33 mg of actin per ml, 0.5 M KCl + 0.02 M borate buffer (pH 7.4) at 0°.

The superprecipitation was measured by the method of EBASHI[14], at 660 mμ with a Spectromon 360 spectrophotometer at room temperature. The conditions of the test were: 0.8 mg of myosin, 0.066 mg of actin per ml, 0.1 mM ATP, 2 mM $MgCl_2$, 5 mM Tris–maleate buffer (pH 7.4), *plus* KCl added in a concentration necessary to reach an ionic strength of 0.1. Superprecipitation was evaluated as the percentual increase in absorbance within 3 min after the addition of ATP. 100% being the absorbance before addition of ATP.

Protein content was measured by the biuret method of GORNALL, BARDAWILL AND DAVID[15].

RESULTS

Myosin was trinitrophenylated in both the presence and absence of ATP. The conditions of trinitrophenylation and the dependence of TNP-lysyl residue formation on TBS concentration are shown in Fig. 1. It is apparent that at the same TBS concentration more TNP-lysine groups are formed in the absence than in the presence of ATP. This observation is in agreement with the results of TONOMURA, YOSHIMURA AND ONISHI[6].

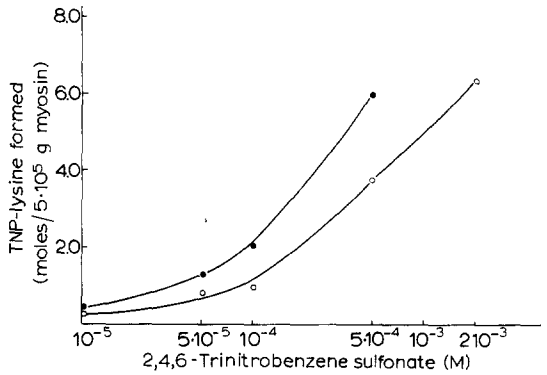

Fig. 1. Trinitrophenylation of myosin in the presence and absence of ATP. Trinitrophenylation in the presence (○) and in the absence (●) of ATP. Details of the treatment see MATERIALS AND METHODS. Abscissa: M TBS; ordinate: moles TNP-lysine formed per $5 \cdot 10^5$ g myosin.

The ATPase activity of trinitrophenylated myosin was measured alternatively in the presence of 5 mM Ca^{2+} or 5 mM EDTA or 2 mM Mg^{2+}. The effect of trinitrophenylation was least observed in the presence of 5 mM Ca^{2+} (Fig. 2). In the absence of ATP during the reaction with TBS, the ATPase activity, as measured at low ionic strength, was found to decrease, but it changed only slightly or not at all when ATP was present. Trinitrophenylation whether ATP was present or not during the reaction of myosin did not cause any appreciable change in the Ca^{2+}-activated ATPase measured at higher ionic strength.

The effect was much more striking when ATPase activity was measured in the presence of EDTA (Fig. 3). The ATPase activity, which in the absence of ATP sharply decreases and almost disappears as the number of TNP-lysine groups increases, shows a far less pronounced change if ATP is present during the incubation with TBS.

A similar effect, but in the opposite sense, was observed in the measured Mg^{2+}-activated ATPase (Fig. 4). The ATPase activity very much increases, particularly at higher ionic strength, with the number of TNP-lysine groups formed, if ATP is absent, but hardly changes if it is present during trinitrophenylation.

The characteristic properties of actomyosins formed from trinitrophenylated myosins were also measured (Table I). No definite change could be observed in the Mg^{2+}-activated ATPase of actomyosin on trinitrophenylating either in the presence or in the absence of ATP (Table I). The activation by actin, however, diminished with the increasing number of TNP-lysine groups, particularly, if the reaction was carried out in the absence of ATP. The decrease in percentual activation can be

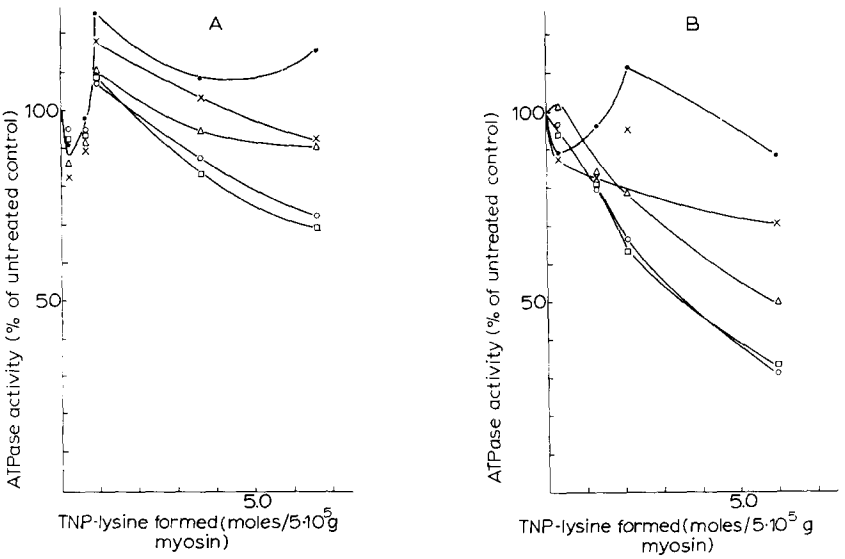

Fig. 2. Effect of trinitrophenylation on the Ca^{2+}-activated ATPase of myosin. Conditions of the enzymic assay: 5 mM $CaCl_2$, 4 mM ATP, 1 mg/ml of myosin, 25 mM Tris–maleate (pH 7.4) and 0.1 M (○); 0.2 M (□); 0.3 M (△); 0.4 M (×); 0.6 M KCl (●). A, ATP present; B, ATP absent during trinitrophenylation. Abscissa: moles TNP-lysine formed per $5 \cdot 10^5$ g myosin; ordinate: ATPase activity expressed in the per cent of the untreated control. Control (100%) activities: 0.1 M: 0.84; 0.2 M: 0.674; 0.3 M: 0.542; 0.4 M: 0.456 and 0.6 M KCl: 0.32 μmole P_i per mg myosin per min.

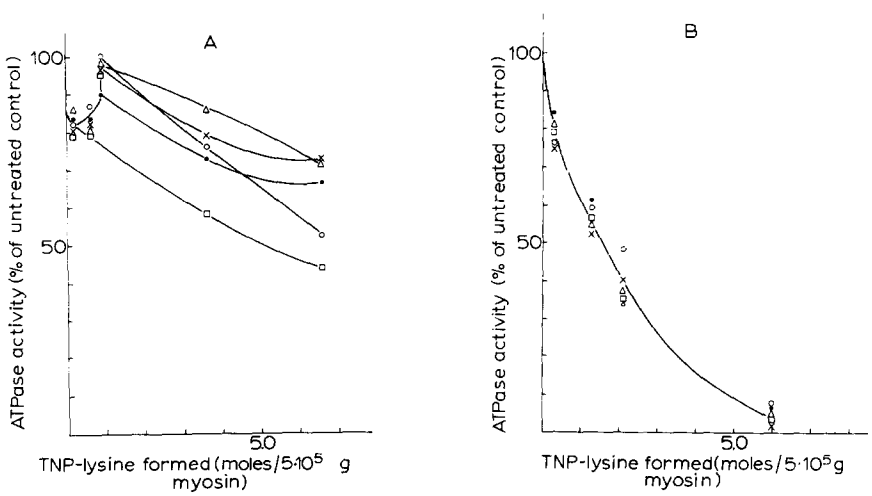

Fig. 3. Effect of trinitrophenylation on the EDTA-activated ATPase of myosin. Conditions of the enzymic assay: 5 mM EDTA, 4 mM ATP, 1 mg/ml of myosin, 25 mM Tris–maleate (pH 7.4) and 0.1 M (○); 0.2 M (□); 0.3 M (△); 0.4 M (×); 0.6 M KCl (●). A, ATP present; B, ATP absent during trinitrophenylation. Abscissa and ordinate see Fig. 2. Control (100%) activities: 0.1 M: 0.0508; 0.2 M: 0.169; 0.3 M: 0.448; 0.4 M: 0.728; and 0.6 M KCl: 1.24 μmoles P_i per mg myosin per min.

attributed to the fact that the Mg^{2+}-activated ATPase activity of myosin which substantially increases because of trinitrophenylation (see also Fig. 4A) cannot be activated any more by actin. This supports the view of TOKUYAMA AND TONOMURA that actin and trinitrophenylation affect the myosin ATPase in a similar way[5].

The superprecipitation of actomyosin reconstituted from trinitrophenylated

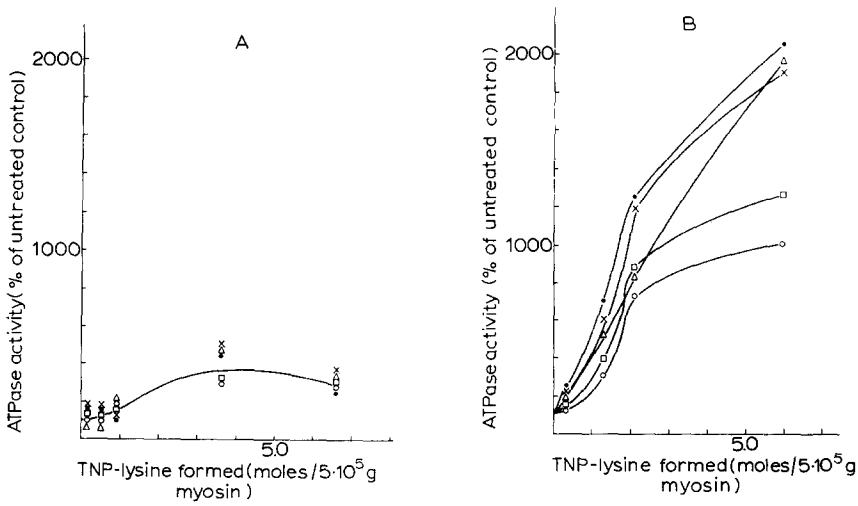

Fig. 4. Effect of trinitrophenylation on the Mg^{2+}-activated ATPase of myosin. Conditions of the enzymic assay: 2 mM $MgCl_2$, 4 mM ATP, 1 mg/ml of myosin, 25 mM Tris–maleate (pH 7.4) and 0.1 M (○); 0.2 M (□); 0.3 M (△); 0.4 M (×); 0.6 M KCl (●). A, ATP present; B, ATP absent during trinitrophenylation. Abscissa and ordinate see Fig. 2. Control (100%) activities: 0.1 M: 0.012; 0.2 M: 0.0104; 0.3 M: 0.0088; 0.4 M: 0.0076; and 0.6 M KCl: 0.0063 μmole P_i per mg myosin per min.

TABLE I

THE CHARACTERISTIC PROPERTIES OF ACTOMYOSIN RECONSTITUTED FROM TRINITROPHENYLATED MYOSIN

Detailed conditions for enzymic assays, determinations of ATP sensitivity and superprecipitation see MATERIALS AND METHODS. ATPase expressed as μmole P_i per mg myosin per min. Percentage activation by actin = actomyosin ATPase/myosin ATPase × 100. Conditions for myosin ATPase see Fig. 4A.

Trinitrophenylation	*moles TNP-lysine per mole myosin*	*Myosin ATPase*	*Actomyosin ATPase*	*Percentage activation by actin*	*ATP sensitivity*	*Superprecipitation*
Not treated	—	0.0121	0.112	925	157	445
ATP present	0.162	0.0114	0.109	955	160	420
	0.598	0.0111	0.085	766	170	370
	1.52	0.0230	0.116	505	—	480
	3.58	0.0302	0.121	400	144	420
	6.62	0.0301	0.115	383	119	472
ATP absent	0.313	0.0232	0.109	470	169	430
	1.27	0.0308	0.082	268	172	400
	2.24	0.0545	0.087	160	—	360
	5.98	0.0851	0.092	108	137	167

myosin did not change very much with the increasing number of TNP-lysine groups (Table I). An appreciable decrease was observed only on trinitrophenylation in the absence of ATP when 5.98 M lysine was trinitrophenylated per mole myosin. ATP sensitivity values (Table I) were rather high and did not significantly decrease with the increasing number of TNP-lysine groups formed when myosin reacted in the presence or absence of ATP.

DISCUSSION

The enzymic properties of myosin trinitrophenylated in the absence of ATP observed in the present experiment are essentially the same as those reported by TONOMURA and co-workers[2,4,5]. The high Mg^{2+}-activated and low EDTA-activated ATPase activity, as well as the inhibition of Ca^{2+}-activated ATPase only at low ionic strength, observed on the trinitrophenylation of myosin seem to indicate that the reacted lysyl residues have some role in the ATPase activity of myosin, but do not provide any information about the localization of these lysyl residues. Considering the fact that the binding of TBS to myosin is suppressed in the presence of ATP or pyrophosphate, TONOMURA, YOSHIMURA AND ONISHI[6] suggested that the lysyl residues specifically reacting with TBS are localized at the active site. STRACHER AND DREIZEN[7], on the other hand, think that ATP may bind to positively charged lysyl residues and compete with TBS for binding sites in the molecule. Our results obtained in the presence of ATP show that ATP specifically prevents the trinitrophenylation of the lysyl residues which have some role in the ATPase activity.

The problem is how these lysyl residues influence the myosin ATPase. The effect of the trinitrophenylation of the lysyl residues in question is very similar to that of actin on myosin ATPase if the activity is measured in the presence of Mg^{2+} at low ionic strength. The actomyosin ATPase is always the same and independent of whether the myosin component of the actomyosin was previously trinitrophenylated or not (*i.e.* the percentual activation by actin is less if actomyosin forms from trinitrophenylated myosin which has an already enhanced ATPase activity in the presence of Mg^{2+}).

Though this finding does not exclude the possibility that these $-NH_2$ groups are parts of the primary active enzymic site, it is improbable that a primary action of the same $-NH_2$ groups is influenced in the same way by complex formation with actin and covalent blocking by trinitrophenylation. On the other hand, such similar action of trinitrophenylation and actin seems plausible, if one assumes the $-NH_2$ groups to influence the ATPase activity by affecting the conformation. A conformation favorable to the ATPase activity in the presence of Mg^{2+} could result from the disappearance of the positive charges of the $-NH_2$ groups and be independent of whether these positive charges are removed either by complex formation with actin or by trinitrophenylation.

The trinitrophenylation of the $-NH_2$ groups of myosin which are protected against trinitrophenylation by ATP does not influence the formation and dissociation of actomyosin (assessed by ATP sensitivity measurements) and inhibits superprecipitation only at an extreme degree of trinitrophenylation. This shows that the $-NH_2$ groups do not play any appreciable part in the actin binding property of myosin. The above observations confirm the widely accepted hypothesis[16–19] that different,

independent sites must be held responsible for the actin binding and enzymic properties of myosin.

The EDTA-activated ATPase is markedly affected by trinitrophenylation, its level decreases even below that of Mg^{2+}-activated ATPase (Table II). The assumption that EDTA activates myosin ATPase by chelating the Mg^{2+} present in the solution[20–22] is consistent with the above results, if one accepts TONOMURA's hypothesis[23] that myosin ATPase is a double headed enzyme which splits ATP essentially in two possible ways. One of them predominates in the absence of Mg^{2+} (K^+- or EDTA-activated ATPase) the other in the presence of Mg^{2+} (Mg^{2+}-inhibited ATPase). Trinitrophenylation influences either of the processes, it was observed to inhibit the former and to enhance the latter.

TABLE II

EDTA-ACTIVATED ATPase TO Mg^{2+}-ACTIVATED ATPase RATIOS OF TRINITROPHENYLATED MYOSINS
Conditions for the enzymic assay see MATERIALS AND METHODS.

Trinitrophenylation	*moles TNP-lysine per mole myosin*	*EDTA-activated ATPase/Mg^{2+}-activated ATPase*				
		0.1 M KCl	*0.2 M KCl*	*0.3 M KCl*	*0.4 M KCl*	*0.6 M KCl*
Not treated	—	4.25	16.25	51.0	96.0	197.0
ATP present	0.162	3.83	11.3	38.0	69.0	128.0
	0.598	3.03	12.15	34.0	72.0	184.0
	0.916	3.16	11.2	30.6	65.5	129.0
	3.58	1.42	3.17	11.35	18.0	26.0
	6.62	0.715	1.93	13.3	23.5	28.1
ATP absent	0.313	2.58	0.73	2.19	3.44	7.25
	1.27	0.75	0.217	5.5	8.45	17.35
	2.06	0.317	0.79	2.01	3.34	6.00
	5.98	0.031	0.042	0.113	0.158	0.296

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REFERENCES

1 T. OKUYAMA AND K. SATAKE, *J. Biochem. Tokyo*, 47 (1960) 454.
2 S. KUBO, S. TOKURA AND Y. TONOMURA, *J. Biol. Chem.*, 235 (1960) 2835.
3 H. TOKUYAMA, S. KUBO AND Y. TONOMURA, *Biochem. Z.*, 345 (1966) 57.
4 S. KITAGAWA, J. YOSHIMURA AND Y. TONOMURA, *J. Biol. Chem.*, 236 (1961) 902.
5 H. TOKUYAMA AND Y. TONOMURA, *J. Biochem. Tokyo*, 62 (1967) 456.
6 Y. TONOMURA, J. YOSHIMURA AND T. ONISHI, *Biochim. Biophys. Acta*, 78 (1963) 698.
7 A. STRACHER AND P. DREIZEN, in D. R. SANADI, *Current Topics in Bioenergetics*, Vol. 1, Academic Press, New York, 1966, p. 153.
8 H. PORTZEHL, G. SCHRAMM AND H. H. WEBER, *Z. Naturforsch.*, 5b (1950) 61.
9 M. BÁRÁNY, N. A. BIRÓ, J. MOLNÁR AND F. B. STRAUB, *Acta Physiol. Hung.*, 5 (1954) 369.
10 W. DRABIKOWSKI AND J. GERGELY, *J. Biol. Chem.*, 238 (1963) 640.
11 W. F. H. M. MOMMAERTS, *J. Biol. Chem.*, 188 (1951) 559.
12 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
13 M. BÁRÁNY, B. NAGY, F. FINKELMAN AND A. CHRAMBACH, *J. Biol. Chem.*, 236 (1961) 2917.

14 S. EBASHI, *J. Biochem. Tokyo*, 50 (1961) 236.
15 A. GORNALL, C. J. BARDAWILL AND N. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
16 M. BÁRÁNY AND K. BÁRÁNY, *Biochim. Biophys. Acta*, 35 (1959) 293.
17 A. STRACHER, *Biochem. Biophys. Res. Commun.*, 13 (1963) 279.
18 S. V. PERRY AND J. COTTERILL, *Biochem. J.*, 92 (1964) 603.
19 A. MÜHLRAD, S. BOSKO AND N. A. BIRÓ, *Biochim. Biophys. Acta*, 132 (1967) 138.
20 A. MÜHLRAD, F. FÁBIÁN AND N. A. BIRÓ, *Biochim. Biophys. Acta*, 89 (1964) 186.
21 G. W. OFFER, *Biochim. Biophys. Acta*, 89 (1964) 566.
22 A. MARTONOSI AND H. MAYER, *J. Biol. Chem.*, 239 (1964) 640.
23 K. IMAMURA, M. TADA AND Y. TONOMURA, *J. Biochem. Tokyo*, 59 (1966) 280.