Biochem. J. (1967) **105**, 1235
Printed in Great Britain

The Effect of Tropomyosin on the Adenosine Triphosphatase Activity of Desensitized Actomyosin

BY M. C. SCHAUB,* S. V. PERRY AND D. J. HARTSHORNE†
Department of Biochemistry, University of Birmingham

(*Received* 19 *July* 1967)

1. Tropomyosin preparations of the Bailey type, and those prepared in the presence of dithiothreitol to prevent oxidation of protein thiol groups, inhibit the Ca^{2+}-activated adenosine triphosphatase (ATPase) of desensitized actomyosin by up to 60%. 2. The inhibitory activity of myofibrillar extracts and tropomyosin survives various agents known to denature proteins but to the action of which tropomyosin is unusually stable, namely heating at 100° and mild tryptic digestion. It is destroyed by prolonged treatment with trypsin. 3. The ethylenedioxybis-(ethyleneamino)tetra-acetic acid (EGTA)-sensitizing factor present in extracts of natural actomyosin and myofibrils could be selectively destroyed, leaving unchanged the inhibitory effect on the Ca^{2+}-activated ATPase. There was no correlation between the EGTA-sensitizing and the Ca^{2+}-activated inhibitory activities of tropomyosin prepared under different conditions. 4. Optimum inhibition was achieved when tropomyosin and the myosin of desensitized actomyosin were present in approximately equimolar proportions. Tropomyosin had no effect on the Ca^{2+}-activated ATPase of myosin measured under similar conditions. 5. Evidence is presented showing that the tropomyosin binds to desensitized actomyosin under the conditions in which the ATPase is inhibited.

In a previous paper (Schaub, Hartshorne & Perry, 1967*a*) the changes in enzymic properties that occurred when preparations of actomyosin extracted as the complex from myofibrils, 'natural' actomyosin (NAM‡), were treated with buffers of low ionic strength were described. The procedure removed about 10% of enzymically inactive protein and converted the actomyosin into a form the enzymic properties of which were comparable with those of actomyosin prepared from individually purified myosin and actin. This preparation was named 'desensitized' actomyosin (DAM) to indicate that, unlike NAM, its Mg^{2+}-activated ATPase was no longer sensitive to low concentrations of EGTA. It was also reported that the specific Ca^{2+}-activated ATPase of DAM was much greater than that of NAM. The sensitivity of the Mg^{2+}-activated ATPase to EGTA was restored and the Ca^{2+}-activated ATPase of DAM decreased to a level corresponding to that of NAM by addition of either the protein fraction removed during desensitization of NAM or a 'soluble protein' fraction extracted by prolonged treatment of myofibrils with 5mM-tris–hydrochloric acid buffer, pH 8·6 (Perry & Corsi, 1958).

In view of the role of Ca^{2+} in the presumed mode of action of the EGTA-sensitizing factor, and the parallel way the two changes in enzymic properties occurred during desensitization, it seemed likely that the desensitization of the Mg^{2+}-activated ATPase to EGTA and the increase in the Ca^{2+}-activated ATPase were related. This possibility was strengthened by the presence of tropomyosin in the low-ionic-strength extracts of NAM and of myofibrils (Perry, 1953; Perry & Corsi, 1958) and the suspected relationship between tropomyosin and the EGTA-sensitizing factor (Ebashi, 1963). On the other hand, the evidence available from the original study suggested that the two effects were due to protein factors of different stability (Schaub, Hartshorne & Perry, 1967*a*,*b*).

The present paper is concerned with the further investigation of the relationship between these two factors. It was demonstrated that tropomyosin preparations with no EGTA-sensitizing factor activity specifically inhibit the Ca^{2+}-activated ATPase of DAM. Also, the investigation provides evidence that the EGTA-sensitizing-factor system

* Present address: Institute of Experimental Gerontology, Basle, Switzerland.

† Present address: The Mellon Institute, Pittsburgh, Pa, U.S.A.

‡ Abbreviations: NAM and DAM, respectively 'natural' and 'desensitized' (see the text) actomyosin: ATPase, adenosine triphosphatase; EGTA, ethylenedioxybis-(ethyleneamino)tetra-acetic acid.

is not identical with tropomyosin alone (cf. Perry, Davies & Hayter, 1966; Watanabe & Staprans, 1966; Hartshorne & Mueller, 1967). Some aspects of these findings have been briefly reported elsewhere (Schaub *et al.* 1967*b*).

METHODS

Preparations of muscle proteins

NAM. NAM was extracted as described by Perry & Corsi (1958) from myofibrils prepared from the back and hind-leg muscles of New Zealand white rabbits by the method of Perry & Zydowo (1959). After two precipitations by the dilution method the actomyosin was normally washed twice with water by centrifugation for 15 min. at 1200*g* and finally stored suspended in 3–4 vol. of 2 mM-tris–HCl buffer, pH 7·6.

DAM. DAM was prepared from NAM as reported elsewhere (Schaub *et al.* 1967*a*) by washing it further at low ionic strength until its Mg^{2+}-activated ATPase was no longer inhibited by Ca^{2+}-chelating reagents. The preparations, stored in 2 mM-tris–HCl buffer, pH 7·6, were used within 3 weeks.

Low-ionic-strength extract from NAM. Extracts were obtained by centrifuging for 20 min. at 33000*g* suspensions of NAM kept in 2 mM-tris–HCl buffer, pH 7·6, for 1–2 days. The supernatant, containing the factors responsible for the observed changes of enzymic behaviour of DAM, was used directly.

'*Soluble protein*' *fraction.* This fraction (Perry, 1953; Perry & Corsi, 1958) was extracted by diluting a rabbit myofibrillar suspension (20–30 mg./ml.) in 0·1 M-KCl in 39 mM-borate buffer, pH 7·0, with 1 vol. of 5 mM-tris–HCl buffer, pH 8·6. The whole suspension was then dialysed against 20 vol. of the tris buffer for 4–5 days at 0° with daily changes of buffer. By centrifuging the viscous suspension for 40 min. at 100000*g* a clear supernatant was obtained that had similar effects on the ATPase activity of DAM to those obtained with the extracts made from NAM at low ionic strength. 'Soluble fraction' preparations from myofibrils of fowl and trout were prepared in a similar way.

Myosin. L-myosin was prepared by the standard method used in these Laboratories (Perry, 1955) with the modification that 10 mM-$Na_4P_2O_7$ and 1 mM-$MgCl_2$ were included in the extraction medium as described by Trayer & Perry (1966).

Actin. Actin was extracted from acetone-dried rabbit muscle fibre prepared by the method of Straub (1943) as modified by Leadbeater & Perry (1963). The fibre was extracted with water in the cold to minimize tropomyosin contamination (Drabikowski & Gergely, 1962). After isoelectric precipitation with 10 mM-sodium acetate buffer, pH 4·7, the actin was polymerized with 0·5 mM-$MgCl_2$ at pH 7 (Martonosi, 1962), sedimented at 100000*g* and dispersed in 10 mM-tris–HCl buffer, pH 7·6, with a hand homogenizer (Hartshorne, Perry & Schaub, 1967). In some cases after isoelectric precipitation the actin was taken through three or four cycles of depolymerization and polymerization as described by Johnson, Harris & Perry (1967). Both actin preparations were shown to be free from detectable amounts of tropomyosin.

Tropomyosin. Tropomyosin was prepared by the method of Bailey (1948) up to the crystallization stage and then twice isoelectrically precipitated. Some preparations were also subjected to ethanol–ether precipitation. For the preparation of tropomyosin possessing EGTA-sensitizing-factor activity, 0·5 mM-dithiothreitol was included in all media (Mueller, 1966*a*).

Estimation of protein concentration

The protein content of actomyosin preparations was determined by the biuret reaction (Layne, 1957), which was standardized by a micro-Kjeldahl procedure. Routine estimations of the concentration of the purified tropomyosin solutions were based on E_{280} measurements, assuming $E^{1\%}_{1cm.}$ 3·0 (Kay & Smillie, 1964).

Enzymic studies

These were carried out in 2 ml. at 25° as described by Perry (1960). The complete incubation system as indicated in the text, less ATP, was brought to 25° and the reaction started by the addition of ATP. Enzymic activities, unless otherwise indicated, were determined in 2·5 mM-tris–ATP in 25 mM-tris–HCl buffer, pH 7·6, containing $MgCl_2$ or $CaCl_2$ (2·5 mM). Specific activities were expressed as μg. of inorganic phosphorus released by 1 mg. of protein in 5 min. under these conditions. For determination of EGTA-sensitivity the Mg^{2+}-activated ATPase was measured in the presence and absence of 1 mM-EGTA, pH 7·6.

Chromatography

Commercial DEAE-cellulose (Whatman P/DE, 100 coarse) was used for chromatography carried out under the general conditions described by Perry & Zydowo (1959).

Reagents

Disodium ATP (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) was converted into tris–ATP by the method of Schwartz, Bachelard & McIlwain (1962). Specially purified tris (C. F. Boehringer und Soehne G.m.b.H.) was used in all experiments. EGTA was supplied by Fisons Ltd., Loughborough, Leics. All other reagents were of analytical grade, and distilled deionized water was used throughout.

RESULTS

Most of the studies reported here were carried out on the protein fraction isolated by prolonged extraction of myofibrils with 5 mM-tris–hydrochloric acid buffer, pH 8·6, previously called the 'soluble fraction' (Perry & Corsi, 1958). This extract has similar effects on the enzymic activity of DAM to those associated with the protein extracted from NAM during conversion into DAM and its study had obvious advantages because it could be more readily obtained in quantity. When 'soluble fractions' were tested on DAM at low ionic strength three distinct effects could be recognized.

(1) The Ca^{2+}-activated ATPase of actomyosin was inhibited by small amounts of 'soluble fraction' when the bivalent cation concentration was in the range 0–10 mM (Fig. 1).

(2) The sensitivity of the Mg^{2+}-activated ATPase

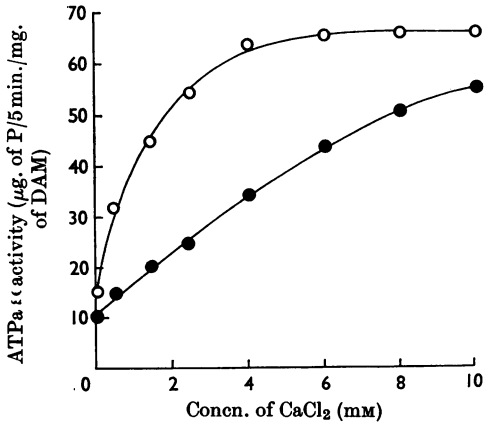

Fig. 1. Effect of 'soluble fraction' on the Ca^{2+}-activated ATPase of DAM. Where indicated, 0·2ml. of supernatant ($E^{1cm.}_{280}$ 0·88) in 10mM-tris–HCl buffer, pH 7·6, obtained after treatment of the 'soluble fraction' with 30%-saturated $(NH_4)_2SO_4$, was added to 0·94mg. of DAM. Standard incubation conditions were as given in the Methods section. ○, DAM; ●, DAM+'soluble fraction'.

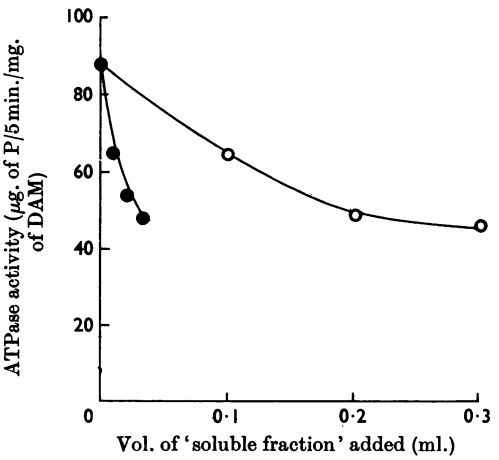

Fig. 2. Comparison of Ca^{2+}-activated ATPase inhibition and sensitization of the Mg^{2+}-activated ATPase to EGTA of DAM by the 'soluble fraction'. The medium contained 25mM-tris–HCl buffer, pH 7·6, 2·5mM-ATP, 0·57mg. of DAM and 'soluble fraction' ($E^{1cm.}_{280}$ 3·0); other additions were: ○, 2·5mM-$MgCl_2$+1mM-EGTA; ●, 2·5mM-$CaCl_2$.

to low concentrations of EGTA was restored (Perry *et al.* 1966). The amounts of 'soluble fraction' required to restore sensitivity to EGTA were usually significantly greater than those needed to produce an effect comparable with that obtained on the Ca^{2+}-activated ATPase (Fig. 2).

(3) Many, although not all, 'soluble fractions'

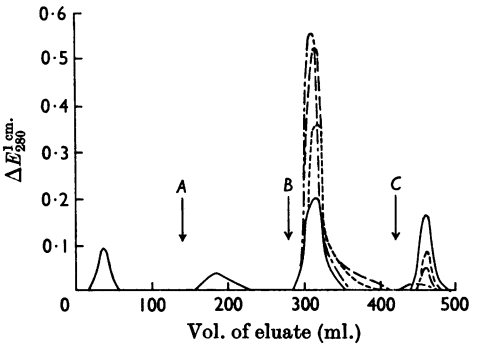

Fig. 3. Chromatography on DEAE-cellulose of tropomyosin and low-ionic-strength extracts of NAM. Samples (7–11ml.) of $E^{1cm.}_{280}$ 1–2 were applied to a column (12cm.×2cm.) of DEAE-cellulose equilibrated against 0·1M-KCl in 10mM-tris–HCl buffer, pH 7·6. Steps to 0·2M-KCl, 0·4M-KCl and 2M-KCl, all in 10mM-tris–HCl buffer, pH 7·6, were applied at points *A*, *B* and *C* respectively. ——, NAM extract at *I* 0·002; ·····, supernatant of NAM extract after 33%-saturated $(NH_4)_2SO_4$ fractionation; —·—, Bailey-type tropomyosin; – – –, tropomyosin prepared and eluted in 0·5mM-dithiothreitol.

produced stimulation of the Mg^{2+}-activated ATPase of DAM. This activation effect, which at the highest concentrations used (1–2mg. of protein/assay) amounted to 10–40%, resembled that obtained with tropomyosin preparations reported by Katz (1964, 1966) and Mueller (1966*b*). It had, however, no apparent relationship to the inhibitory effect on the Ca^{2+}-activated ATPase or to the EGTA-sensitizing factor.

Investigation was directed particularly to the factors responsible for effects (1) and (2); these factors were both non-diffusible and could be precipitated by 30–70%-saturated ammonium sulphate.

Evidence for identity of tropomyosin and the factor inhibiting the Ca^{2+}*-activated ATPase.* When the 'soluble fraction' or the protein separated from NAM on desensitization was brought to 50% saturation with ammonium sulphate the specific inhibitory activity on the Ca^{2+}-activated ATPase was increased five- to ten-fold in the supernatant, where the bulk of the tropomyosin, which is invariably present in these extracts, would be expected to be found. Non-identity of tropomyosin with the EGTA-sensitizing factor was implied by the fact that most of the latter factor was found in the precipitate under these conditions.

Chromatography on DEAE-cellulose of low-ionic-strength extracts of myofibrils or NAM under the conditions described elsewhere (Perry *et al.* 1966) resolved the material into four fractions eluted successively at 0·1M-, 0·2M-, 0·4M- and

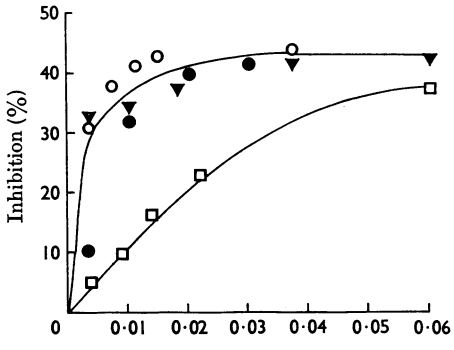

Fig. 4. Effect of fractions obtained in DEAE-cellulose chromatography of NAM extracts and tropomyosin on the Ca^{2+}-activated ATPase of DAM. In all cases fractions were eluted between 0·2M- and 0·4M-KCl in 10mM-tris–HCl buffer as in Fig. 3. Standard assay conditions were used with 0·78mg. of DAM. Eluates were tested at E_{280} 1·0. □, NAM extract at I 0·002; ○, supernatant of NAM extract after precipitation with 33%-saturated $(NH_4)_2SO_4$; ●, Bailey-type tropomyosin; ▲, tropomyosin prepared and eluted in the presence of 0·5mM-dithiothreitol.

2·0M-potassium chloride in 10mM-tris–hydrochloric acid buffer, pH7·6 (Fig. 3). The peak eluted by 0·4M-potassium chloride in 10mM-tris–hydrochloric acid buffer, pH7·6, was consistently found to be the only one that produced inhibition of the Ca^{2+}-activated ATPase of DAM. Tropomyosin, prepared either as described by Bailey (1948) or in the presence of dithiothreitol as described by Mueller (1966*a*) and reprecipitated isoelectrically twice, was eluted sharply by the step to 0·4M-potassium chloride in 10mM-tris–hydrochloric acid buffer, pH7·6 (Perry & Zydowo, 1959) (Fig. 3). In neither type of tropomyosin preparation was material eluted at lower ionic strength, whereas, with extracts from NAM, peaks were eluted at 0·1M- and 0·2M-potassium chloride in 10mM-tris–hydrochloric acid buffer. The latter two peaks were no longer obtained if the protein precipitated by 33%-saturated ammonium sulphate was removed before application of NAM extracts to the DEAE-cellulose column. The fractions eluted by 0·4M-potassium chloride in 10mM-tris–hydrochloric acid buffer, pH7·6, from tropomyosin preparations and from NAM extracts possessed comparable activity as inhibitor of the Ca^{2+}-activated ATPase when compared on an E_{280} basis, and as would be expected this fraction was much more effective than the original NAM extract (Fig. 4).

No difference in inhibitory activity was found between standard Bailey-type tropomyosin and that prepared in the presence of thiol compounds (Mueller, 1966*a*). For this reason, unless otherwise stated, Bailey-type tropomyosin was used for most of the studies described here. The tropomyosin preparations sedimented as a single symmetrical peak both in 1M-potassium chloride in 5mM-tris–hydrochloric acid buffer, pH7·6, and in 1M-potassium chloride in 0·1N-potassium hydroxide, pH13.

In all cases if tropomyosin preparations were precipitated with 50%-saturated ammonium sulphate the specific inhibitory activity was similar in supernatant and precipitate. In those tropomyosin preparations prepared to preserve EGTA-sensitizing activity (Mueller, 1966*a*) the EGTA-sensitizing activity was likewise not significantly fractionated by 50%-saturated ammonium sulphate, in striking contrast with the results obtained on the 'soluble fraction'.

It has been reported elsewhere (Schaub *et al.* 1967*a*,*b*) that the factor responsible for the inhibitory effect on the Ca^{2+}-activated ATPase was less sensitive to tryptic digestion than that responsible for sensitizing the Mg^{2+}-activated ATPase to EGTA. After prolonged digestion of the 'soluble fraction' with trypsin at 25° its inhibitory action on the Ca^{2+}-activated ATPase was also destroyed.

After heating tropomyosin for 10min. at 100° at pH values in the range 2·7–11 and subsequently adjusting the pH to 7·0 the inhibitory activity was not lost. Likewise inhibitory activity was unimpaired when tropomyosin was precipitated with 5% (w/v) trichloroacetic acid, redissolved by adjusting to pH7 and the solution equilibrated by dialysis against 10mM-tris–hydrochloric acid buffer, pH7·6. Treatment with 4·2M-guanidine hydrochloride for 30min. and subsequent dialysis against 10mM-tris–hydrochloric acid buffer, pH7·6, to remove the reagent also did not significantly impair the inhibitory activity of tropomyosin. Under these conditions tropomyosin has been shown to regain up to 80% of its original helicity (Noelken & Holtzer, 1964).

Although tropomyosin preparations consistently inhibited the Ca^{2+}-activated ATPase of DAM, their effect on the Mg^{2+}-activated ATPase was somewhat variable. Preparations that had been less vigorously purified usually stimulated the Mg^{2+}-activated ATPase, and much higher concentrations of tropomyosin were required to produce this effect compared with that obtained on the Ca^{2+}-activated ATPase (Fig. 5). The effect was obtained whether or not the tropomyosin preparations possessed EGTA-sensitizing activity. It survived ammonium sulphate precipitation, but was diminished if the number of isoelectric precipitations was increased and appeared to be lost when tropomyosin preparations were frozen, freeze-dried or stored at −20° for long periods. The inhibitory effect of tropomyosin

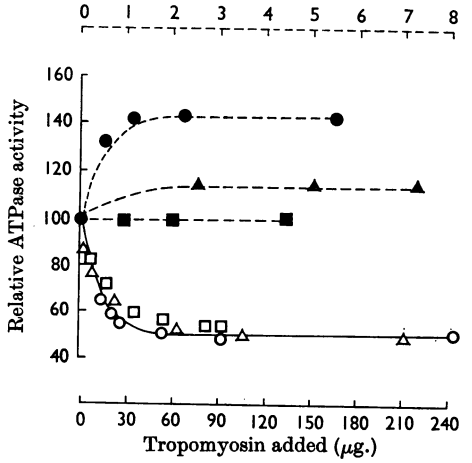

Fig. 5. Effect of various tropomyosin preparations on the Mg^{2+}- and Ca^{2+}-activated ATPases of DAM. The medium contained 25mM-tris–HCl buffer, pH 7·6, 2·5mM-ATP and about 0·7mg. of DAM. ●, ▲ and ■, 2·5mM-$MgCl_2$ present; ○, △ and □, 2·5mM-$CaCl_2$ present. ● and ○, Bailey-type tropomyosin, precipitated once; ▲ and △, Bailey-type tropomyosin, precipitated three times; ■ and □, tropomyosin frozen and thawed twice. It should be noted that the upper tropomyosin scale refers to the Mg^{2+}-activated ATPase.

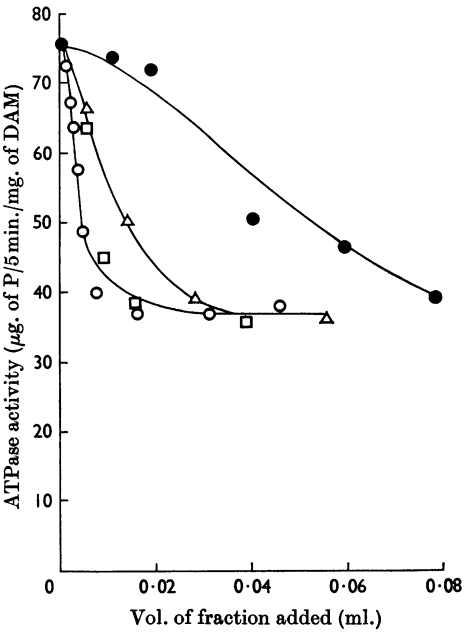

Fig. 6. Inhibitory action on the Ca^{2+}-activated ATPase of DAM of 'soluble fractions' of rabbit, trout and fowl myofibrils. Standard conditions were used with 2·5mM-$CaCl_2$ and 0·7mg. of DAM. Fractionated 'soluble fraction' is the supernatant after precipitation with 50%-saturated $(NH_4)_2SO_4$ in each case. Fractions were adjusted to E_{280} 1·0 before testing. ●, 'Soluble fraction' from rabbit; ○, fractionated 'soluble fraction' from rabbit; □, fractionated 'soluble fraction' from fowl; △, fractionated 'soluble fraction' from trout.

on the Ca^{2+}-activated ATPase was not affected by any of these procedures. The activating effect of tropomyosin on the Mg^{2+}-activated ATPase was considered to be independent of the other effects studied and was not further investigated.

Dialysed sarcoplasm had no influence on the Ca^{2+}-activated ATPase of DAM, nor did the factor isolated from myofibrils that inhibits the Mg^{2+}-activated ATPase of DAM (Hartshorne, Perry & Davies, 1966; Hartshorne *et al.* 1967).

The solubility, stability and chromatographic properties strongly suggested that tropomyosin was the factor responsible for the inhibitory action on the Ca^{2+}-activated ATPase of DAM. Also, the effect was general to skeletal muscle and not simply a feature of rabbit muscle, on the protein systems of which most of the studies were carried out. Similar results were obtained with 'soluble fraction' prepared from the myofibrils of fowl and trout skeletal muscle and tested on DAM prepared from rabbit skeletal muscle (Fig. 6).

Effect of purified tropomyosin preparations on the Ca^{2+}*-activated ATPase of DAM.* Tropomyosin prepared by the method of Bailey (1948), subjected to three isoelectric precipitations and which possessed no EGTA-sensitizing activity, inhibited the Ca^{2+}-activated ATPase of DAM up to 60% at low ionic strengths. In the standard assay conditions (see the Methods section) 1·5μg. of tropomyosin produced a significant inhibition (more than 10%) of the Ca^{2+}-activated ATPase of DAM. As the concentration of tropomyosin increased inhibition rose to a maximum and then flattened off at this value, even though the tropomyosin concentration was increased well above the minimum 40–50μg. required to give the maximum effect (Fig. 7). The maximum inhibition obtained was usually 40–60%, the precise value obtained with a given tropomyosin preparation depending on the DAM preparation used. The concentration of tropomyosin at which the ATPase became relatively insensitive to further amounts of tropomyosin corresponded in all cases to an approximately equimolar relationship between it and myosin.

The superprecipitation behaviour of DAM with Ca^{2+} as activating ion in the presence of tropomyosin was retarded compared with control preparations without tropomyosin, as would be expected in view of the close correlation between enzymic activity and superprecipitation under these conditions.

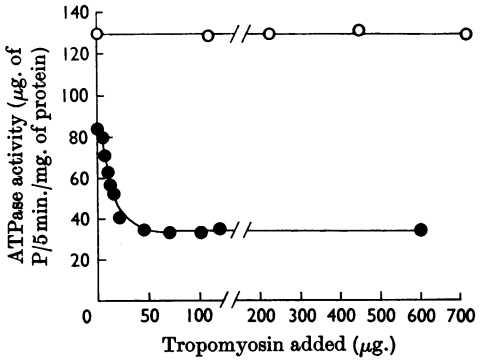

Fig. 7. Effect of Bailey-type tropomyosin on the Ca^{2+}-activated ATPase of myosin and DAM. Standard assay conditions were used with 2·5mM-$CaCl_2$. ○, 0·24mg. of myosin; ●, 0·54mg. of DAM.

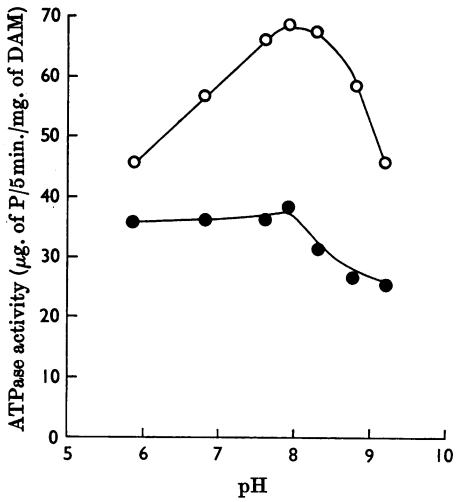

Fig. 8. Effect of pH on the inhibitory action of tropomyosin on the Ca^{2+}-activated ATPase of DAM. Standard assay conditions were used with 0·61mg. of DAM. ●, 555μg. of tropomyosin; ○, no tropomyosin.

The inhibitory effect of tropomyosin was apparent over the pH range 6–9 with the maximal effect at pH values about 8·0 (Fig. 8). The effect was sensitive to ionic strength and decreased progressively with increasing concentrations of potassium chloride. It was completely abolished when 150mM-potassium chloride was present in the assay (Fig. 9).

The effect was apparent over the range 0–10mM-calcium chloride when the ATP concentration was kept constant at 2·5mM. If the calcium

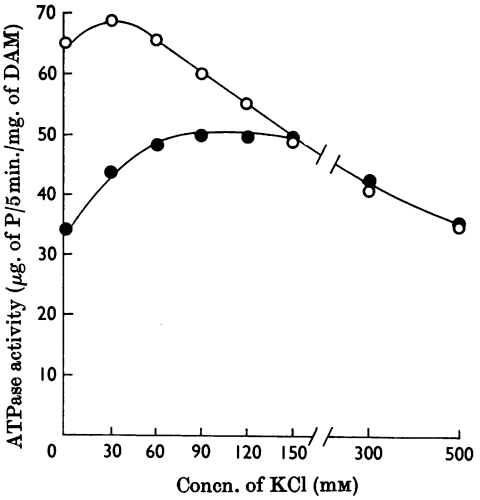

Fig. 9. Effect of KCl on the inhibitory action of tropomyosin on the Ca^{2+}-activated ATPase of DAM. Standard assay conditions were used with 0·88mg. of DAM. ●, 555μg. of tropomyosin; ○, no tropomyosin.

chloride concentration was kept constant at 2·5mM and the substrate concentration varied, the actomyosin ATPase was maximally inhibited at approximately equimolar concentrations of substrate and activator. At substrate concentrations higher than 7·5mM the effect disappeared.

In contrast with the effect with DAM no inhibitory effect on the Ca^{2+}-activated ATPase of myosin could be demonstrated (Fig. 7). Unless special precautions were taken to remove tropomyosin from actin preparations, addition of increasing amounts of the latter protein to myosin produced a slight decrease in the Ca^{2+}-activated ATPase. This did not occur when actin carefully freed of tropomyosin was added to myosin or DAM (two to three times as much actin as DAM) and suggests that the decrease in Ca^{2+}-activated ATPase obtained on addition of actin to myosin previously reported (Perry, 1967) is probably due to tropomyosin contamination.

Interaction of tropomyosin and actomyosin. The conditions of low ionic strength required to remove tropomyosin from NAM suggest certain unusual features of the interaction between these proteins, for electrostatic interaction would be expected to be maximal under these conditions. The conditions for interaction of tropomyosin with DAM are shown in Table 1, which illustrates that neither tropomyosin of the Bailey type nor preparations purified in the presence of a thiol compound (to keep the thiol groups of the protein intact) can be removed from DAM by washing with 50–80mM-tris-

Table 1. *Binding ability of Bailey-type and dithiothreitol-treated tropomyosin to DAM*

Bailey-type tropomyosin was used after three isoelectric precipitations, dithiothreitol-treated tropomyosin after two isoelectric precipitations in the presence of 0·5 mM-dithiothreitol. Solutions were stored at −20°. After the addition of tropomyosin to 10 ml. of DAM (6·46 mg./ml.) the samples were brought to 15 ml. and to the appropriate buffer concentrations. The samples were then centrifuged for 15 min. at 33000*g*, the supernatants discarded and the sediments resuspended in the original volume of buffer. This procedure was repeated twice. Enzymic estimations were performed before and after washing under standard assay conditions on samples and corresponding controls without addition of tropomyosin. The specific activities were based on biuret estimations before and after the washing procedure.

Type of tropomyosin added	Inhibition of Ca^{2+}-activated ATPase of DAM with tropomyosin added in excess (%)	Concn. of tris–HCl, pH 7·6, used for washings (mM)	Inhibition after washings (%)
Bailey-type	37	water	7
Bailey-type	29	5	16
Bailey-type	35	50	33
Dithiothreitol-treated	32	70	36
Bailey-type	30	80	31
Dithiothreitol-treated	39	70+0·1 mM-EDTA	36

Table 2. *Effect of tropomyosin on the* Ca^{2+}*-activated ATPase of DAM in the presence of actin in excess*

Standard assay conditions were as given in the Methods section. Tropomyosin (55·5 μg. or just enough to cause maximal inhibition of the 0·608 mg. of DAM used per assay) was first mixed with appropriate amounts of actin in the reaction medium less DAM and ATP, then 0·608 mg. of DAM was added and finally the reaction was started by adding the substrate.

Actin added per assay (mg.)	Tropomyosin as % of the actotropomyosin mixture added	Inhibition of Ca^{2+}-activated ATPase of DAM (%)
0	100	50
0·29	12·5	36
0·58	6·6	15–25
0·87	4·2	15–25
1·26	3·6	10–20
2·79	2·0	10–20
4·63	1·2	0–5

hydrochloric acid buffer, pH 7·6. as the inhibitory action of the tropomyosin on the Ca^{2+}-activated ATPase survives these procedures. Extraction with 70 mM-tris–hydrochloric acid buffer containing EDTA (0·1 mM) likewise did not remove the inhibitory effect of the tropomyosin on DAM, implying that traces of bivalent cations were not required for the interaction. If the buffer concentration was decreased below 5 mM the tropomyosin was readily removed by three washings, as judged by the loss of the inhibitory effect.

From evidence available on interaction of tropomyosin with actin (Laki, Maruyama & Kominz, 1962; Martonosi, 1962; Maruyama, 1964) it might be expected that excess of actin would decrease the inhibitory action of tropomyosin by competing with actomyosin for it. Table 2 shows that this is indeed the case, for when F-actin is added to DAM the inhibitory effect of a given amount of tropomyosin is diminished.

DISCUSSION

The effect of tropomyosin on the enzymic activity of actomyosin adds the purified form of this protein to the growing list of protein factors of myofibrillar origin that have been reported to modify in some way the enzymic behaviour of actomyosin systems. Up to the moment effects on the ATPase activity of actomyosin due to the following protein factors have been distinguished.

(1) The EGTA-sensitizing factor, which sensitizes the Mg^{2+}-activated ATPase of actomyosin to Ca^{2+}-chelating agents.

(2) The inhibitory factor, which produces inhibition of the Mg^{2+}-activated ATPase of desensitized actomyosin (Hartshorne *et al.* 1966).

(3) Tropomyosin, which produces inhibition of Ca^{2+}-activated ATPase of desensitized actomyosin.

(4) The factor stimulating the Mg^{2+}-activated ATPase of actomyosin, which is associated with certain tropomyosin preparations but not identical with tropomyosin.

So far as factors (1), (2) and (3) are concerned there are suggestions that the observed effects may be different aspects of the complexity of the mechanism involved in the control of the myofibrillar ATPase. Effect (4), although reported by several groups (Katz, 1964, 1966; Mueller, 1966*b*; Hartshorne *et al.* 1967), has not been systematically studied and there is as yet no indication that it is related to the other effects listed.

It is noteworthy that effects due to factors (2) and (3) can only be demonstrated with actomyosin that has been purified so that its Mg^{2+}-activated ATPase is no longer sensitive to low concentrations of EGTA. Effects (1) and (4) have been associated with tropomyosin preparations, but the work described here indicates that effect (3) is the only one that appears to be a property of this protein alone. The survival of the inhibitory effect under rather extreme conditions to which tropomyosin is unusually stable compared with most proteins is good evidence for ascribing this effect on the Ca^{2+}-activated ATPase to tropomyosin. Additional support for this view is given by the small amounts that are effective and by the stoicheiometry of the effect.

The experimental findings indicating that tropomyosin is not identical with the EGTA-sensitizing factor support other evidence of non-identity (Perry *et al.* 1966; Ebashi & Kodama, 1966; Watanabe & Staprans, 1966). They do not, however, exclude the possibility that tropomyosin may be associated with another protein factor, troponin, which Ebashi & Kodama (1966) have reported as essential in addition to tropomyosin for EGTA to inhibit the superprecipitation of trypsin-treated actomyosin preparations. Evidence has also been obtained (S. V. Perry, M. C. Schaub & V. Darby, unpublished work) that tropomyosin enhances the sensitization of the Mg^{2+}-activated ATPase of DAM produced by a tropomyosin-free protein fraction obtained by DEAE-cellulose chromatography of a myofibrillar extract (Perry *et al.* 1966). In the presence of Bailey-type tropomyosin alone the system is completely insensitive to EGTA.

Thus the evidence suggests that a complex system of which tropomyosin is a component is required for EGTA inhibition of both the superprecipitation and the ATPase activities of actomyosin. The inhibitory effect described here is the first report of a biochemical activity for tropomyosin that appears to be a property of purified tropomyosin itself rather than in association with other protein fractions. It is therefore attractive to suppose that the inhibitory effect is in some way related to the role of tropomyosin in the EGTA-sensitizing system. It should be stressed, however, that the tropomyosin effect is exhibited on the Ca^{2+}-activated ATPase, whereas the EGTA-sensitivity is a property of the Mg^{2+}-activated system. Nevertheless, EGTA-sensitivity implies a role for Ca^{2+}, and if tropomyosin was involved its role might be reflected by its effect on the Ca^{2+}-activated ATPase of actomyosin. It is, however, unlikely that tropomyosin inhibits the ATPase through lowering the Ca^{2+} concentration by simply binding the bivalent cation. Under the conditions of assay used for inhibition 0·5 μM-tropomyosin gave maximal effect in a system containing 2·5 mM-calcium chloride, i.e. approx. 5000 Ca^{2+} ions/mol. of tropomyosin.

In view of the stoicheiometry of the effect it is much more likely that the tropomyosin exerts its action at the active centres of the myosin molecule, although the effect cannot be simple as it is not shown on myosin alone but only when actin is also present. Pertinent to these findings is the report of Kominz & Maruyama (1967), who found by direct estimation that 'native' tropomyosin was not bound to myosin but to actomyosin at the level of more than 1 mol./mol. of actin present. The enzymic studies imply that in some way the tropomyosin is involved in the interaction of actin with the active centres of myosin, and are strong support for the view that the property of tropomyosin described here is of fundamental significance in the biological function of the myofibril. The tropomyosin effect is sensitive to ionic strength and pH in a similar way to the Mg^{2+}-activated ATPase of actomyosin, implying that in addition to the requirement for actin both phenomena may involve other common features. When the events *in situ* are considered it is perhaps significant that the usually accepted values for the protein composition suggest that there are about 1–2 mol. of tropomyosin/mol. of myosin in the myofibril, i.e. enough under optimum conditions to saturate the system and produce the maximal effect on the Ca^{2+}-activated ATPase.

M.C.S. was supported by the Swiss Academy of Medical Sciences.

REFERENCES

Bailey, K. (1948). *Biochem. J.* **43**, 271.
Drabikowski, W. & Gergely, J. (1962). *J. biol. Chem.* **237**, 3412.
Ebashi, S. (1963). *Nature, Lond.*, **200**, 1010.
Ebashi, S. & Kodama, A. (1966). *J. Biochem., Tokyo*, **59**, 425.
Hartshorne, D. J. & Mueller, H. (1967). *J. biol. Chem.* **242**, 3089.
Hartshorne, D. J., Perry, S. V. & Davies, V. (1966). *Nature, Lond.*, **209**, 1352.
Hartshorne, D. J., Perry, S. V. & Schaub, M. C. (1967). *Biochem. J.* **104**, 907.
Johnson, P., Harris, C. I. & Perry, S. V. (1967). *Biochem. J.* **105**, 361.
Katz, A. M. (1964). *J. biol. Chem.* **239**, 3304.
Katz, A. M. (1966). *J. biol. Chem.* **241**, 1522.
Kay, C. M. & Smillie, L. B. (1964). In *Biochemistry of Muscle Contraction*, p. 379. Ed. by Gergely, J. London: J. and A. Churchill Ltd.
Kominz, D. R. & Maruyama, K. (1967). *J. Biochem., Tokyo*, **61**, 269.
Laki, K., Maruyama, K. & Kominz, D. R. (1962). *Arch. Biochem. Biophys.* **98**, 323.

Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 450. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Leadbeater, L. & Perry, S. V. (1963). *Biochem. J.* **87**, 233.
Martonosi, A. (1962). *J. biol. Chem.* **237**, 2795.
Maruyama, K. (1964). *Arch. Biochem. Biophys.* **105**, 142.
Mueller, H. (1966*a*). *Nature, Lond.*, **209**, 1128.
Mueller, H. (1966*b*). *Biochem. Z.* **345**, 300.
Noelken, M. & Holtzer, A. M. (1964). In *Biochemistry of Muscle Contraction*, p. 374. Ed. by Gergely, J. London: J. and A. Churchill Ltd.
Perry, S. V. (1953). *Biochem. J.* **55**, 114.
Perry, S. V. (1955). In *Methods in Enzymology*, vol. 2, p. 582. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Perry, S. V. (1960). *Biochem. J.* **74**, 94.
Perry, S. V. (1967). *Progr. Biophys. molec. Biol.* **17**, 327.
Perry, S. V. & Corsi, A. (1958). *Biochem. J.* **68**, 5.
Perry, S. V., Davies, V. & Hayter, D. (1966). *Biochem. J.* **99**, 1c.
Perry, S. V. & Zydowo, M. (1959). *Biochem. J.* **71**, 220.
Schaub, M. C., Hartshorne, D. J. & Perry, S. V. (1967*a*). *Biochem. J.* **104**, 263.
Schaub, M. C., Hartshorne, D. J. & Perry, S. V. (1967*b*). *Nature, Lond.*, **215**, 635.
Schwartz, A., Bachelard, H. S. & McIlwain, H. (1962). *Biochem. J.* **84**, 626.
Straub, F. B. (1943). *Stud. Inst. med. Chem. Univ. Szeged*, **3**, 23.
Trayer, I. P. & Perry, S. V. (1966). *Biochem. Z.* **345**, 87.
Watanabe, S. & Staprans, I. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 572.