The Regulatory Proteins of the Myofibril

SEPARATION AND BIOLOGICAL ACTIVITY OF THE COMPONENTS OF INHIBITORY-FACTOR PREPARATIONS

By J. M. WILKINSON, S. V. PERRY, H. A. COLE and I. P. TRAYER

*Department of Biochemistry, University of Birmingham,
Birmingham B15 2TT, U.K.*

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1. Inhibitory-factor preparations isolated from myofibrils were shown to consist principally of proteins with molecular weights of 37000 and 23000. Under certain preparative procedures an additional component of molecular weight 14000 was present. 2. The 23000-dalton protein, the inhibitory factor, was the major active component. Its activity was enhanced by tropomyosin. 3. The 14000-dalton component also possessed inhibitory activity, although less than that of the 23000-dalton component when compared on a molar basis. Its activity was not always enhanced by tropomyosin. The 14000-dalton component could not be detected in whole fresh myofibrils and the limited evidence available is compatible with its formation during the preparation of the troponin complex. 4. The 37000-dalton component could not replace the inhibitory factor, calcium-sensitizing factor or tropomyosin as components of the relaxing-protein system. 5. All three components had distinctive amino acid compositions, particularly in their cysteine content.

Earlier studies from this laboratory (Schaub & Perry, 1969, 1971) described the isolation from troponin preparations of an inhibitory factor which was active in inhibiting the Mg^{2+} -activated ATPase* activity of desensitized actomyosin. A similar preparation has been described by Hartshorne & Mueller (1968) and named by them troponin B. Because of the pronounced tendency of inhibitory-factor preparations to aggregate at neutral pH values, the molecular weight and homogeneity of preparations were studied by gel filtration in 6M-guanidine hydrochloride after their cysteine residues had been carboxymethylated with iodo[1- ^{14}C]acetic acid (Schaub & Perry, 1971). Under these conditions proteins are considered to lose all residual non-covalent structure (Fish *et al.*, 1969). The eluted protein was detected by measurement of radioactivity, and a single fairly symmetrical peak was found, from the elution volume of which a molecular weight of 23000 was deduced. Any other components that did not contain cysteine, however, would not have been detected and their presence could not be ruled out.

Reinvestigation of the homogeneity of the inhibitory-factor preparations to explore this possibility has shown that some preparations contain, in addition to the major biologically active component, a smaller protein of molecular weight about

14000, which is effective as an inhibitor and which contains no cysteine. In addition, all troponin-complex and most inhibitory-factor preparations contain a hitherto uncharacterized protein of molecular weight 37000, which is also low in cysteine content. As yet no specific role has been ascribed to the latter protein.

Some of the findings described have been briefly reported elsewhere (Wilkinson *et al.*, 1971).

Methods

Preparative procedures

Unless otherwise stated all preparations were carried out at 1–2°C.

Myofibrils. Myofibrils were prepared from mixed rabbit skeletal muscle by the method of Perry & Zydowo (1959). Care was taken to remove cell fragments by low-speed centrifugation and, after initial separation from the muscle residue after the second homogenization, the myofibrils were washed four times by centrifugation and resuspension. Myofibrils were stored in 0.1M-KCl–39mM-sodium borate buffer, pH 7.0.

Troponin complex. A low-ionic-strength extract was prepared directly from myofibrils without the prior extraction with Weber's solution described by Schaub & Perry (1971). In general this procedure gave better yields and was more convenient. A suspension of well-washed myofibrils prepared as described

* Abbreviations: ATPase, adenosine triphosphatase; SE-Sephadex, sulphoethyl-Sephadex; QAE-Sephadex, diethyl-(2-hydroxypropyl)aminoethyl-Sephadex.

above in 0.1M-KCl–39mM-sodium borate buffer, pH7.0 (20–30mg/ml), was blended for a few seconds in a Waring Blendor with 5 vol. of 15mM-2-mercaptoethanol. The myofibrils were sedimented by centrifugation at 2000g for 30min and the supernatant was discarded. The myofibrils were resuspended in 6 vol. of 15mM-2-mercaptoethanol, and the diluted resuspended myofibrils dialysed for 2 days against 10 vol. of 15mM-2-mercaptoethanol. The extremely viscous suspension was then diluted with 1 vol. of dialysis fluid and centrifuged for 40min at 90000g. The yield of clear viscous supernatant could be increased by resuspending the sediment in 1–2 vol. of dialysis fluid, adjusting the pH to 8.0 with 1M-tris base and repeating the centrifugation after the suspension had been standing overnight. The combined supernatants were either freeze-dried for storage or used immediately for troponin preparation as described by Schaub & Perry (1971).

Inhibitory factor. Two methods of preparation of the inhibitory factor from the troponin complex were used. These are referred to as type 1 and type 2 in the remainder of the paper and were obtained as follows.

(a) Type 1 preparations. These were made by chromatography on SE-Sephadex in 6M-urea essentially as described by Schaub & Perry (1969). Troponin (250mg; 10mg/ml) was applied directly to the SE-Sephadex column (16cm×4cm) equilibrated with 6M-urea–33mM-sodium citrate (pH6.0)–0.5mM-dithiothreitol without prior chromatography under the same conditions in the absence of urea (Schaub & Perry, 1969). The impurities that would have been removed by the latter procedure accumulated in the calcium-sensitizing-factor fraction, which was further purified if necessary by isoelectric precipitation at pH4.6 in the presence of 1.0M-KCl and chromatography on QAE-Sephadex (Schaub *et al.*, 1972). Gel electrophoresis at pH8.6 indicated that type 1 preparations were usually free of tropomyosin and calcium-sensitizing factor. Finally the preparation was dialysed against 15mM-tris–HCl buffer, pH7.4, and freeze-dried for storage.

(b) Type 2 preparations. These were made by a modification of the acid-fractionation method of Hartshorne & Mueller (1968). Troponin preparations dissolved in 15mM-tris–HCl buffer, pH7.4, containing 15mM-2-mercaptoethanol and 0.1mM-CaCl₂, were dialysed against three changes of 1.2M-KCl–0.1M-HCl. The precipitate formed during the dialysis was removed by centrifugation and the supernatant was dialysed against 0.01M-HCl and freeze-dried.

Actin. This was extracted from acetone-dried fibre (Leadbeater & Perry, 1963) as described by Hartshorne *et al.* (1967).

Myosin. This was prepared from mixed rabbit skeletal muscle by the method described by Trayer & Perry (1966) with the modification that 15mM-2-mercaptoethanol was included in all extraction and

dialysis fluids. It was converted into heavy mero-myosin by chymotryptic digestion by the method previously described (Leadbeater & Perry, 1963).

Tropomyosin. This was prepared essentially by the method of Bailey (1948, 1951). After an initial isoelectric precipitation of the 0.1M-KCl extract of the dried fibre, the precipitate was collected, dissolved at pH7.0, salted out between 41 and 70%-satd. ammonium sulphate and stored as a paste at –20°C. When required, portions of the paste were dissolved in 10mM-tris–HCl buffer, pH7.4, and dialysed against several changes of this buffer. The tropomyosin solution was made 1.0M with respect to KCl, adjusted to pH4.6 with 0.1M-HCl and, after the solution had been standing for 1h at 0°C, the precipitate was collected by centrifugation. The tropomyosin was redissolved in water, adjusted to pH7.0 with 0.1M-NaOH and dialysed overnight against 1.0M-KCl. After a second isoelectric precipitation the tropomyosin was precipitated between 46 and 65%-satd. ammonium sulphate. The tropomyosin (1–2mg/ml) was further purified by precipitation between 53 and 60%-satd. ammonium sulphate. This was followed by a third isoelectric precipitation in the presence of 1M-KCl. The last-named two stages were repeated until the tropomyosin migrated as a single band on polyacrylamide-gel electrophoresis at pH8.6 in 6M-urea, when more than 20–30µg was loaded on to the gel. With lighter loading this band could be resolved into two closely spaced bands. On electrophoresis in sodium dodecyl sulphate a similar picture was observed although resolution into the double band was more difficult to observe. Electrophoresis in urea and sodium dodecyl sulphate indicated the absence of the 37000-dalton component from all the tropomyosin preparations used in the enzymic studies. Finally preparations were dialysed against several changes of 10mM-tris–HCl buffer, pH7.4, freeze-dried and stored at –20°C.

Enzymic assays

ATPase was assayed essentially as described by Schaub & Perry (1969). The standard assay mixture consisted of 0.3–0.8mg of actomyosin, 25mM-tris–HCl buffer, pH7.6, 2.5mM-MgCl₂ and 2.5mM-tris–ATP. Incubations were carried out for 5min at 25°C. Specific activity was expressed as the amount (µg) of myofibrillar protein required to produce maximum inhibition of the Mg²⁺-stimulated ATPase in 1mg of desensitized actomyosin assayed under standard conditions. Maximum inhibition was taken as the extent of inhibition when further addition of inhibitory factor gave no further increase of inhibition of the Mg²⁺-stimulated ATPase. The amount of inhibitory factor required was determined by extrapolation from the linear portion of the response curve.

Gel filtration of inhibitory-factor preparations

For the separation of components of the inhibitory factor by gel filtration, Sephadex G-75, G-100 or G-200 was equilibrated with 0.2M-sodium formate buffer, pH3.3, containing 6M-urea. 2-Mercaptoethanol (15mM) was included in the buffers used for proteins that had not been carboxymethylated. Samples were dissolved in the eluting buffer, to which sucrose was added to approx. 20% (w/v), and up to 2ml containing 3–10mg of protein/ml was loaded on a Whatman precision column (1.5cm × 90cm). Elution was carried out at 4–8ml/h, usually at 4°C, and 2–3ml fractions were collected.

Chromatography of type 2 inhibitory-factor preparations

Type 2 preparations were further purified by chromatography on CM-cellulose by the method of Senshu & Iwai (1970). CM-cellulose (Whatman CM32) was washed with 0.5M-NaOH, then 0.5M-HCl, and finally equilibrated with a buffer containing aq. 70% (v/v) ethanol, 2.6M-formic acid and 0.1M-sodium formate. Columns (1.8cm × 34cm) were washed with several column volumes of starting buffer before application of the sample and developed at room temperature with a linearly decreasing concentration of ethanol from 70% to 48% (v/v). The total volume of the gradient was 1 litre. Freeze-dried protein samples were dissolved in aq. 80% (v/v) ethanol–2.6M-formic acid to a concentration of 20mg/ml and

columns were loaded with up to 400mg of protein. Fractions were pooled, dialysed against 0.01M-HCl, concentrated by ultrafiltration on an Amicon UM10 membrane and freeze-dried.

Polyacrylamide-gel electrophoresis

pH8.5 gel. This was carried out as described by Schaub & Perry (1969), with 6M-urea–15mM-2-mercaptoethanol in the gels.

pH3.2 gel. The procedure used was as described by Panyim & Chalkley (1969). In all cases 6.25M-urea–7.5% (w/v) acrylamide was used in the gels, which were run in 6mm × 100mm tubes. Methyl Green (British Drug Houses Ltd., Poole, Dorset, U.K.) was used as a marker dye to follow the pre-electrophoresis and was also included in the samples. Samples (1mg/ml) were dissolved in 0.9M-acetic acid–6M-urea and 20μl was applied to each gel.

The pH8.5 and pH3.2 gels were stained with 0.6% Amido Black in water–ethanol–acetic acid (5:3:1, by vol.) for at least 2h and destained either by prolonged washing in 7% (v/v) acetic acid or electrophoretically with the apparatus described by Ward (1970), again with 7% acetic acid as the destaining solution.

Sodium dodecyl sulphate gel. The method employed was essentially identical with that described by Weber & Osborn (1969). Throughout this study 10% (w/v) acrylamide gels were employed in 6mm × 150mm glass columns. Freeze-dried samples were dissolved at a concentration of 1–2mg/ml in 1% sodium

Fig. 1. Gel filtration of a type 1 inhibitory-factor preparation

A Sephadex G-75 column (1.5cm × 90cm) was used, eluted with 6M-urea–0.2M-sodium formate–15mM-2-mercaptoethanol, pH3.3. Protein solution (3ml, with E_{280} 11.0) in eluting buffer was applied to the column.

dodecyl sulphate – 1% 2-mercaptoethanol – 0.01 M-sodium phosphate buffer, pH 7.0, and incubated at 37°C for 12–16 h before application to the gel. Electrophoresis was performed at 20–25°C at a constant current of 8–9 mA/tube for 8 h, by which time the marker dye (0.05% Bromophenol Blue in water) had migrated to within 1 cm of the bottom of the gel. The gels were stained in a solution of Coomassie Brilliant Blue (1.25 g dissolved in a mixture of 454 ml of 50% methanol and 46 ml of acetic acid) for 2–6 h and destained for 24–36 h in a solution containing 14% (v/v) acetic acid and 5% (v/v) methanol. The molecular weights of the protein components were determined by comparison of their mobilities with those of proteins of known molecular weight as described by Weber & Osborn (1969). Sodium dodecyl sulphate was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Coomassie Brilliant Blue R was obtained from George T. Gurr Ltd., London S.W.6, U.K.

Molecular weights by gel filtration

Molecular weights were determined by the method of Fish *et al.* (1969) at room temperature (21°C). V_0 and V_i as defined by Fish *et al.* (1969) were determined with Blue Dextran and Dnp-glycine respectively. The position of the protein eluate was determined by measuring the extinction at 280 nm.

Carboxymethylation of proteins

Samples (5–10 mg) of freeze-dried material were dissolved in 1 ml of 0.4 M-tris-HCl buffer, pH 8.2, containing 6 M-guanidine hydrochloride and 10 mM-dithiothreitol. After incubation at 37°C for 3 h they were cooled to 20°C and 0.02 ml of iodo[14 C]acetic acid (1 mCi/ml) was added. After 5 min the samples were cooled to 0°C and made 22 mM with respect to freshly dissolved ether-recrystallized iodoacetic acid that had been neutralized with 2 M-tris base. After standing for 1 h at 0°C, the samples were dialysed exhaustively against the appropriate solvent.

Amino acid analysis

This was performed by the method of Spackman *et al.* (1958) with a Beckman model 120B amino acid analyser fitted with a Locarte automatic loader. All amino acids were separated on the 50 cm column by sequential elution with sodium citrate buffers, pH 3.25 and 4.25 (0.2 M) and pH 6.65 (1.0 M). Duplicate samples were hydrolysed for 24 and 72 h and the amounts of threonine and serine corrected for losses by extrapolation to zero time of hydrolysis. Cysteine was determined as cysteic acid by the performic acid oxidation method of Moore (1963).

Results

Previous experience (Schaub & Perry, 1971) indicated that unless fractionation of [14 C]carboxymethylated inhibitory-factor preparations was attempted under strongly dissociating conditions, e.g. in the presence of 6 M-guanidine hydrochloride, gel filtration gave a complex elution pattern owing to the marked tendency of the protein to aggregate. Preparative gel filtration of the inhibitory-factor preparations (see the Methods section) was therefore carried out on Sephadex G-75 in 6 M-urea–0.2 M-sodium formate (pH 3.3)–15 mM-2-mercaptoethanol and the eluted protein was followed by measuring the E_{280} of fractions. Under these conditions, type I preparations gave two main peaks on elution, peak I at the void volume and a well-defined more retarded component, peak II, which was somewhat variable in amount in different preparations (Fig. 1). The latter peak was present in most preparations, whether of type 1 or 2, although only traces were present in preparations obtained by the latter method. In general with type 1 preparations the amount of peak II present in the total protein eluted was rather variable but usually was less than 50% of the whole preparation. The few inhibitory-factor preparations containing 40–50% or more of peak II material appeared to be more soluble in 10 mM-tris-HCl,

Fig. 2. Inhibitory activity on the Mg^{2+} -stimulated ATPase of desensitized actomyosin of fractions from a type 1 inhibitory-factor preparation

Peaks I and II were obtained by gel filtration under conditions described in Fig. 1. Assays were carried out under standard conditions (see the Methods section) with 0.50 mg of desensitized actomyosin. No troponin was added. The relative ATPase activity is the activity in the presence of the inhibitory factor expressed as a percentage of the activity in its absence. ○, Whole preparation; ●, peak I; △, peak II.

pH 7.6, than the preparations consisting principally of peak I material. Each peak, when rechromatographed separately, was eluted in the same position as it was when the original inhibitory preparation was chromatographed. The less-retarded component was usually eluted as a single peak, but often a slight shoulder appeared in the trailing side (Fig. 1). This shoulder was more pronounced when material from type 2 preparations was run.

Biological activity of fractions obtained by gel filtration of inhibitory-factor preparations

Peaks I and II obtained by Sephadex chromatography in urea-formate of type 1 inhibitory-factor preparations (Fig. 1) both possessed inhibitory activity towards the Mg^{2+} -activated ATPase of desensitized actomyosin (Fig. 2). When compared on a nitrogen basis peak II material was 1.5–2 times more effective in producing 50% inhibition of the Mg^{2+} -activated ATPase of a given desensitized actomyosin preparation than the original or peak I materials. In preparations containing relatively low amounts of peak II material little difference could be found between the specific activity of the original inhibitory-factor preparations and the peak I material derived from it.

Full inhibitory activity was usually obtained with the material from peaks I and II, and with the original inhibitory factor in the absence of tropomyosin, and addition of this protein did not further increase the inhibition. In some cases larger amounts of tropomyosin decreased the extent of inhibition. The lack of requirement for tropomyosin was not due to the presence of significant amounts of this protein in the desensitized actomyosin, as the latter preparation did not give a precipitin band in the Ouchterlony procedure when allowed to diffuse against anti-tropomyosin in 0.15M- or 1.0M-KCl (F. J. Wilson, unpublished work). Small amounts of material that migrated with a mobility similar to that of tropomyosin could sometimes be detected, however, when large amounts (600 μ g) of desensitized actomyosin were loaded on to sodium dodecyl sulphate gels and subjected to electrophoresis.

Purified troponin from which the inhibitory-factor preparation type I was obtained required the addition of tropomyosin for restoration of the Ca^{2+} -sensitivity of the Mg^{2+} -stimulated ATPase when tested against the same desensitized actomyosin as used to assay the inhibitory factor. A similar lack of requirement of tropomyosin for full inhibitory activity was shown when the inhibitory-factor type 1 was tested on actoheavy meromyosin prepared from actin and chymotrypsin-prepared heavy meromyosin. Gel-electrophoresis studies or immunochemical tests (also kindly carried out by Dr. F. J. Wilson) gave no

Fig. 3. Polyacrylamide-gel electrophoresis of inhibitory-factor preparations and fractions

Approx. 20 μ g of protein was applied in each case to 7.5% gels containing 6.25M-urea–0.9M-acetic acid, pH 3.2. (a) Type 1 inhibitory-factor preparation; (b) peak I from type 1 inhibitory-factor preparation (see Fig. 1); (c) peak II from type 1 inhibitory-factor preparation; (d) type 2 inhibitory-factor preparation; (e) '37000 component'; (f) '23000 component'. See the Results section for explanation of bands A, B and C.

evidence of significant amounts of tropomyosin in the inhibitory-factor preparations.

It was noted that on addition of type 1 inhibitory factor to actin solutions in 25mM-tris–HCl, pH 7.6, turbidity developed and actin was precipitated from solution. The effectiveness of the inhibitory factor in precipitating actin varied somewhat from preparation to preparation, but usually most of the actin in solution was precipitated by about 10–20% of its weight of inhibitory factor. Similar turbidity changes were obtained at low ionic strength on addition of inhibitory factor to solutions of tropomyosin, heavy meromyosin and bovine serum albumin. With the last-named three proteins, however, precipitation did not appear to be as complete as with actin. It is likely that at pH 7.6 the interaction is largely of an electrostatic type between the positively charged inhibitor and the other negatively charged proteins, for the effect did not occur if 50–60mM-KCl was added to the buffer.

Material from both peaks I and II retained the ability to precipitate actin and although the relative effectiveness, as judged by the turbidity produced by a given amount of protein, was somewhat variable, in two out of three experiments peak II material was more effective in precipitating actin than either peak I material or the original preparation.

Electrophoresis of inhibitory-factor preparations

The gel-filtration studies on Sephadex G-75 or G-100 indicated that heterogeneity in size could be

detected in type 1 inhibitory-factor preparations. It was not clear, however, whether the observed heterogeneity was due to polymeric forms of a single active subunit or to the presence of several different molecular species. It was also noted that the specific activities of type 1 inhibitory-factor preparations, the fractions from it, and type 2 preparations varied and were often significantly lower than the inhibitory-factor preparations obtained previously from natural actomyosin (Schaub & Perry, 1971) and purified by chromatography on SE-Sephadex.

These observations suggested that the less-active inhibitory-factor preparations either contained material less effective in the enzymic test than the '23000 component' or possibly appreciable amounts of inactive protein. The electrophoretic behaviour of both preparations on polyacrylamide gels was therefore studied under conditions favouring dissociation, namely in 0.9M-acetic acid (pH 3.2)–6.25M-urea and also in sodium dodecyl sulphate.

Type 2 inhibitory-factor preparations usually migrated at pH 3.2 in a reproducible pattern (Fig. 3). This consisted of a slow band, A, and a faster band, B, which occasionally appeared as a doublet. Minor bands were sometimes apparent in such preparations,

but some of these disappeared in prolonged storage at pH 3.2 in 6M-urea before electrophoresis.

Type 1 preparations gave a much less sharply defined picture on electrophoresis. A band of the same mobility as band A was always present, but the band pattern in the region where band B migrated was often complex and apparently consisted of a series of components of different electrophoretic mobilities. In addition all type 1 inhibitor preparations contained a third band, C, which moved faster than the material corresponding to bands A and B (Fig. 3).

When both types of inhibitory-factor preparation were examined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate some of the differences apparent between them disappeared (Fig. 4). Type 2 preparations consisted principally of two main components whose molecular weights were calculated to be 37400 and 23600 by comparison with the mobilities of protein standards of known molecular weights. Type 1 preparations also consisted mainly of 37000- and 23000-dalton components but with an additional component of molecular weight 14000. The similarity of the band pictures of the two preparations on sodium dodecyl sulphate gels suggested that the apparent differences on electro-

Fig. 4. Sodium dodecyl sulphate-gel electrophoresis of inhibitory-factor preparations and components

Protein was applied to 10% polyacrylamide gels containing 0.1% sodium dodecyl sulphate, 0.1% 2-mercaptoethanol, 0.01M-sodium phosphate buffer, pH 7.0. (a) 300 μ g of whole myofibrils; (b) 60 μ g of type 1 inhibitory-factor preparation; (c) 60 μ g of type 2 inhibitory-factor preparation; (d) 40 μ g of '37000 component'; (e) 40 μ g of '23000 component'; (f) 50 μ g of peak II on gel filtration in 6M-urea–0.2M-sodium formate–15mM-mercaptoethanol, pH 3.3. See Fig. 1 for the method of preparing peak II. Bands A, B and C correspond to those illustrated in Fig. 3.

phoresis at pH3.2 in 6.25M-urea might arise from micro-heterogeneity owing to modification during the preparative procedures. This micro-heterogeneity would not be observed on gel electrophoresis in the presence of sodium dodecyl sulphate, where mobility is determined by size. Thus the main difference between the two preparations was the increased amount of material of 14000 daltons present in the type 1 inhibitory-factor preparations. On careful examination, however, traces of the low-molecular-weight material could also be detected in type 2 preparations.

When peak II material obtained from type 1 preparations by chromatography on Sephadex G-75 was examined by gel electrophoresis in sodium dodecyl sulphate and at pH3.2 it was shown to correspond to band C and have a molecular weight of 14000. Similarly it was shown that the two major peaks isolated from type 2 preparations by chromatography on CM-cellulose (see below) corresponded to bands B and A with molecular weights of 23000 and 37000 (see Figs. 3 and 4).

In addition to these three major components, sodium dodecyl sulphate gels of inhibitory-factor preparations showed several minor bands of molecular weights 29500, 27000, 22000, 17000, 16000 and 12000. No attempt, however, was made to isolate these components (see Fig. 4).

When freshly prepared whole myofibrils were examined by sodium dodecyl sulphate-gel electrophoresis, bands corresponding to the '23000 component' and '37000 component' were clearly apparent (Figs. 4 and 5). On the other hand, even when 0.8–1.0mg of total myofibrillar protein was applied to the sodium dodecyl sulphate gels no significant amount of '14000 component' could be detected.

The results obtained from molecular-weight determinations by sodium dodecyl sulphate-gel electrophoresis and gel filtration in 6M-guanidinium chloride are summarized in Table 1.

Biological activity of '23000 component' and '37000 component'

Electrophoretic studies (Schaub & Perry, 1969, 1971) indicated that inhibitory-factor preparations consisted principally of basic protein of isoelectric point between pH8 and 10. A procedure similar to that used by Senshu & Iwai (1970) for the fractionation of histones was therefore evaluated for the separation of the '37000 and 23000 components' in type 2 inhibitory-factor preparations. When such preparations were applied to a column of CM-cellulose equilibrated with 2.6M-formic acid–0.1M-sodium formate in aq. 70% (v/v) ethanol and subsequently eluted with a linear gradient of decreasing ethanol concentration, fractionation into two main peaks, corresponding to the '23000 com-

Fig. 5. Diagram of main bands seen on sodium dodecyl sulphate-gel electrophoresis of whole myofibrils isolated from rabbit skeletal muscle

For experimental details see the text and Perrie & Perry (1970) for light-chain nomenclature (MI_1 etc.).

ponent' and '37000 component' respectively, was obtained (Fig. 6). Components isolated by this procedure and also by repeated gel filtration on Sephadex G-150 in 8M-urea – 0.2M-sodium formate – 0.5mM-dithiothreitol, pH3.3, were tested for biological activity after monitoring for homogeneity by sodium dodecyl sulphate-gel electrophoresis.

Of the two proteins only the '23000 component' had any significant inhibitory activity. Practically all the preparations of '23000 component', and particularly the most active, required tropomyosin for full inhibitory activity. The mean specific activities (see the Methods section) of the '23000 component' were $201 \pm 112 \mu\text{g}$ in the absence of tropomyosin and $40 \pm 8.2 \mu\text{g}$ in the presence of optimum amounts of the protein. The best preparations had a specific activity of $35 \mu\text{g}$ in the presence of optimum amounts of tropomyosin. In general the less the amount of minor band material that could be detected in the preparations the greater was their inhibitory activity in the presence of tropomyosin. The low activity without added tropomyosin could not be ascribed to tropomyosin contamination of the preparation for this protein could not be detected either electro-

Table 1. *Molecular weights of components of the troponin complex*

Experimental details are given in the Methods section. Standard deviations are given where calculated, with numbers of determinations in parentheses.

Component	Mol.wt.	
	By electrophoresis with sodium dodecyl sulphate	By gel filtration in 6M-guanidinium chloride
Inhibitory factor	37400 ± 1000 (46)	35600
	23600 ± 600 (38)	23000* (2)
	14400 ± 800 (39)	
Calcium-sensitizing factor	18600 ± 750 (26)	17900* (2)

* Average of results obtained in this investigation and that of Schaub & Perry (1971).

Fig. 6. *Chromatography of a type 2 inhibitory-factor preparation*

Protein (270 mg) was applied to a column (1.8 cm × 34 cm) of CM-cellulose in aq. 70% (v/v) ethanol–2.6 M-formic acid–0.1 M-sodium formate. The column was eluted with a linearly decreasing gradient to aq. 48% (v/v) ethanol. Fractions eluted between (1) 490 ml and 610 ml and (2) 680 ml and 820 ml were pooled and corresponded to the ‘23000 component’ and ‘37000 component’ respectively.

phoretically or immunochemically (kindly carried out by Dr. F. J. Wilson) in the preparations with inhibitory activity. Carboxymethylation with iodoacetic acid of the ‘23000 component’ decreased its activity slightly but did not alter its requirement for tropomyosin for maximum activity.

When increasing amounts of ‘23000 component’ were added to desensitized actomyosin a lag phase was often observed in response to low amounts. The reason for this is not clear, but could be explained by the presence of a trace of calcium-sensitizing factor in the desensitized actomyosin preparation. Such an explanation was supported by the observation that some desensitized actomyosin preparations when heavily loaded (600 µg) showed a faint band on sodium dodecyl sulphate–gel electrophoresis corresponding in molecular weight to

that of the calcium-sensitizing factor. Any calcium-sensitizing factor present would neutralize added inhibitory factor and thus prevent its action on the Mg²⁺-activated ATPase until the inhibitory factor was present in excess. Another feature of the assay system that was noted was a slight activation of the Mg²⁺-stimulated ATPase of desensitized actomyosin (see Fig. 8) by tropomyosin alone. The effect has been commented on elsewhere (Schaub *et al.*, 1967), and like the lag effect described above it appeared to be a feature of the desensitized actomyosin preparations. Neither of these properties occurred consistently, and preliminary studies suggest that the effects could be eliminated if, after the precipitation with Weber’s solution, the actomyosin was dissolved in 0.6 M-KCl and reprecipitated again before proceeding with the washing of the precipitate. In the later

stages of the work all desensitized actomyosin preparations were made this way.

Whereas the '23000 component' had a fairly consistent requirement for tropomyosin for full inhibitory activity, the '14000 component' was much more variable in response. Although generally of lower specific activity on a nitrogen basis than the '23000 component', activity of many preparations was relatively unchanged by tropomyosin. This effect was intrinsic to the '14000 component' preparation, for when such preparations were compared with the '23000 component' by assaying with the same sample of desensitized actomyosin and tropomyosin, only the activity of the '23000 component' was enhanced significantly by the latter protein (Fig. 8). By using the same assay system it was also shown that troponin needed to be supplemented with tropomyosin to restore calcium sensitivity to the Mg^{2+} -stimulated ATPase.

The '37000 component' sometimes showed a very slight inhibitory activity that was enhanced by the addition of tropomyosin. Although this activity was usually considered to be due to minor contamination

with the '23000 component', it should be mentioned, however, that some preparations had more inhibitory activity than would be expected from the amount of

Fig. 7. Effect of tropomyosin on the activity of the inhibitory factor

Conditions are described in the Methods section; 0.50mg of desensitized actomyosin was used. Relative ATPase activity is expressed as in Fig. 2. Δ, '23000 component'; ●, '23000 component' + 50 μg of tropomyosin.

Fig. 8. Comparison of the effect of tropomyosin on the inhibitory activities of the '23000 component' and '14000 component'

Assays were carried out under standard conditions with 0.8mg of desensitized actomyosin and 50 μg of tropomyosin. The same samples of tropomyosin and desensitized actomyosin were used for both experiments. Relative ATPase activity is expressed as in Fig. 2. (a) '23000 component'; (b) '14000 component'. Δ, Without tropomyosin; ●, with tropomyosin.

contamination with the '23000 component' estimated to be present from the density of staining of gels after sodium dodecyl sulphate-gel electrophoresis.

The '37000 component' would not replace tropomyosin in conferring inhibitory activity to the '23000 component', or in restoring the EGTA [ethanedioxybis(ethylamine)tetra-acetate]-sensitivity of troponin when tested on the Mg^{2+} -stimulated ATPase of desensitized actomyosin.

The '37000 component' was apparently not essential for the reconstitution of the relaxing-protein system. Preparations containing the '23000 component' and the calcium-sensitizing factor ('18000 component'), which had not been separated during their preparation but which were free of '37000 component', restored EGTA-sensitivity to the Mg^{2+} -stimulated ATPase activity of desensitized actomyosin. In preliminary experiments no striking decrease in the amounts of these components required to restore EGTA-sensitivity was obtained by pre-incubating them with the '37000 component' in 6M-urea (cf. Greaser & Gergely, 1971*b*).

Amino acid composition

The amino acid compositions of the isolated components of the inhibitory factor are presented in Table 2. As judged by the electrophoretograms

obtained in sodium dodecyl sulphate over a range of protein concentrations (20–200 μ g) the preparations of '23000 component' and '37000 component' analysed were estimated to be at least 90% pure.

In assessing the analytical results for the '14000 component' it should be noted that the preparations usually contained small amounts of components that were of slightly faster and slower mobilities than the main band obtained on gel electrophoresis at pH 3.2 (Fig. 3). The composition of two preparations that were analysed differed somewhat, particularly in the content of aspartate and glutamate, and the results presented in Table 2 are an average of these two analyses. The presence of contaminants was also observed on electrophoresis in the presence of sodium dodecyl sulphate (Fig. 4) and by comparison with known standards the molecular weights of the material represented by the additional faint bands were estimated to be 17000, 16000 and 12000. Unlike the '23000 and 37000 components' the band corresponding to the '14000 component' was rather diffuse in both the electrophoretic systems employed. The amounts of additional components in the 14000-molecular-weight fraction varied between preparations but was always minor.

The absence of cysteine from the '14000 component' is of particular significance and may explain why this component was not detected when [^{14}C]-

Table 2. Amino acid compositions of components of inhibitory-factor preparations

Experimental details are given in the Methods section.

Component (daltons) ...	Content (mol of residue/mol)			Content (mol/10 ⁵ g)		
	37000	23000	14000†	37000	23000	14000‡
Lys	42.7	24.2	17.2	115.2	105.3	122.8
His	6.4	3.9	1.9	17.4	17.0	13.6
Arg	27.4	15.2	9.2	73.9	66.1	65.7
Asp	26.6	18.8	12.8	71.8	81.8	91.4
Thr*	7.1	4.0	5.5	19.2	17.4	39.3
Ser*	11.0	10.4	6.0	29.7	45.2	42.8
Glu	76.2	37.6	18.4	205.7	163.6	131.4
Pro	11.1	5.6	4.3	30.0	24.4	30.7
Gly	10.5	9.7	7.2	28.4	42.2	51.4
Ala	33.7	17.1	11.4	91.0	74.4	81.4
Val	13.9	8.7	4.7	38.9	37.8	33.6
Met	5.6	9.2	3.2	15.3	40.0	22.8
Ile	9.4	5.2	4.6	25.2	22.6	32.8
Leu	23.8	20.0	12.2	64.2	87.0	87.1
Tyr	4.8	2.5	2.4	12.9	10.9	17.1
Phe	5.6	3.3	2.8	15.3	14.4	20.0
Cys†	0.6	3.2	—	1.6	13.9	—

* Values extrapolated to zero time of hydrolysis.

† Determined as cysteic acid after oxidation.

‡ Average of two different preparations.

Fig. 9. Gel filtration of a type 1 inhibitory-factor preparation previously alkylated with iodo[^{14}C]acetate. A portion (1 ml) of protein solution (E_{280} 5.6) was applied to a Sephadex column (1.5 cm $\times$ 90 cm) in 6M-urea–0.2M-sodium formate, pH 3.3. ●, E_{280} ; ○, radioactivity.

carboxymethylated inhibitory factor was eluted from Sephadex G-200 by 6M-guanidinium chloride and the column eluates were assayed by radioactivity measurements (Schaub & Perry, 1971). To confirm the validity of this explanation type 1 inhibitory factor was carboxymethylated with iodo[^{14}C]acetate and fractionated on Sephadex G-75 in the urea-formate system (Fig. 9). Under these circumstances the radioactivity was eluted mainly associated with the protein peak I. No significant amount of radioactivity was associated with peak II.

The two peaks of radioactivity observed within protein peak I represented partial resolution of the components having molecular weights of 37000 and 23000 by gel filtration in the urea-formate system. The main retarded radioactive peak contained most of the radioactivity and represented the '23000 component' of higher specific radioactivity (Table 1). The distribution of the radioactivity between these components was confirmed by electrophoresis of the [^{14}C]carboxymethylated type I inhibitory-factor preparation in sodium dodecyl sulphate. The bands corresponding to the components of molecular weights 37000, 23000 and 14000 were cut out and their radioactivities measured. No significant radioactivity was present in the 14000-daltons band, whereas the '23000 component' contained about fourfold the radioactivity of the '37000 component'.

Further gel-filtration studies of radioactively labelled preparations suggest that the '37000 component' in fact contains no cysteine and that that found by analysis and suggested by the results of sodium dodecyl sulphate-gel electrophoresis was probably due to slight impurity in the preparation and non-specific labelling during alkylation respectively.

Relationship between the components of the inhibitory-factor preparations

The fact that both the '23000' and '14000 components' possess inhibitory activity raises the question of the relationship between them and the '37000 component'. In contrast with the '23000' and '37000 components', no significant amounts of '14000 component' could be detected on sodium dodecyl sulphate-gel electrophoresis of whole myofibrils (Figs. 4 and 5). This observation suggested that the '14000 component' was produced during the preparation of the inhibitory-factor preparations.

The amino acid analysis results are such that, with the exception of the threonine value for the '23000 component', the '14000 component' could be derived from either the '23000 component' or the '37000 component'. It is not compatible, however, with the derivation of one molecule of each of the '14000' and

'23000 components' from one molecule of the '37000 component'.

No significant increase in the amount of the '14000 component' present in a type 1 inhibitory-factor preparation, which was initially rather deficient in this component, was observed after the preparation was left at 0°C for 10 days in the presence of 0.01 M-HCl. Rechromatography of peak I (Fig. 1) on Sephadex G-75 in 6 M-urea-0.2 M-sodium formate-15 mM-2-mercaptoethanol, pH 3.3, failed to produce any material that was eluted in the position of peak II. These observations suggest that if any interconversion took place it was not due simply to the acid conditions during preparation. This conclusion is further supported by the fact that type 1 inhibitory-factor preparations contain more '14000 component' than do type 2 preparations, which are subjected to lower pH values during the fractionation procedure.

The possibility of the formation of the '14000 component' by bacterial proteolytic action on the '23000 component' was unlikely, as the yields of the lower-molecular-weight component were not decreased by taking special care to avoid bacterial contamination during preparation and by inhibiting the action of any bacteria present by the addition of a few drops of toluene to all the solutions used.

When sodium dodecyl sulphate-gel electrophoresis was performed at each stage of preparation of troponin and inhibitory factor it was noted that the '14000 component' and the other minor bands sometimes associated with both troponin and inhibitory-factor preparations (i.e. those with mobilities corresponding to molecular weights 29500, 27000, 22000, 17000, 16000 and 12000; see Fig. 4) first appeared in the supernatants after removal of tropomyosin (from the troponin preparations) by isoelectric precipitation at pH 4.6. If the preparation was left with stirring in the cold-room at this stage and fractions were taken for electrophoretic analysis at increasing time-intervals, the amounts of the '23000' and '37000 components' gradually decreased. At the same time there was an increase in the amounts of the minor components described above and particularly in the material present in the diffusely stained area of the gel corresponding to a molecular weight of 14000. None of the minor bands appearing after the period at pH 4.6 could be positively identified with those present in sodium dodecyl sulphate-gels of the whole fresh myofibrils from which these preparations were made (Fig. 4). The conditions of the isoelectric precipitation (pH 4.5-4.7, 1 M-KCl) suggested that acid proteases of the cathepsin type may have been responsible for the interconversions noted. This explanation seemed likely, as low but significant activity of the cathepsin-D type, assayed as described by Iodice *et al.* (1966), was found in the low-ionic-strength extract from myofibrils.

Discussion

The present study confirms that the major active protein component present in inhibitory-factor preparations, whether made from troponin by chromatographic fractionation in urea or by an acid-precipitation procedure (troponin B), has a molecular weight of 23000. This component we designate the inhibitory factor. Although some preparations had a slight inhibitory effect on the Mg^{2+} -stimulated ATPase of desensitized actomyosin in the absence of tropomyosin their activity was enhanced severalfold by the addition of the latter protein. With the most active preparations at optimum conditions one part by weight of purified inhibitory factor produced 50% of the maximum inhibition obtainable with the Mg^{2+} -stimulated ATPase of 50-60 times its weight of desensitized actomyosin. This activity is only slightly more than that of the most active preparation described by Schaub & Perry (1971), but about twice the average activity of such preparations. The latter preparations were presumed homogeneous, for they were eluted from Sephadex G-200 in 6 M-guanidine hydrochloride as a single peak of radioactivity after carboxymethylation with iodo[^{14}C]acetate. The reason for this apparent homogeneity, despite the fact that the preparations no doubt contained inactive '37000 component' and possibly '14000 component', is now clear. The '37000 component' probably contains no cysteine and the '14000 component' certainly contains none. Therefore after carboxymethylation with iodo[^{14}C]acetate they would not be readily detected by radioactivity measurements on the eluate obtained by gel filtration, for, as the electrophoresis experiments indicate, most of the radioactivity is localized in the '23000 component'.

The preliminary results suggest that the '14000 component' in type I preparations is derived from one of the other components of the relaxing-protein system by the action of muscle cathepsins, although muscle is not normally considered as a tissue rich in proteolytic activity. Formation from the '23000 component' seems very likely from the studies of biological activity, but the '37000 component' and tropomyosin are also possible precursors if amino acid analysis results alone are taken into consideration. Until sufficient information is available to determine the best inhibitor to use, it follows that to minimize catheptic activity during the preparation of the troponin complex and its components the isoelectric precipitation stage should be carried out rapidly and in the cold.

If the '14000 component' is derived from the '23000 component' its formation involves the loss of that part of the inhibitory-factor molecule that contains the cysteine residues. These residues, as has been shown elsewhere (Schaub & Perry, 1971), can be carboxymethylated without destruction of bio-

Table 3. *Molecular weights and possible relationships between components of troponin complex reported by different investigators*

Components in a horizontal row are considered to be identical. Asterisks are used to indicate less certainty of identity with components in the first column.

Biological activity	Schaub & Perry (1971); Wilkinson <i>et al.</i> (1971)	Greaser & Gergely (1971a,b)	Ebashi <i>et al.</i> (1971)	Hartshorne & Pyun (1971)
ATPase-inhibitory activity	14000 23000, inhibitory factor 37000	14000, troponin 1 24000, troponin 2 35000, troponin 3	22000, troponin II* (fraction II) 40000, troponin I* (fraction I)	Troponin B (26000 34000 39000)
Calcium-binding activity	18000, calcium sensitizing factor	19000–22000, troponin 4	17000, fraction III	18500, troponin A

logical activity. The conversion does apparently lead to some loss of inhibitory activity and the product has a variable requirement for tropomyosin for full inhibitory activity. Caution is necessary, however, in comparing the specific inhibitory activities of the '23000 component' and '14000 component', as some preparations of the latter contained about 30% of protein of slightly different molecular weight (see Figs. 3 and 4). The variable requirement for tropomyosin for maximum activity of inhibitory-factor preparation type 1, peaks I and II isolated from it, and the '23000' and '14000 components' is a puzzling feature for which we have no explanation at the moment. We do not believe that these differences are due to tropomyosin contamination because they persist when fractions with inhibitory activity, in which no trace of tropomyosin can be detected, are compared against the same desensitized actomyosin and the same sample of tropomyosin. This observation suggests that the differences are features of the inhibitory-factor preparations themselves. The difference in tropomyosin requirement of the '23000 component' and '14000 component' is not due to the loss of thiol groups, for carboxymethylated '23000 component' requires tropomyosin for full activity. We have previously reported anomalies in the tropomyosin requirement of troponin (Perry *et al.*, 1966) and the inhibitory factor (Schaub & Perry, 1969).

The component of molecular weight 37000 is a consistent component of inhibitory-factor preparations made from the myofibril of skeletal muscle. To our knowledge the isolation and purification of a protein of this molecular weight from the myofibril has not been previously reported, although there have been brief reports of bands corresponding to molecular weights of 34000 and 39000 (Hartshorne & Pyun, 1971) on sodium dodecyl sulphate–gel electrophoresis of troponin fractions. Scopes & Penney (1971), however, in their study of the sodium dodecyl sulphate–gel electrophoresis of the whole myofibril did not identify a '37000 component'. Also since this

work was completed Greaser & Gergely (1971a,b) have reported the isolation of a troponin fraction 3, which they consider to be required together with troponin fractions 2 and 4 to reconstitute the relaxing-protein system. Although troponin fraction 3 has a reported molecular weight of 35000 it may be identical with the '37000 component', for its amino acid analysis (J. Gergely, personal communication) is very similar. The troponin I component described by Ebashi *et al.* (1971) has a similar amino acid composition to the '37000 component', although in some respects its biological function resembles the inhibitory factor. Further detailed investigation is required before a role can be firmly assigned to the '37000 component'. The relationship presumed to exist between the proteins which we consider to be present in troponin and the fractions reported by other investigators are summarized in Table 3. Quite good correlation exists between our components and the troponin fractions reported by Greaser & Gergely (1971b) but identities with the fractions reported by Ebashi *et al.* (1971) are less apparent.

The differences in molecular weight reported by investigators for the various fractions seem rather greater than would be expected from the accuracy of the sodium dodecyl sulphate method that was generally used for their determination. It is possible that they are the consequence of muscle catheptic activity, the effect of which investigators have not been sufficiently aware. The values for molecular weights that we have adopted are the averages of our results obtained by sodium dodecyl sulphate–gel electrophoresis and gel filtration, taken to the nearest thousand.

From our studies we conclude that preparations of inhibitory factor, troponin B and the troponin complex vary, according to the procedure used in their preparation, in the amounts of the '37000', '23000' and '14000 components' that they contain. The conditions of isoelectric precipitation particularly favour formation of the '14000 component', probably through

catheptic activity. The variable composition may be the reason for the variations in specific inhibitory activity of different inhibitory-factor preparations. In particular the fact that the activity of the '14000 component' is often not enhanced by tropomyosin also explains reports (Schaub & Perry, 1969, 1971) that the activity of some inhibitory-factor preparations was much less responsive to the addition of tropomyosin than others.

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