Biochem. J. (1971) **123**, 367–377
Printed in Great Britain

The Regulatory Proteins of the Myofibril

CHARACTERIZATION AND PROPERTIES OF THE INHIBITORY FACTOR (TROPONIN B)

BY M. C. SCHAUB* AND S. V. PERRY
Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

(*Received* 28 *January* 1971)

1. Gel-filtration results indicate that the major component of inhibitory-factor preparations isolated by dissociation of the troponin complex consisted of a protein of subunit weight 23000 daltons. By the same procedure a molecular weight of 18000 was obtained for the calcium-sensitizing factor. 2. The inhibitory factor is specific for the actomyosin type of ATPase and ITPase. It is effective on desensitized actomyosin, natural actomyosin and intact myofibrils. 3. For inhibition, the actomyosin ATPase must be stimulated by Mg^{2+}, Ca^{2+} or Mn^{2+}. The Co^{2+}-, Cd^{2+}- or Zn^{2+}-stimulated ATPases are not affected. 4. Biological activity is stable to treatment with dissociating agents, heat, pH 11, pH 1 and carboxymethylation. 5. Increasing amounts of actin, but not myosin or tropomyosin, progressively neutralize the inhibitory activity when added to desensitized actomyosin or myofibrils.

During earlier studies (Perry, Davies & Hayter, 1966) it was noted that under certain conditions extracts from myofibrils developed the ability to inhibit the Mg^{2+}-stimulated ATPase† of desensitized actomyosin in the absence of EGTA. Investigation of this effect led to the isolation from myofibrils of a protein factor that inhibited both the Mg^{2+}-stimulated ATPase and the superprecipitation of desensitized actomyosin in the absence of EGTA and was not identical with any of the then known muscle proteins (Hartshorne, Perry & Davies, 1966; Hartshorne, Perry & Schaub, 1967). Although the preparations were obviously far from homogeneous the inhibitory factor was highly specific for the Mg^{2+}-stimulated ATPase of desensitized actomyosin and the suggestion was made (Perry, 1967; Hartshorne *et al.* 1967) that it was derived from the troponin complex.

Subsequently Hartshorne & Mueller (1968) and Hartshorne, Theiner & Mueller (1969) reported the splitting of troponin by treatment with 0.1M-hydrochloric acid in potassium chloride. Independently in this laboratory fractionation of troponin was carried out by chromatography in the presence of dissociating agents to give two proteins, which were named the calcium-sensitizing factor and the inhibitory factor respectively. The latter fraction was clearly a much purer preparation of the inhibitory-protein factor described earlier (Hartshorne *et al.* 1966) and similar in properties to the troponin B prepared by Hartshorne *et al.* (1969). Although initially the latter authors did not identify troponin B with the inhibitory factor, Hartshorne (1970) has acknowledged this fact. The present paper is concerned with the further characterization and study of the enzymic properties of the inhibitory factor.

* Present address: The Institute of Pharmacology, University of Zurich, Zurich, Switzerland.

† Abbreviations: ATPase, adenosine triphosphatase; ITPase, inosine triphosphatase; SE-Sephadex, sulphoethyl-Sephadex; EGTA, ethanedioxybis(ethylamine)-tetra-acetate.

METHODS

Myofibrils. Myofibrils were prepared from longissimus dorsi and back leg muscles of the rabbit by the method of Perry & Zydowo (1959). Washed suspensions were used directly for extraction of actomyosin. Myofibrils which were used for enzymic tests were washed twice with water containing 15mM-2-mercaptoethanol and stored in 1mM-dithiothreitol–10mM-KCl–10mM-tris-HCl buffer, pH 7.4.

Natural actomyosin. This was prepared from washed myofibrils by a slight modification of the procedure of Perry & Corsi (1958). The myofibril suspension was blended for a few seconds in a Waring blender with 7 vol. of Weber's solution (0.6M-KCl–10mM-Na_2CO_3–40mM-$NaHCO_3$) containing 15mM-2-mercaptoethanol. After standing for 10min the suspension was centrifuged for 20min at 1200*g*. The turbid supernatant was slowly diluted 1:10 with water containing 15mM-2-mercaptoethanol and after 30–60min the sediment of normal actomyosin was collected by centrifugation. It was washed twice with 50mM-KCl containing 15mM-2-mercaptoethanol and finally suspended in 10mM-tris–HCl buffer, pH 7.4.

Desensitized actomyosin. After the initial preparation of normal actomyosin outlined above the precipitate was washed twice with water (rather than 50mM-KCl) containing 15mM-2-mercaptoethanol, and desensitized actomyosin prepared essentially as described earlier (Schaub, Hartshorne & Perry, 1967). Average Mg^{2+}-stimulated ATPase activity under standard conditions was 77.2± 11.0μg of P/5min per mg of desensitized actomyosin.

Troponin complex. The starting material for troponin preparation was a low-ionic-strength extract of myofibrils possessing relaxing-protein-system activity (Schaub & Perry, 1969). This extract was prepared by treating washed myofibrils in 0.1M-KCl–39mM-sodium borate buffer, pH7.0, with 7vol. of Weber's solution as for the preparation of normal actomyosin. After 30min the whole extract was diluted with 6–7vol. of water containing 15mM-2-mercaptoethanol and left for 1h. The precipitate was collected by centrifugation, washed twice with 15mM-2-mercaptoethanol and dialysed with one change of dialysis fluid for 24h against 10vol. of 15mM-2-mercaptoethanol–2mM-tris–HCl buffer, pH7.4. The viscous suspension was diluted with 1vol. of dialysis fluid, centrifuged for 30–40min at 55000*g* and the clear viscous supernatant collected. The yield could be increased by resuspending the sediment in dialysis fluid and repeating the centrifugation. The supernatant was used for preparation of the troponin complex as described below or stored at −20°C after freeze-drying. This preparation had relaxing-protein-system activity without the addition of tropomyosin.

The freeze-dried low-ionic-strength extract was suspended in 1M-KCl–2mM-dithiothreitol, the pH adjusted to 7.6–8.0 by addition of dil. HCl or NaOH and the solution stirred for 2h. Any undissolved material was removed by centrifugation to give a solution containing 2–4mg of protein/ml. By the addition of 0.1M-HCl the pH was adjusted to 4.5–4.7 and the suspension left for 1h. The precipitate formed was removed by centrifugation and resuspended in 1M-KCl–2mM-dithiothreitol, the pH readjusted to 8.0 with 2M-tris solution and the mixture stirred for 1h. The isoelectric precipitation was repeated as described above and the precipitate obtained was discarded or occasionally used for the preparation of tropomyosin. The combined supernatants from the isoelectric-precipitation steps were adjusted to pH7.0 with 2M-tris solution and the troponin complex was precipitated by 70%-satd. $(NH_4)_2SO_4$. The precipitate was dispersed in 15mM-2-mercaptoethanol–10mM-tris–HCl buffer, pH7.4, and dialysed against several changes of the same solution until free of $(NH_4)_2SO_4$. For the last change of dialysis fluid the 2-mercaptoethanol was replaced by 0.5mM-dithiothreitol. A slight precipitate sometimes formed during dialysis but as this was active the whole suspension was freeze-dried and stored at −20°C.

If electrophoresis in 6M-urea (Schaub & Perry, 1969) indicated that the troponin was contaminated with tropomyosin the isoelectric-precipitation stage was repeated. When an excess of slower-moving material was apparent on electrophoresis this was removed by precipitation with 0.5mM-dithiothreitol–10mM-$MgCl_2$–10mM-tris–HCl buffer, pH7.4.

Inhibitory and calcium-sensitizing factors. After an initial purification by chromatography on SE-Sephadex the troponin was dissociated into the inhibitory and calcium-sensitizing factors by chromatography on SE-Sephadex in urea as described by Schaub & Perry (1969). The separated factors were dialysed against 10mM-tris–HCl buffer, pH7.4, containing 0.5mM-dithiothreitol, freeze-dried and stored at −20°C.

Tropomyosin. Tropomyosin was prepared by a procedure described by Bailey (1948, 1951) modified by carrying out all of the three isoelectric precipitations in the presence of 1M-KCl to remove the troponin complex. The first was carried out by the addition of 0.1vol. of 1M-sodium acetate buffer, pH4.6, to the initial 1M-KCl extract of the dried fibre. The precipitate was collected, dissolved at pH7.0 and salted out between 40%-satd. and 70%-satd. $(NH_4)_2SO_4$ and a second isoelectric precipitation carried out under the same conditions as the first. The final isoelectric precipitation was carried out after precipitation of the tropomyosin between 50%-satd. and 65%-satd. $(NH_4)_2SO_4$. Finally the tropomyosin was dialysed against water, freeze-dried and stored at −20°C.

Actin. Actin was extracted with acetone-dried fibres prepared as described by Leadbeater & Perry (1963) with the modification that all solutions employed contained 15mM-2-mercaptoethanol or 0.5mM-dithiothreitol. The acetone-dried powder was extracted with 15vol. of 0.5mM-ATP (sodium salt), pH7.0, for 30min, the suspension filtered and the clear extract adjusted to pH4.6 with 10mM-sodium acetate buffer. The actin precipitate was separated by centrifugation, adjusted to pH7.0 with a little saturated $NaHCO_3$ soln. and further purified to remove any contaminating relaxing-protein-system components as described by Hartshorne *et al.* (1967).

Myosin. Myosin was prepared from mixed rabbit skeletal muscle by the method described by Trayer & Perry (1966) with 15mM-2-mercaptoethanol in all extraction and dialysis fluids. All protein preparative procedures were carried out at 1–2°C.

Column chromatography. Gel filtration on Sephadex G-200 was carried out essentially as described by Fish, Mann & Tanford (1969) at room temperature (21°C) on a chromatography column (90cm×1.5cm). The column was filled to a bed volume of 141.0ml under a pressure differential of 15–20cm of solvent and subsequently operated continuously flowing downwards, under the same pressure. Once a month the solvent was passed upwards through the column for 1–2 days to prevent the tendency for the gel bed to pack more tightly under continuous downward flow.

For determination of molecular weight in the guanidine hydrochloride medium, the latter was previously purified by the method of Nozaki & Tanford (1967). Blue Dextran 200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used at frequent intervals to determine the column void volume, V_0. The proteins used as standards are given in Table 1. All proteins except myoglobin were fully reduced and carboxymethylated before gel filtration. Most proteins were labelled with iodo[^{14}C]acetate. In the eluate fractions protein was detected by its E_{280}, cytochrome *c* at 405nm, or by counting the radioactivity of portions of the column fractions in a Nuclear–Chicago liquid-scintillation Counter, model Unilux II. Portions (0.3ml) were added to 6ml of scintillator [4g of 2,5-diphenyloxazole and 50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l of toluene] and 3ml of Triton X-100 and vigorously shaken. The clear samples had to be counted within about 30min since on

Table 1. *Proteins used as standards for gel-filtration experiments*

Protein	Molecular or subunit weight	Reference
Pepsin	35000	Bovey & Yanari (1960)
Tropomyosin	34000	Woods (1966, 1967, 1969)
Bence-Jones protein (κ-chain)	23500	Dayhoff & Eck (1969)
Myoglobin (apoprotein)	17200	Edmundson & Hirs (1962)
Ribonuclease	13700	Hirs, Moore & Stein (1960)
Cytochrome *c* (including ferrohaem)	12400	Margoliash (1962)

standing precipitation occurred. Larger as well as smaller samples than 0.3ml of the column fractions containing 6M-guanidine hydrochloride were precipitated when added to the scintillation fluid. Addition of water also led to precipitation. The radioactivity of samples was counted for 1–20min, depending on their radioactivity, at approx. 45% efficiency. No corrections were made for quenching by the guanidine hydrochloride.

In the acid-solvent system low concentrations of protein in the column fractions could be measured by their E_{360} after precipitation by the addition of 0.4ml of 25% (w/v) trichloroacetic acid/2ml of fractions (Davison, 1968).

Enzyme assays. Assays of ATPase were in general carried out by incubation as described by Schaub & Perry (1969) for 5min at 25°C. The standard incubation mixtures were 0.3–0.6mg of actomyosin or myofibrils, 25mM-tris–HCl buffer, pH7.6, 2.5mM-tris–ATP, 2.5mM-$MgCl_2$ or -$CaCl_2$ as indicated.

Determination of protein concentration. Protein was determined by an ultra-micro method involving nesslerization (Strauch, 1965) assuming a N content of 16%.

Electrophoresis. Disc electrophoresis on polyacrylamide gel was carried out as described by Schaub & Perry (1969).

Amino acid analyses. Amino acid analysis was carried out in the Beckman model 120B and Unichrome amino acid analysers. The amounts of amino acid were estimated by the height–width method described by Spackman, Stein & Moore (1958).

Tryptophan was determined by a modification suggested by Melamed & Green (1963) of the method of Beaven & Holiday (1952) as described by Crumpton & Wilkinson (1963).

Cysteine was determined as cysteic acid by the modification suggested by Moore (1963) of the method of Schram, Moore & Bigwood (1954).

Carboxymethylation of proteins. Protein samples (10–20mg/ml) were dissolved in 6M-guanidine hydrochloride–0.5M-tris–HCl buffer (pH8.4)–10mM-EDTA–100mM-2-mercaptoethanol (Crestfield, Moore & Stein, 1963; Miller & Metzger, 1965). After deaeration with N_2, the sample vessels were capped, incubated for 3–5h at 37°C, cooled to 0°C and iodoacetic acid was added to 100mM. Freshly prepared iodoacetic acid, recrystallized from ether, was dissolved in 1M-NaOH and the pH adjusted to 8.5 before addition. After being left for 20min the samples were dialysed exhaustively against 100mM-KCl and subsequently against the appropriate solvent.

For radioactive labelling the proteins were reduced under the same conditions except that the 100mM-2-mercaptoethanol was replaced with 10mM-dithiothreitol. Then 0.02ml of iodo[^{14}C]acetic acid (1mCi/ml) was added and the samples were left for 20min at 0°C. Subsequently the samples were made 50mM with respect to 2-mercaptoethanol and left for 60min at 37°C, again before more unlabelled iodoacetic acid was added as described above.

Reagents. Tris–ATP and tris–ITP were prepared from the di- and tri-sodium salts respectively (Schwartz, Bachelard & McIlwain, 1962). Chemicals were of analytical grade when obtainable. Distilled deionized water was used throughout. Trypsin (2×recrystallized), ribonuclease (5× recrystallized) and cytochrome *c* (horse heart, type III) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.; pepsin (2×recrystallized) was from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; $^{45}CaCl_2$ and iodo[2-^{14}C]acetic acid were from The Radiochemical Centre, Amersham, Bucks., U.K.; myoglobin (apoprotein) and the κ-chain of Bence-Jones protein were kindly given by Dr J. M. Wilkinson.

RESULTS

Properties of the inhibitory-factor preparations. In general at low ionic strength and physiological pH values the inhibitory factor was not very soluble. Clear solutions of freeze-dried inhibitory-factor preparations containing 2–4 mg/ml could be obtained by dissolution in 40mM-tris–HCl buffer, pH7.4. With higher protein concentrations either dissolution was not complete or precipitates formed on dialysis against solutions of ionic strength 0.01–0.20. Although the precipitates that appeared in these solutions had normal inhibitory activity they were usually removed by centrifugation from solutions of the inhibitory factor before enzyme assays were carried out. The precipitate was soluble below pH2.0 and above pH10. It dissolved readily in 5mM-tris–ATP, as reported by Hartshorne & Mueller (1968) for their troponin B preparations.

The inhibitory action of the factor was remarkably stable to treatments that normally destroy the biological activity of proteins. Solutions of the inhibitory factor could be frozen and thawed repeatedly without any loss of activity, and heating in 10mM-tris–HCl buffer, pH7.4, at 100°C for periods of up to 2h had little effect on its inhibitory action. Activity was little affected by precipitation with 7% (w/v) trichloroacetic acid or treatment with 6M-urea or 6M-guanidine hydrochloride for

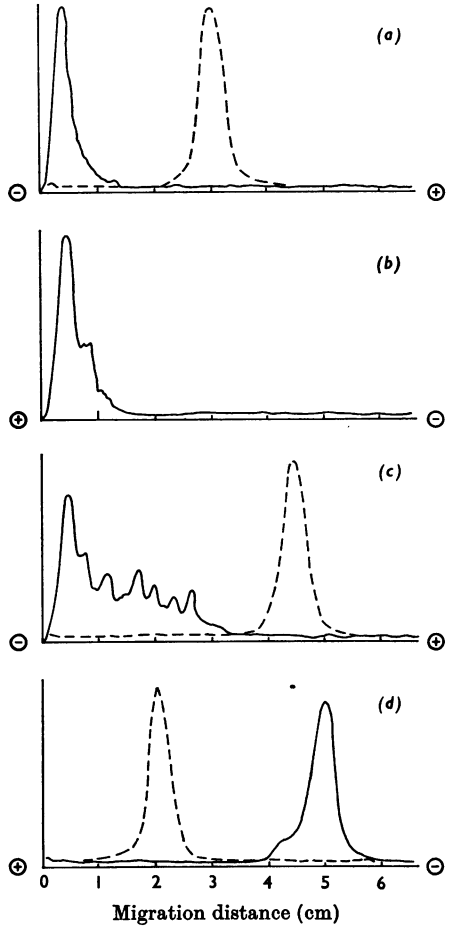

Fig. 1. Densitometric traces of electrophoretograms of inhibitory factor and tropomyosin. Samples (150–200 μg) of protein were applied to 8% polyacrylamide gels. (*a*) Tris–glycine buffer, pH 8.5; (*b*) as for (*a*) but with cathode at the bottom of the gel columns; (*c*) glycine–NaOH buffer, pH 11.3; (*d*) Tris–acetate buffer, pH 3.1. Urea (6M) was present in all cases. ——, Inhibitory factor; ----, tropomyosin.

several days at room temperature providing that inhibitory-factor preparations were subsequently dialysed exhaustively against buffer solutions of low ionic strength and pH 7.4. The inhibitory effect, however, was destroyed completely within 10 min by digestion at 20°C with trypsin at a ratio of 1:10.

Electrophoretic studies on inhibitory factor. All electrophoretic runs were performed in the presence of 6M-urea (Schaub & Perry, 1969) for in the absence of high concentrations of urea the usual contaminants of the inhibitory factor, tropomyosin and calcium-sensitizing factor, either do not enter the gel or complex with the inhibitory factor. On electrophoresis at pH 8.6 in 8% polyacrylamide gels little migration of the inhibitory-factor protein took place, for it stained as a diffuse band close to the origin (Schaub & Perry, 1969). A similar picture was obtained when the current was reversed by making the lower vessel the anode rather than the upper, as was the usual procedure (Figs. 1*a*,*b*). The absence of any faster-migrating bands under these conditions established that the inhibitory-factor preparations were free of contaminating tropomyosin or calcium-sensitizing factor for both these proteins migrate under these conditions (Schaub & Perry, 1969). At pH 8.6 the electrophoretic pattern was essentially unchanged when the urea concentration was increased to 10M. As the pH was raised to 11.3 an increasing proportion of the protein migrated towards the anode as several distinct fine bands (Fig. 1*c*). If electrophoresis was performed at pH 3.1, all the protein entered the gels and migrated towards the cathode mainly as one broad band (Fig. 1*d*), but sometimes this band was accompanied by one or two slightly slower-migrating bands. The isoelectric point of the inhibitory factor could not be determined precisely by electrophoretic studies because of the tendency of the protein to remain at the origin of the gels at mildly alkaline pH values, but was estimated to be in the range pH 8–10. Tropomyosin that was used for comparative purposes migrated as a single band (Fig. 1).

Molecular weights of inhibitory and calcium-sensitizing factors by gel filtration. Inhibitory factor was eluted from Sephadex G-100 in 20mM-sodium hydroxide–0.1M-tris, pH 12, partly as a peak running closely behind Blue Dextran and partly in a more retarded peak variable in relative size. On Sephadex G-200 in 0.2M-potassium chloride–20mM-hydrochloric acid, pH 1.8, the inhibitory factor was eluted as a heterogeneous peak between Blue Dextran and the marker protein pepsin (Fig. 2). These results indicated that even though the inhibitory factor was soluble at extreme pH values there was some polydispersity.

Some difficulty was experienced in detecting the protein peak eluted on gel filtration of the inhibitory factor on Sephadex G-200 in 6M-guanidine-hydrochloride for molecular-weight determination as described by Fish *et al.* (1969). To aid detection, the inhibitory factor was carboxymethylated with iodo[^{14}C]acetic acid so that low concentrations of protein could readily be detected in the eluate by measuring radioactivity. When the gel filtration of carboxymethylated-inhibitory factor was followed in this manner it was eluted as a single peak (Fig. 3). Under identical conditions the calcium-sensitizing factor, also carboxymethylated with iodo[^{14}C]-acetate, was retarded on the column to a greater

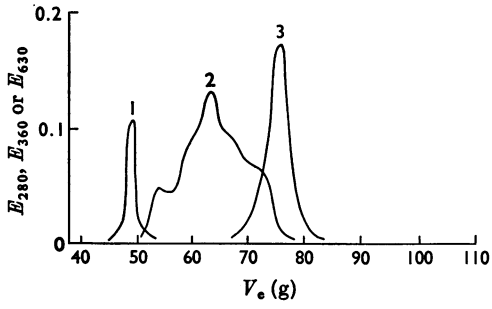

Fig. 2. Gel filtration of carboxymethylated inhibitory factor on Sephadex G-200 at pH 1.8. Samples (0.1–0.2 ml) containing 0.3–2.0 mg were applied to a column (88 cm × 1.5 cm) and eluted with 20 mM-HCl–200 mM-KCl, pH 1.8, at a flow rate of 2.5 ml/cm²/h. The elution position of Blue Dextran was followed by its E_{630}, pepsin by the E_{280} and the inhibitory factor by E_{360} after precipitation with trichloroacetic acid as described in the Methods section. Peak 1, Blue Dextran; peak 2, inhibitory factor; peak 3, pepsin.

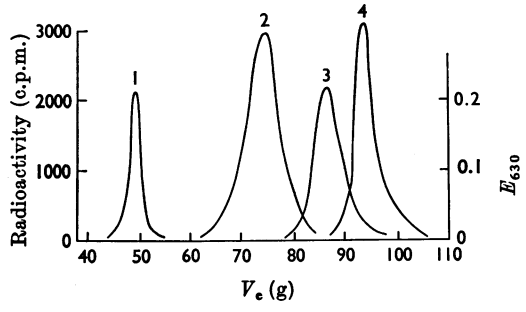

Fig. 3. Gel filtration in guanidine hydrochloride of the inhibitory and calcium-sensitizing factors carboxymethylated with iodo[^{14}C]acetate. Conditions in general were as for Fig. 2. Elution was carried out with 6 M-guanidine hydrochloride–20 mM-tris–HCl, pH 6.0. Elution of proteins was followed by measurement of radioactivity, and Blue Dextran by its E_{630}. Peak 1, Blue Dextran; peak 2, pepsin; peak 3, inhibitory factor; peak 4, calcium-sensitizing factor.

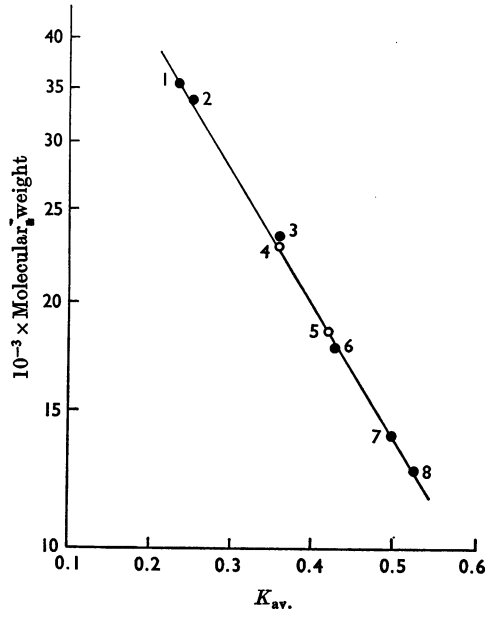

Fig. 4. Graphical interpretation of elution results obtained for gel filtration of carboxymethylated proteins on Sephadex G-200 in 6 M-guanidine hydrochloride–20 mM-tris–HCl, pH 6.0: semi-logarithmic plot of the molecular weight as function of the partition coefficient, $K_{av.}$ (Laurent & Killander, 1964). The proteins corresponding to the points on the graph are: 1, pepsin; 2, tropomyosin; 3, Bence-Jones protein (κ chain); 4, inhibitory factor; 5, calcium-sensitizing factor; 6, myoglobin; 7, ribonuclease; 8, cytochrome *c*.

extent than the inhibitory factor but was likewise eluted as a single peak.

To obtain maximum precision for molecular-weight determination the weight rather than volume of fractions was used as the measure of the eluted position of the applied proteins (Andrews, 1964). The results were treated by plotting the logarithm of molecular weight against either relative elution volume, V_e/V_0 (Andrews, 1965), or the partition coefficient, $K_{av.}$ (Laurent & Killander, 1964) (Fig. 4). In both cases the results obtained with proteins of known molecular weight lay on a straight line over the range 12000–35000. From the graph the molecular weights 22900 ± 800 and 18400 ± 600 were obtained for the carboxymethylated-inhibitory factor and calcium-sensitizing factors respectively.

Amino acid composition of inhibitory factor. The amino acid content of the inhibitory factor was very similar to that reported by Hartshorne & Mueller (1968) for troponin B. For comparison Hartshorne & Mueller's (1968) results calculated for a molecular weight of 23000 are also included in Table 2. The ratio of polar to non-polar residues of 2.4 is high and similar to those of myosin and tropomyosin (Hatch, 1965). It is worth noting that the inhibitory factor is the most basic of the characterized myofibrillar proteins, containing 197 total basic residues/10^5 g compared with 150–160/10^5 g for myosin and tropomyosin (Kominz, Saad, Gladner & Laki, 1957; Lowey & Cohen, 1962). No methylated derivatives of histidine or lysine could be detected on analyses by the procedures described elsewhere (Hardy, Harris, Perry & Stone, 1970).

The u.v. spectrum of the inhibitory factor was

Table 2. *Amino acid content of the inhibitory factor*

Results are means of duplicate analyses of two preparations after hydrolysis for 24 and 72h. The composition was calculated by adding up residue weights and relating to 23000g on the assumption that recovery is 100%.

Amino acid	Content (mol/23000g) Schaub & Perry (this paper)	Hartshorne & Mueller (1968)
Lys	28.1	23.0
His	3.7	3.1
Arg	17.0	14.7
Asp	17.7	21.4
Thr	5.9	5.8
Ser	9.2	8.4
Glu	38.4	40.2
Pro	6.8	7.0
Gly	9.5	10.3
Ala	18.3	18.7
Val	8.2	8.1
Met	5.5	7.0
Ile	6.4	7.2
Leu	18.0	16.1
Tyr	3.1	2.9
Phe	3.9	6.3
Trp	1.1	—
Cys	1.9	—

typical of that of a protein with a maximum at 278nm and an inflexion around 290nm due to tryptophan, of which there appears to be approximately one residue per mol. No significant differences were apparent in the spectra measured in 40mM-tris–HCl, pH 7.4, and in 20mM-hydrochloric acid. Neither phosphorus nor neutral carbohydrate were present in significant amounts in the inhibitory factor.

Effect of the inhibitory factor on the enzymic properties of actomyosin. The inhibitory factor alone inhibited the Mg^{2+}-stimulated ATPase activity of desensitized actomyosin, but usually, although not invariably, the addition of tropomyosin significantly increased the extent of inhibition. The inhibitory factor also strongly inhibited the Mg^{2+}-stimulated ATPase activity not only of normal actomyosin but also of myofibrils (Fig. 5). The addition of tropomyosin was without effect in these systems, presumably because they were already saturated with tropomyosin. Addition of 1mM-EGTA, or calcium chloride up to concentrations of 0.5mM, did not affect the inhibitory action on the myofibrillar ATPase.

The minimum amount of inhibitory factor required for maximal effect on the ATPase of desensitized actomyosin or myofibrils was estimated by extrapolation from the initial linear part of the curve obtained by plotting inhibition against amounts of added inhibitory factor. As might be expected the inhibitory factor was less effective on the ATPase of myofibrils than on that of desensitized actomyosin. On average 66 and 136μg of inhibitory factor were needed for maximum inhibition of 1mg of desensitized actomyosin or myofibrils respectively (Table 3).

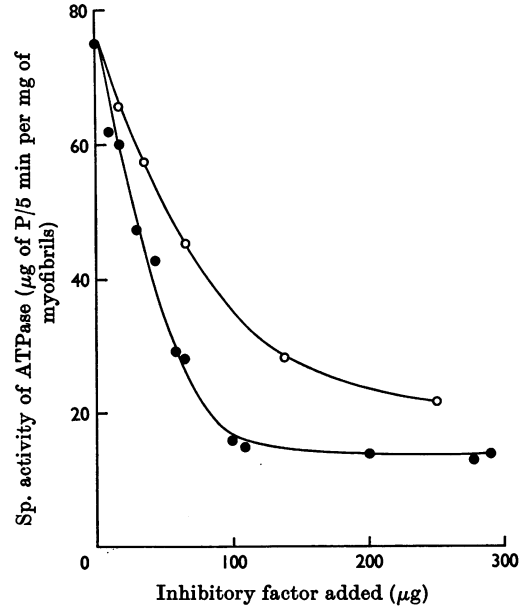

Fig. 5. Effect of inhibitory factor on the Mg^{2+}-stimulated ATPase activity of myofibrils. ATPase assays were carried out under standard conditions on 0.32mg of myofibrils. ●, + inhibitory factor; ○, + carboxymethylated inhibitory factor.

Table 3. *Estimated amounts of inhibitory factor required for maximal inhibition of the* Mg^{2+}*-stimulated* ATPase *activity of desensitized actomyosin and myofibrils*

The results were obtained by plotting the percentage inhibition of the Mg^{2+}-stimulated ATPase activity against the amount of inhibitory factor added to a given preparation of desensitized actomyosin or myofibrils. The amount required for maximum inhibition was determined by extrapolation from the initial linear portion of the response graph. Assays were carried out on desensitized actomyosin in each case in the presence of the optimum tropomyosin concentration. This was determined on each preparation and usually was equal to the amount of the inhibitory factor present. Values in parentheses are standard deviations.

	Amount of inhibitory factor required for maximum inhibition (μg/mg of protein)	
Preparation no.	Desensitized actomyosin	Myofibrils
1	37	63
2	65	124
3	72	88
4	54	157
5	95	163
6	83	175
7	61	167
8	60	154
Average	65.8 (±17.8)	136.4 (±40.8)

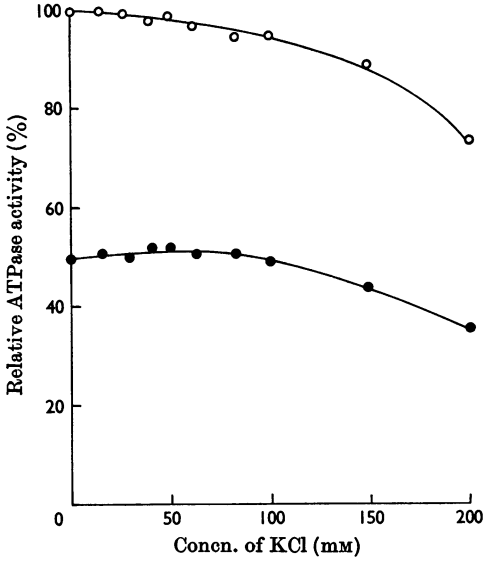

Fig. 6. Effect of increasing KCl concentration on the action of inhibitory factor on the Mg^{2+}-stimulated ATPase activity of myofibrils. ATPase assays were carried out under standard conditions on 0.45 mg of myofibrils. Relative activity is expressed as a percentage of that obtained with myofibrils alone under standard assay conditions. ●, + 55 μg of inhibitory factor/assay mixture; ○, no inhibitory factor.

Intact thiol groups seemed not to be essential for the inhibitory effect, as blocking of the two cysteine residues/mol of the inhibitory factor by carboxymethylation only slightly decreased its activity (Fig. 5).

With myofibrils, the Mg^{2+}-stimulated ATPase activity of which is less susceptible to increasing salt concentrations than that of desensitized actomyosin, the inhibitory effect was little changed by the addition of up to 200 mM-potassium chloride to the incubation medium (Fig. 6). When tested in the presence of tropomyosin the inhibitory effect was also exerted in the range pH 6–9, the full pH range over which desensitized actomyosin possessed high Mg^{2+}-stimulated ATPase activity (Fig. 7).

During preincubation of the reaction mixture used for the enzyme tests, complete with the exception of ATP, it was observed that the inhibitory factor consistently formed a slight precipitate that dissolved on addition of ATP to start the enzymic reaction. The precipitation was probably due to the magnesium chloride present, which was previously shown to precipitate crude inhibitory-factor preparations (Hartshorne *et al.* 1967). As in this earlier investigation it was shown that under some conditions Mg^{2+} modified the activity of the crude inhibitory-factor preparations, the effect of magnesium chloride on the activity of the purified inhibitory factor was further investigated. Precautions were taken to ensure a constant ionic strength in the enzymic tests by the addition of compensating amounts of potassium chloride. Although the Mg^{2+}-stimulated ATPase activity of desensitized actomyosin decreased slightly when the concentration of magnesium chloride exceeded that of the ATP, the percentage inhibition was constant in the presence of up to 7.5 mM-magnesium

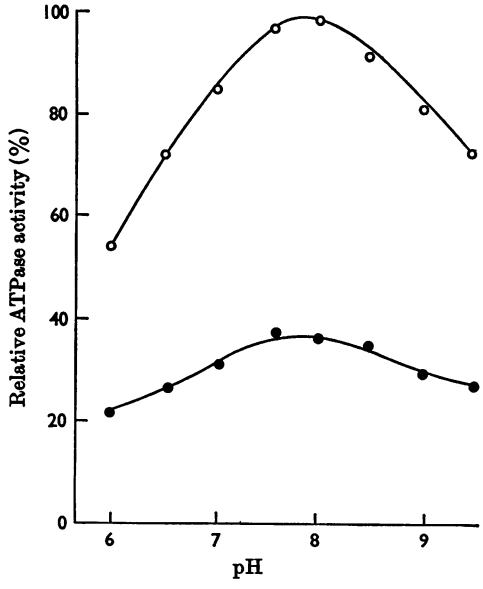

Fig. 7. Effect of pH on the inhibitory action on the Mg^{2+}-stimulated ATPase activity of desensitized actomyosin. Assays were carried out in 50mM-tris–maleate buffer, 2.5mM-tris–ATP and 2.5mM-$MgCl_2$ on 0.54mg of desensitized actomyosin and 100μg of tropomyosin. Relative activity is expressed as a percentage of that obtained at maximum activation in the absence of inhibitory factor. ●, 48μg of inhibitory factor; ○, no inhibitory factor.

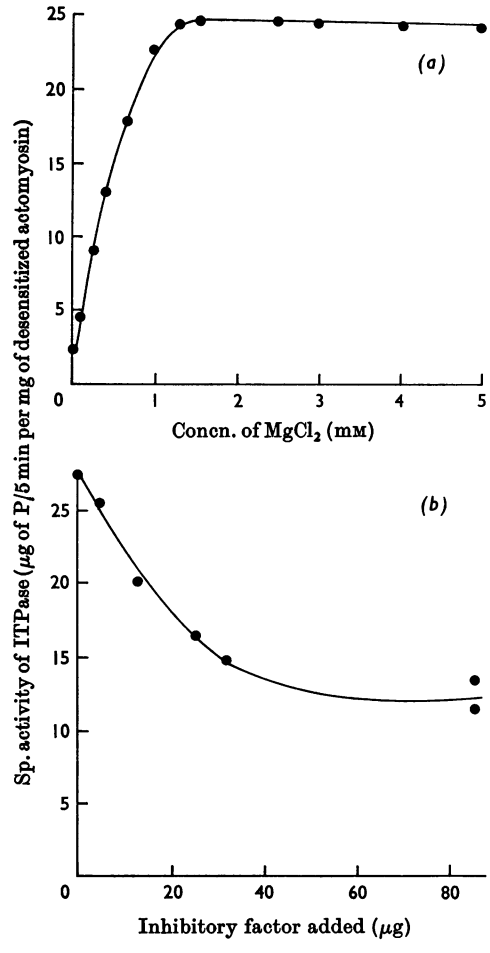

Fig. 8. Effects of the concentration of $MgCl_2$ and of the inhibitory factor on the Mg^{2+}-stimulated ITPase activity of desensitized actomyosin. Assays were carried out in 2.5mM-tris–ITP, 25mM-tris–HCl buffer, pH7.6, and 50mM-KCl on 0.71mg of desensitized actomyosin. (*a*) Concentration of $MgCl_2$ varied; (*b*) increasing amounts of inhibitory factor in the presence of 1.5mM-$MgCl_2$.

chloride. Precipitation of the inhibitory factor also occurred with 0.5–5.0mM-cobalt sulphate, zinc chloride, manganese chloride, cadmium acetate and to a lesser extent with calcium chloride. All these bivalent cations stimulate the ATPase activity of the actomyosin type equally well in the desensitized actomyosin system, provided they are at the optimum concentration in the presence of 2.5mM-ATP (Schaub & Ermini, 1969).

The effect of the inhibitory factor was tested in the presence of different bivalent cations in the absence of tropomyosin. This protein was omitted from all the tests since when tested alone it inhibits the Ca^{2+}- and Cd^{2+}-stimulated ATPase activities of desensitized actomyosin (Schaub & Ermini, 1969) and the results with the different cations would not have been comparable. The inhibitory factor inhibited the ATPase activity of desensitized actomyosin when stimulated by magnesium chloride, cobalt sulphate or manganese chloride. The Zn^{2+}-, Cd^{2+}- and Ca^{2+}-stimulated ATPase activities were not affected by the inhibitory factor. Similar results were obtained with myofibrils. It should be mentioned that the ATPase activities of myofibrils stimulated by those bivalent cations which were not affected by the inhibitory factor, i.e. zinc chloride, cadmium acetate and calcium chloride, were considerably lower than those obtained in the presence of magnesium chloride, cobalt sulphate or manganese chloride.

Under optimum conditions, i.e. 2.5mM-magnesium chloride and 50mM-potassium chloride in the presence of 2.5mM-ITP, the Mg^{2+}-stimulated ITPase activity of desensitized actomyosin was on average only one-third of that obtained with ATP as substrate (Fig. 8*a*). Nevertheless, the inhibitory factor inhibited the Mg^{2+}-stimulated ITPase activity of desensitized actomyosin by up to 60% at

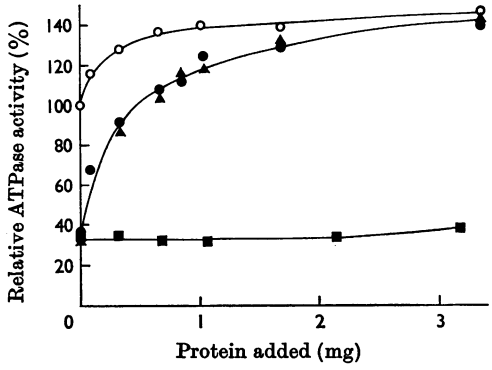

Fig. 9. Effects of actin and tropomyosin on the action of the inhibitory factor on the Mg^{2+}-stimulated ATPase of desensitized actomyosin. Assays were done under standard conditions with 2.5 mM-$MgCl_2$ and 0.46 mg of desensitized actomyosin with the following additions: ○, increasing amounts of actin; ●, increasing amounts of actin + 111 μg of inhibitory factor; ▲, increasing amounts of actin + 111 μg of inhibitory factor + 1 mM-EGTA; ■, increasing amounts of tropomyosin + 111 μg of inhibitory factor. Relative activity expressed as for Fig. 6.

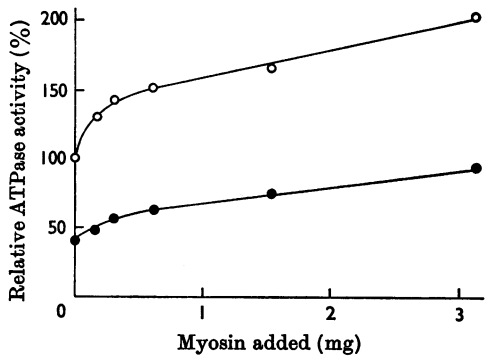

Fig. 10. Effect of myosin on the action of inhibitory factor on the Mg^{2+}-stimulated ATPase of desensitized actomyosin. Assays were done under standard conditions with 2.5 mM-$MgCl_2$ and 0.46 mg of desensitized actomyosin. ○, No inhibitory factor; ●, 56 μg of inhibitory factor. Relative activity expressed as for Fig. 6.

roughly the same concentrations as were required for maximal inhibition of the ATPase activity (Fig. 8*b*). These tests were performed in the absence of tropomyosin, as the latter protein itself has a pronounced effect on the Mg^{2+}-stimulated ITPase activity of desensitized actomyosin, increasing the requirement for magnesium chloride by about 10 times (Schaub, 1970).

Neither the Ca^{2+}-stimulated ATPase nor the ATPase activity of myosin stimulated by 5 mM-EDTA in the presence of 1 M-potassium chloride (Seidel, 1969) were inhibited by the inhibitory factor. The inhibitory factor did not have any effect on the 'basal' or 'extra' ATPase activity of fragmented sarcoplasmic reticulum. The Mg^{2+}-stimulated ATPase activities of mitochondria prepared from liver and from muscle were also not inhibited. The inhibitory factor had no effect on the formation of ATP from phosphocreatine and ADP catalysed at pH 7.6 by creatine kinase isolated from rabbit skeletal muscle.

Interaction of inhibitory factor with other myofibrillar proteins. In an attempt to determine whether any special relationship existed between the inhibitory factor and the major myofibrillar proteins, actin and myosin, the effects of adding additional amounts of these proteins to inhibited actomyosin ATPase systems were investigated. The complexities of the enzymic system present difficulties in interpretation and experimental design, for the inhibitory factor requires actin, myosin and tropomyosin, all of which interact among themselves, for maximal activity. To simplify the situation the effect of actin and myosin on the action of the inhibitory factor in the absence of tropomyosin was studied. This was possible, since excess of inhibitory factor inhibits without additional tropomyosin and the inhibition is usually not modified by further addition of tropomyosin. Figs. 9 and 10 show that increasing amounts of actin added to desensitized actomyosin neutralized the inhibitory action, whereas addition of myosin did not significantly affect the extent of inhibition. In the latter case enzymic activity increased in a somewhat parallel manner both in the absence and presence of inhibitory factor as the amount of added myosin was increased.

Similar results were obtained with myofibrils, although in this case it appeared that rather more actin was required to neutralize a given amount of inhibitory factor than with desensitized actomyosin. This might be explained as being due to the endogenous inhibitory factor present as part of the troponin complex in the intact myofibril.

The effect could not be explained as being due to calcium-sensitizing factor present in the added actin, for the addition of EGTA to the desensitized actomyosin system containing inhibitory factor and excess of actin produced no inhibition (Fig. 9).

DISCUSSION

Although the pronounced tendency of the inhibitory factor to aggregate produces difficulties in determining precisely the molecular homogeneity of the preparation, the evidence for gel filtration in 6 M-guanidine hydrochloride suggests that the preparation is fairly homogeneous with regard to subunit size. It should be pointed out, however, that as

the protein eluted was monitored by its radioactivity after carboxymethylation with iodo[^{14}C]-acetate, protein in the eluate that was not radioactive would not be detected. This possibility is relevant to the present study for preliminary evidence (Wilkinson, Perry, Cole & Trayer, 1971) has been obtained that in addition to the main active component that becomes radioactive after treatment with iodo[^{14}C]acetate some inhibitory-factor preparations contain a small amount (usually less than about 20%) of a lower-molecular-weight protein. This protein is active as an inhibitor of the Mg^{2+}-stimulated ATPase of desensitized actomyosin and contains little or no cysteine. It therefore would not be readily detected under the conditions used for molecular-weight determination. Nevertheless the evidence indicates that the main active component of inhibitory-factor preparations has a subunit weight of 23000.

A value for the minimum molecular weight can also be calculated by assuming that to be effective one molecule of the inhibitory factor combines either with each actin monomer or each active centre of the myosin ATPase. On this basis, from the results obtained with desensitized actomyosin and presented in Table 3 the inhibitory factor would have a molecular weight of 19000 or 23000 depending on whether it acted on each ATPase site of myosin or each actin monomer respectively. For the purposes of this calculation the desensitized actomyosin is estimated to contain 13% actin, as judged from analyses for 3-methylhistidine (S. V. Perry & C. I. Harris, unpublished work). These conclusions, together with the results of preliminary fluorescence-polarization measurements carried out by F. W. J. Teale of this department, agree well with the view that the main molecular species of the inhibitory-factor preparation has a molecular weight of 23000.

The stability of the inhibiting factor is remarkable and suggests that the tertiary structure is a very stable one. The high content of polar residues may be of significance for this property and also for the strong tendency of the inhibitory factor to form aggregates of the subunit. It is not possible from this study, however, to decide whether the active form is the subunit itself or some polymeric form of it. The evidence would suggest that at physiological pH values and low ionic strength the inhibitory factor does not exist in the monomer form. Extreme dissociating conditions are required for complete dissociation to take place.

The action of the inhibitory factor is highly specific for the ATPase of the actomyosin type, for the enzymic properties of myosin alone and other ATPase systems were not affected. Further, the enzymic activity of actomyosin and of myofibrils was inhibited by the inhibitory factor at all pH values or salt concentrations at which Mg^{2+} produced appreciable stimulation of the ATPase activity. The specificity with respect to the bivalent cations is not limited to Mg^{2+}, as the Co^{2+}- or the Mn^{2+}-stimulated ATPase activities were also inhibited by the inhibitory factor. Its action is not restricted to the hydrolysis of ATP, for the ITPase activity of actomyosin was inhibited under appropriate conditions.

This is one of the few aspects in which the properties of the inhibitory factor differed from those of the crude inhibitory-factor preparations, which were earlier reported not to be effective with ITP as substrate (Hartshorne *et al.* 1967). Presumably the presence of other proteins in the crude inhibitory-factor preparations may modify the action of the inhibitory factor on the ITPase and account for the lack of inhibition previously reported. Certainly considerable amounts of other protein were present in the crude inhibitory-factor preparations, for they possessed only 4–5% of the specific activity of the inhibitory factor prepared by dissociation of the troponin complex.

It is notable in this respect that the ITPase activities of myofibrils can be inhibited by chelation of trace amounts of Ca^{2+} (Weber, 1969) contrary to earlier reports that relaxation of model systems could only be obtained with ATP as substrate (Hasselbach, 1956; Bendall, 1958). Caution must be used in the interpretation of results with ITP as substrate, since it has been reported that tropomyosin itself has a pronounced inhibitory effect on the ITPase activity of actomyosin at concentrations of magnesium chloride at which it was fully activated in the absence of tropomyosin (Schaub, 1970).

The highly specific action of the inhibitory factor on the actomyosin ATPase suggests that it should provide a useful probe for detection of enzymes of this type in various other systems. Its precise specificity and the special role of actin in the enzymic mechanism indicates that the inhibitory factor acts by preventing the interaction of actin at the enzymic site of myosin. The interaction enables myosin to utilize $MgATP^{2-}$ as substrate but when blocked by the inhibitory factor the hydrolysis of $MgATP^{2-}$ decreases to a low value. Specific interaction between actin and the inhibitory factor is suggested by the experiments in which on addition of the major myofibrillar proteins to actomyosin systems actin was the only protein that neutralized the inhibitory factor's action. This experiment implies that actin is able to bind the inhibitory factor and render it ineffective and supports the hypothesis that the factor exerts its specific effect on this protein.

We are grateful to Dr J. M. Wilkinson and Miss Sue Brewer for carrying out the amino acid analyses. The work

was supported in part by research grants from the Medical Research Council and the Muscular Dystrophy Associations of America Inc.

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