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The Interaction of the Calcium-Binding Protein (Troponin C) with Bivalent Cations and the Inhibitory Protein (Troponin I)

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1. The molecular weight of the calcium-binding protein of rabbit white skeletal muscle was estimated to be 18500 by sedimentation equilibrium and electrophoresis in sodium dodecyl sulphate. 2. Addition of 2 Ca^{2+} ions per molecule produced reversible changes in the u.v.-absorption spectrum that are interpreted as arising from conformational changes in the structure of the protein. 3. Cd^{2+} was almost as effective as Ca^{2+} in producing the spectral changes. Other bivalent metal ions, particularly Mg^{2+}, were less effective. 4. Binding of Ca^{2+} by the calcium-binding protein produced an increase in mobility to the anode on electrophoresis in 6M-urea at pH 8.6. The Ca^{2+}-saturated form of the protein was more retarded on gel filtration than the Ca^{2+}-free form. 5. In the presence of Ca^{2+} the calcium-binding protein formed an equimolar complex with the inhibitory protein. This complex was stable in 8M-urea and in the pH range 7.0–8.6. 6. An isotope-dilution method for the measurement of the content of calcium-binding protein in whole muscle is described. In rabbit psoas muscle the ratio of actin monomers to molecules of calcium-binding protein was approx. 7:1. Similar values were obtained for red skeletal and cardiac muscle. 7. Evidence is presented indicating that in the rabbit the inhibitory protein of the troponin complex of red skeletal and cardiac muscles is different from the inhibitory protein of white skeletal muscle.

It is now well established that the target protein for Ca^{2+} in the troponin complex is the calcium-binding protein, troponin C (Hartshorne & Mueller, 1968; Schaub & Perry, 1969; Wilkinson *et al.*, 1971, 1972; Greaser & Gergely, 1971; Ebashi *et al.*, 1971; Ebashi, 1972; Drabikowski *et al.*, 1971; Murray & Kay, 1971). The changes in physical properties that have been reported as associated with the binding of Ca^{2+} by the troponin complex (Fuchs, 1971; Wakabayashi & Ebashi, 1968) probably reflect modifications in the structure of the calcium-binding-protein component itself. Changes in the calcium-binding protein must, however, induce responses in the associated components of the troponin–tropomyosin system as it functions *in situ*. Not only does the calcium-binding protein influence the function of the other components of the troponin complex but the interactions of these components may also modify the properties of the calcium-binding protein. For example, there is preliminary evidence that the inhibitory protein (troponin I) may influence the Ca^{2+}-binding properties of the calcium-binding protein (Potter & Gergely, 1972).

Han & Benson (1970) and Fuchs (1971) have produced evidence of conformational change on Ca^{2+} binding by the troponin complex from studies of fluorescence changes and thiol-group reactivity respectively. For the reasons stated above it cannot be concluded with certainty that the changes observed with the complex necessarily reflect changes in the calcium-binding protein alone. Nevertheless, studies involving measurement of circular dichroism, optical rotatory dispersion and fluorescence indicate that conformational changes can be induced in isolated calcium-binding-protein preparations by Ca^{2+} (Murray & Kay, 1972; Van Eerd & Kawasaki, 1972). It has been suggested that the changes in electrophoretic mobility that occur on removal of Ca^{2+} from the calcium-binding protein are also due to conformational changes (Schaub *et al.*, 1972; Perry *et al.*, 1972). The changes occurring in the isolated calcium-binding protein must presumably be responsible for part at least of the conformational changes observed to occur in the complex as a whole.

The present study is an investigation of the interaction of the calcium-binding protein with bivalent cations and other components of the troponin complex. New evidence is presented for a conformational change in the calcium-binding protein when it binds Ca^{2+}. It is also shown that the calcium-binding protein binds specifically to the inhibitory protein in the presence of Ca^{2+}. The techniques used in these studies have led to the development of a simple accurate method of determining the concentration of calcium-binding protein in muscle. Some aspects of this work have been briefly reported (Head & Perry, 1973; Perry *et al.*, 1972).

Methods

Preparation of the troponin complex and its components

The troponin complex was prepared essentially by the method of Ebashi *et al.* (1971). Individual components were isolated from the complex by a modification of the method of Schaub & Perry (1969). Frequently troponin was isolated containing small amounts of tropomyosin which interfered with subsequent fractionation. This protein was conveniently removed by fractionation on SP (sulphopropyl)-Sephadex in 25mM-cacodylate–HCl buffer (pH6.5)–1mM-dithiothreitol–0.1mM-$CaCl_2$. Cacodylate was used as a buffer because of its low affinity for Ca^{2+} ions compared with citrate or phosphate, as used in the original method. Usually about 400mg of troponin in 20ml of buffer were applied to a column of 15cm×3cm. Tropomyosin was eluted in the void volume, and on application of a 0–0.4M-KCl gradient in the buffer the troponin complex was eluted at an ionic strength of approx. 0.2 (Schaub & Perry, 1969). The troponin thus isolated was dialysed against 25mM-cacodylate–HCl buffer (pH6.5)–6M-urea–1mM-dithiothreitol–0.1mM-$CaCl_2$ and rerun on SP-Sephadex (column size as above) in this buffer. Under these conditions a complex of the calcium-binding protein and the inhibitory protein was eluted in the void volume and troponin T (the '37000 component'; Wilkinson *et al.*, 1972) at an ionic strength of about 0.25 on application of a 0–0.4M-KCl gradient. The complex of calcium-binding and inhibitory proteins was dialysed against 25mM-cacodylate–HCl (pH6.5)–6M-urea–1mM-dithiothreitol–5mM-EDTA and rerun on SP-Sephadex (column size as above) equilibrated with this buffer. The calcium-binding protein was eluted in the void volume and the inhibitory protein at an ionic strength of about 0.2 by subsequent application of a 0–0.4M-KCl gradient.

Proteolytic breakdown by endogenous cathepsins, traces of which are very difficult to remove from troponin preparations and to which the '37000 component' and the inhibitory protein are particularly susceptible, was minimized by carrying out all procedures at 4°C. The calcium-binding protein appeared much less sensitive to endogenous proteolysis.

Electrophoresis

Polyacrylamide-gel electrophoresis was carried out on 8% polyacrylamide slab gels (Perrie *et al.*, 1973) by using a continuous buffer system of 25mM-Tris–80mM-glycine, pH8.6. As a routine 6M-urea was included in the gel. When non-dissociating conditions were required the urea was replaced by 40% (v/v) glycerol (Perrie & Perry, 1970).

Electrophoresis in the presence of 0.1% sodium dodecyl sulphate was carried out by the method of Weber & Osborn (1969) in 100mM-sodium phosphate buffer, pH7.0, unless otherwise stated.

Ultraviolet absorption

U.v-absorption spectra were measured and recorded by using a Pye–Unicam SP1800 spectrophotometer in conjunction with a Unicam AR25 linear recorder: 1cm-light-path silica cuvettes were used throughout.

Ultracentrifugation

These studies were carried out on a Beckman model E ultracentrifuge equipped with Rayleigh interference optics. Molecular-weight determinations were made by using the long-column, meniscus-depletion, sedimentation-equilibrium technique as described by Chervenka (1970). A 12mm double-sector cell with interference window holders and a double-sector, capillary, synthetic-boundary centre piece was used with an An-D rotor for these studies.

Cross-linking

Dimethyl suberimidate was used as a cross-linking agent as described by Davies & Stark (1970).

Gel filtration

Gel filtration on Sephadex G-200 was carried out on a column (90cm×1.5cm) packed under a pressure of 20cm of solvent at 21°C and operated by continuous downward flow at the packing pressure and temperature. Flow rates depended on the solvent used and varied from 4 to 9g/h.

Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) and dinitrophenylglycine were included with each sample to determine the void and total accessible volume of the column respectively. Samples were dissolved in the elution buffer and sufficient sucrose was added to stabilize them during layering. Samples were applied in not more than 0.25ml and the eluate was collected in 1–2g fractions. Protein content was determined by measurement of the E_{280} or E_{230} or, with samples of known specific radioactivity, by measuring radioactivity of portions of the fractions. Blue Dextran 2000 and dinitrophenylglycine were determined by measurement of E_{630} and E_{360} respectively.

Carbamoylmethylation of calcium-binding protein

An aqueous solution of calcium-binding protein (1mg/ml) was dialysed for 15h against 50mM-potassium phosphate buffer (pH8.2)–5mM-dithiothreitol, followed by dialysis for a similar period against the

same buffer in which the dithiothreitol concentration was lowered to 0.5mM. Guanidinium chloride was added to a concentration of 5M and with N_2 bubbling through the solution the pH was maintained at 8.2 by addition of 0.1M-NaOH. The reaction was started by the addition of 10μCi of iodo[1-^{14}C]acetamide (The Radiochemical Centre, Amersham, Bucks., U.K.; specific radioactivity 58mCi/mmol) to each ml of sample. After 5min unlabelled iodoacetamide, previously recrystallized from water, was added to give four times molar excess over the total thiol groups present in the sample. The reaction was halted after 1h by the addition of 2-mercaptoethanol to a concentration of 50mM. The sample was then dialysed against repeated changes of 20mM-potassium phosphate buffer, pH7.0, until no radioactivity above background could be detected in the diffusion medium. A final dialysis against water, adjusted to pH7.0 by the addition of saturated $NaHCO_3$ solution, was carried out before specific-radioactivity measurements.

Determination of the amount of calcium-binding protein in muscle by isotope dilution

Weighed samples of freshly excised psoas major muscles of the rabbit were dispersed in approx. 5vol. of 6M-urea by using a small Waring Blendor-type homogenizer. The protein concentration of the suspension was measured by the Folin method (Lowry *et al.*, 1951), with total muscle protein as standard, on a portion diluted 100-fold in 0.1M-NaOH. All measurements of the quantity of the muscle suspension were made by weighing, to avoid pipetting errors. The sample of the calcium-binding protein used for the assays was carbamoylmethylated with iodo[1-^{14}C]acetamide and shown to migrate as a single band on electrophoresis in sodium dodecyl sulphate at pH7.0 and in 6M-urea at pH8.6. The specific radioactivity of ^{14}C-labelled carbamoylmethylated calcium-binding protein was measured on the protein dissolved in water, by mixing 0.5–1.0ml of a 100μg/ml solution of the labelled calcium-binding protein (1.53×10^6d.p.m./mg) made up to 6M-urea with up to 10g of muscle suspension in 6M-urea–4mM-EGTA [ethanedioxybis(ethylamine)-tetra-acetate]–50mM-2-mercaptoethanol containing up to 400mg of protein. Equilibrium was achieved by mechanical shaking at 4°C and little difference in results was obtained when this was carried out for more than 1h. A representative sample of pure calcium-binding protein (usually about 1mg) was then isolated from the muscle suspension by preparative polyacrylamide-gel electrophoresis at pH8.6. This was carried out by a procedure developed by Dr. T. Hirabayashi (unpublished work) using a modified form of the apparatus described by Perrie *et al.* (1973). Separation was carried out at 21°C in a 8% gel (1cm×6cm×20cm) containing 6M-urea–25mM-Tris–80mM-glycine, pH8.6. The gel was pre-run for 3h at 50mA, and the total sample of about 10ml of suspension containing ^{14}C-labelled calcium-binding protein applied and run into the gel at 10–20mA for 2h. Electrophoresis was then carried out at 40mA for 4h. The calcium-binding protein, which is the fastest-migrating component of the whole muscle under these conditions, was then located by cutting 2.5mm-thick vertical sections from the centre and both ends of the gel, staining with Amido Black stain (0.6% Amido Black in water–ethanol–acetic acid, 5:3:1, by vol.) for 5min and destained electrophoretically. Thus the position of the band of the calcium-binding protein was determined, so that it could be cut from the remainder of the slab. The gel remaining after removal of this slice was stained for 1h and destained electrophoretically to confirm the sectioning. The protein was removed from the slice of gel by electrophoresis through a glass sinter into dialysis tubing by using the same buffer as for preparative electrophoresis. The calcium-binding protein obtained in this way was dialysed exhaustively against water at 4°C to remove the Tris–glycine buffer completely before measurement of specific radioactivity.

All measurements for the specific radioactivity were made at least in triplicate. Radioactivity measurements were at 10000 counts or above.

Determination of protein

Protein determinations were based on the measurement of non-dialysable N by an ultra-micro method involving nesslerization (Strauch, 1965) assuming a N content of 16%. Where the presence of nitrogen-containing buffers or reagents precluded the use of this method the Folin method (Lowry *et al.*, 1951) was used. All solutions used were tested for interference with the Folin reaction. A standard curve was obtained with exhaustively dialysed samples of calcium-binding protein of known N content determined by nesslerization.

Radioactivity measurements

Known volumes of aqueous solutions of radioactively labelled proteins were made up to 1ml with water and mixed with 3ml of Triton X-100 and 6ml of scintillator [4g of 2,5-diphenyloxazole and 50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene]. Scintillation counting was carried out in a Phillips liquid-scintillation counter model PW4510, with automatic background subtraction.

Determination of carbohydrate

Carbohydrate was measured by the phenol–H_2SO_4 reaction of Dubois *et al.* (1956), sufficient protein

being used to detect 1 residue of monosaccharide per molecule of calcium-binding protein.

Results

Properties of the calcium-binding protein

Calcium-binding protein prepared as described in the Methods section migrated as a single band when samples of up to 150μg were examined by electrophoresis in sodium dodecyl sulphate, pH7.0 (Plate 1*a*) and in 8M-urea, pH8.6. The protein also sedimented as a single component on ultracentrifugation and was eluted as a single peak on gel filtration (see below).

On electrophoresis in sodium dodecyl sulphate–sodium phosphate buffer, pH7.0, the calcium-binding protein migrated with the mobility corresponding to that expected for a protein of molecular weight 18500 by comparison with protein standards with molecular weights in the range 14000–77000 (Plate 1*a*). If the sodium dodecyl sulphate electrophoresis of calcium-binding protein was carried out in the presence of Tris–borate, pH7.0 (Perrie *et al.*, 1973), it migrated as a protein of apparent molecular weight 22000, barely separated from the inhibitory protein of molecular weight 23000 (Plate 1*a*).

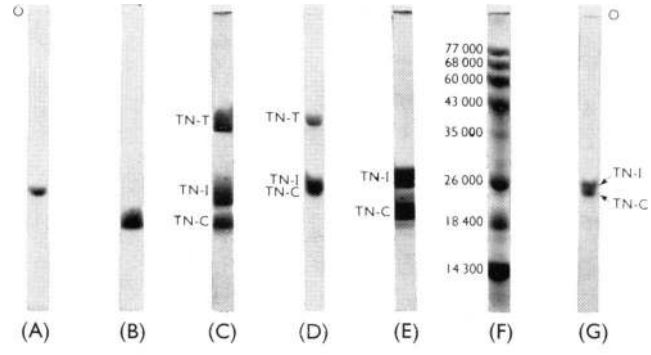
The molecular weight obtained from electrophoresis in sodium dodecyl sulphate and 100mM-sodium phosphate buffer, pH7.0, was identical with that obtained by sedimentation-equilibrium ultracentrifugation studies. The log of the fringe displacement gave a straight line when plotted against the square of the radial distance. By using a partial specific volume of 0.72, calculated from the amino acid analysis (Schachman, 1957) (Table 1) a molecular weight of 18500±980 was obtained from sedimentation-equilibrium runs carried out at 42040 rev./min in 0.1M-KCl–20mM-Tris–HCl (pH7.6)–1mM-dithiothreitol and in 25mM-Tris–80mM-glycine (pH8.5)–1mM-dithiothreitol. This value was not significantly changed when $CaCl_2$ up to 0.1mM was added to the buffer.

On electrophoresis of a sample of the calcium-binding protein on 8% polyacrylamide gel in 8M-urea at pH8.5 in the presence of 0.1mM-$CaCl_2$ the single band obtained migrated with 60% of the mobility of Bromophenol Blue (Plate 1*b*). When another portion of the same sample was run under conditions identical except for the replacement of the $CaCl_2$ by 2mM-EGTA the band migrated approximately 10% slower (Plate 1*b*). No difference in molecular weight in the absence or presence of Ca^{2+} could be detected on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate or by sedimentation-equilibrium studies. The change in mobility was opposite to that which would be expected for a change in charge resulting from the binding of Ca^{2+} and it seemed likely that this change in mobility reflected an alteration in conformation

Table 1. *Amino acid analysis of the calcium-binding protein*

Results are the means of duplicate analyses of six preparations after 24 and 72h hydrolysis. The composition was calculated by adding up residue weights and relating them to 18500g, assuming 100% recovery. Analyses were carried out as described by Wilkinson *et al.* (1972). Cysteine was measured as cysteic acid after oxidation of protein with performic acid (Moore, 1963). The tryptophan estimate was based on fluorescence at 350nm on excitation at 280nm.

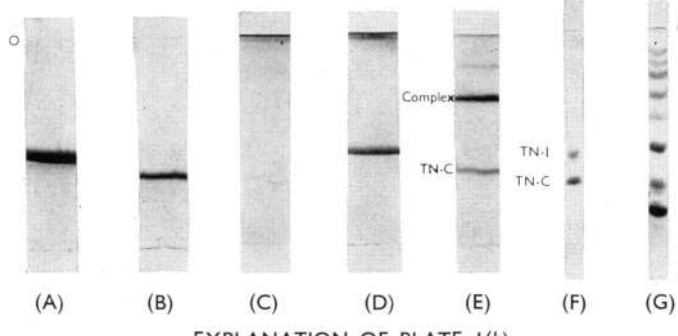
	Content (residues/18500g)			
	This study	Hartshorne & Pyun (1971)	Murray & Kay (1972)	Greaser *et al.* (1972)
Lys	9.9	9.5	10.8	9.5
His	1.2	1.1	1.5	1.1
Arg	7.3	6.7	6.8	7.0
Asx	23.0	21.1	21.3	23.5
Thr	7.5	6.7	6.1	5.7
Ser	8.4	7.7	7.0	6.8
Glx	33.5	26.3	27.6	35.2
Pro	0.9	1.4	3.4	2.0
Gly	11.7	13.3	12.5	12.8
Ala	13.8	13.2	13.5	12.9
Val	6.9	7.1	8.2	7.1
Met	8.4	11.3	8.5	9.3
Ile	9.8	9.4	9.0	9.1
Leu	10.1	9.7	10.8	9.1
Tyr	2.3	2.0	2.5	1.9
Phe	9.4	10.6	9.2	9.3
Trp	0	—	—	0
Cys	0.9	—	—	0.9



EXPLANATION OF PLATE 1(*a*)

Polyacrylamide-gel electrophoresis of the calcium-binding protein and the troponin complex in the presence of sodium dodecyl sulphate

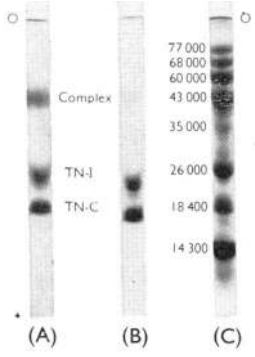
Electrophoresis was carried out in 10% polyacrylamide–0.1% sodium dodecyl sulphate; 100mM-sodium phosphate, pH 7.0 (phosphate), or 82.5mM-Tris–400mM-borate, pH 7.0 (Tris–borate), were used as indicated. Abbreviations: TN-C, calcium-binding protein; TN-I, inhibitory protein; TN-T, 37000 component. O marks the origin. (A) 10μg of calcium-binding protein (Tris–borate); (B) 20μg of calcium-binding protein (phosphate); (C) 50μg of troponin complex (phosphate); (D) 40μg of troponin complex (Tris–borate); (E) 50μg of 1:1 molar mixture of the inhibitory and calcium-binding proteins (phosphate); (F) standard protein mixture containing transferrin (77000), bovine serum albumin (68000), catalase (60000), ovalbumin (43000), pepsin (35000), chymotrypsin (25700), β-lactoglobulin (18400), lysozyme (14300) (Tris–borate); (G) 20μg of 1:1 molar mixture of inhibitory and calcium-binding proteins (Tris–borate).



EXPLANATION OF PLATE 1(*b*)

Effect of Ca^{2+} on the electrophoretic mobility of the calcium-binding protein in the absence and the presence of inhibitory protein

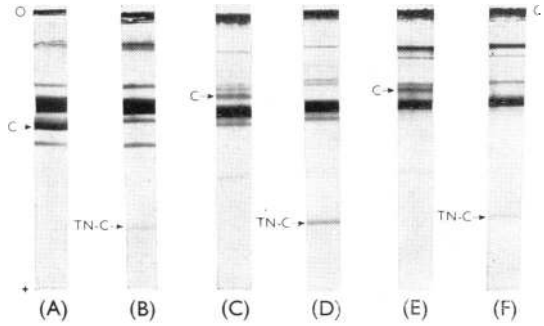
Electrophoretic runs (A) to (E) were carried out in 8% polyacrylamide–25mM-Tris–80mM-glycine (pH 8.6)–6M-urea; samples were applied dissolved in 6M-urea–50mM-2-mercaptoethanol. Runs (F) and (G) were on 10% polyacrylamide–0.1% sodium dodecyl sulphate–100mM-sodium phosphate, pH 7.0; samples were applied in 1% sodium dodecyl sulphate–10mM-sodium phosphate buffer (pH 7.0)–50mM-2-mercaptoethanol. Abbreviations are as for Plate 1(*a*). (A) 25μg of calcium-binding protein with 2mM-EGTA in sample; (B) 15μg of calcium-binding protein with 0.1mM-$CaCl_2$ in sample; (C) 20μg of inhibitory protein; (D) 20μg of inhibitory protein + 30μg of calcium-binding protein + 2mM-EGTA in sample; (E) 20μg of inhibitory protein + 30μg of calcium-binding protein with 0.1mM-$CaCl_2$ in sample; (F) band of complex extracted from urea gel, pH 8.6, and applied to sodium dodecyl sulphate gel; (G) protein standards as for Plate 1(*a*), gel (F).



EXPLANATION OF PLATE 2(*a*)

Cross-linking of the calcium-binding protein to the inhibitory protein with dimethyl suberimidate

Protein (2mg/ml) was treated with dimethyl suberimidate (2mg/ml); then sodium dodecyl sulphate and 2-mercaptoethanol were added to 1% and 50mM respectively. Polyacrylamide-gel electrophoresis was performed in 0.1% sodium dodecyl sulphate–100mM-sodium phosphate buffer, pH 7.0. Abbreviations are as for Plate 1(*a*). (A) 50μg of equimolar mixture of inhibitory protein and calcium-binding protein cross-linked in the presence of 0.1 mM-$CaCl_2$; (B) as (A) but with 2mM-EGTA in place of $CaCl_2$; (C) standard proteins as for Plate 1(*a*), gel (F).



EXPLANATION OF PLATE 2(*b*)

Polyacrylamide-gel electrophoresis of extracts of rabbit red and white muscle in the absence and the presence of $CaCl_2$

Electrophoresis was carried out in 8% polyacrylamide–25mM-Tris–80mM-glycine (pH 8.6)–6M-urea. Approx. 1 vol. of whole muscle was dissolved in 5 vol. of 6M-urea–50mM-2-mercaptoethanol and about 0.1 ml of the suspension was applied to the gel with additions indicated. C, Complex of inhibitory and calcium-binding proteins; TN-C, troponin C, calcium-binding protein. (A) Psoas, 0.1 mM-$CaCl_2$; (B) psoas, 2mM-EGTA; (C) soleus, 0.1 mM-$CaCl_2$; (D) soleus, 2mM-EGTA; (E) cardiac, 0.1 mM-$CaCl_2$; (F) cardiac, 2mM-EGTA.

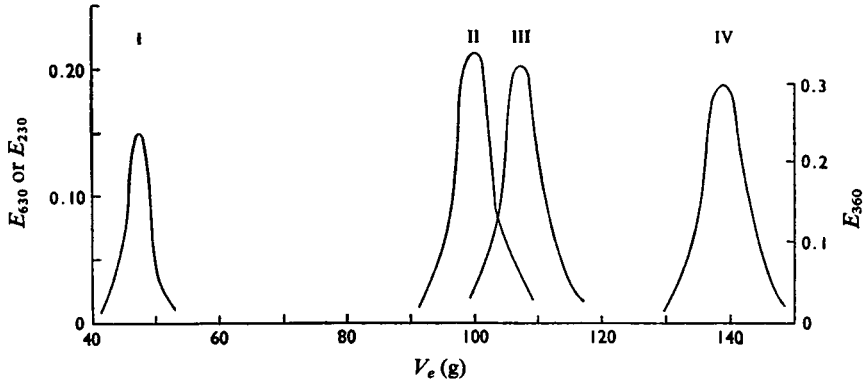


Fig. 1. *Gel filtration of calcium-binding protein in the presence and absence of $CaCl_2$*

Protein (10mg) in 0.25ml of 0.1M-KCl–20mM-Tris–HCl (pH7.6)–1mM-dithiothreitol was applied to a column of Sephadex G-200 previously equilibrated against the buffer, 20°C. Peaks: I, Blue Dextran (E_{630}); II, calcium-binding protein with 5mM-EGTA included in buffer (E_{230}); III, calcium-binding protein with 0.1mM-Ca^{2+} included in buffer (E_{230}); IV, dinitrophenylglycine (E_{360}).

of the protein molecule. Further evidence of such a change was given by the dependence of the elution position on gel filtration on the absence or presence of low concentrations of Ca^{2+}. In a typical experiment when the calcium-binding protein was run on a Sephadex G-200 column (90cm × 1.5cm) in the presence of 0.1M KCl–20mM-Tris–HCl (pH7.6)1mM-dithiothreitol–0.1mM-$CaCl_2$ the elution peak was at 107g. On inclusion of 5mM-EGTA in the column buffer the peak of eluted calcium-binding protein was at 100g (Fig. 1).

U.v.-absorption spectrum

The u.v.-absorption spectrum of calcium-binding protein is characteristic of a protein with low tyrosine and tryptophan and high phenylalanine content (Hartshorne & Pyun, 1971; Schaub *et al.*, 1972). Fig. 2 compares the u.v. spectrum obtained at pH7.6 with that of a phenylalanine/tyrosine mixture in the molar ratio 9:2. In the protein the fine structure of the spectra of these amino acids is subject to a long-wave shift of approx. 1nm. The absorption spectrum of the calcium-binding protein in this region showed a characteristic change as the free Ca^{2+} concentration fell from 0.1mM to 0.1 μM (cf. Van Eerd & Kawasaki, 1972) producing the difference spectrum illustrated in Fig. 3. An identical difference spectrum was obtained if the Ca^{2+} was removed from the calcium-binding protein by passage through a column of Chelex 100 (Bio-Rad Laboratories, St. Albans, Herts., U.K.) in 20mM-Tris–15mM-HCl, pH7.6, or by treatment with EGTA.

The shoulder at 249nm and maxima at 253, 259, 265 and 269nm in the difference spectrum appear to arise from increases in fine structure of the phenyl-

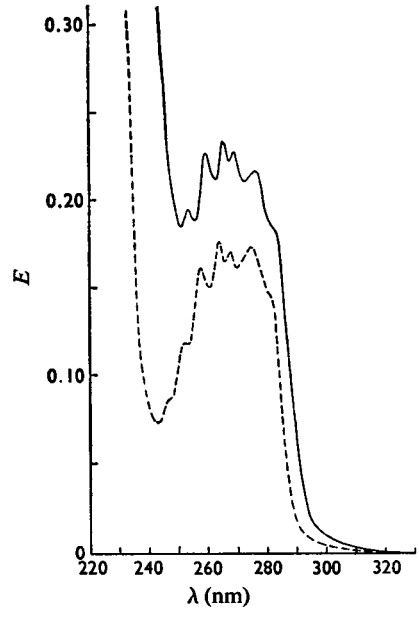


Fig. 2. *U.v.-absorption spectrum of calcium-binding protein and a mixture of tyrosine and phenylalanine*

Protein and amino acids were dissolved in 0.1M-KCl–20mM-Tris – 15mM-HCl (pH7.6) – 0.1mM-$CaCl_2$. For further details see the text. ——, Calcium-binding protein (1mg/ml); ----, 0.11mM-tyrosine + 0.49mM-phenylalanine.

alanine absorption spectrum. The maxima at 276nm and 285nm correspond to those arising from perturbations of the environment of tyrosine (Herskovits,

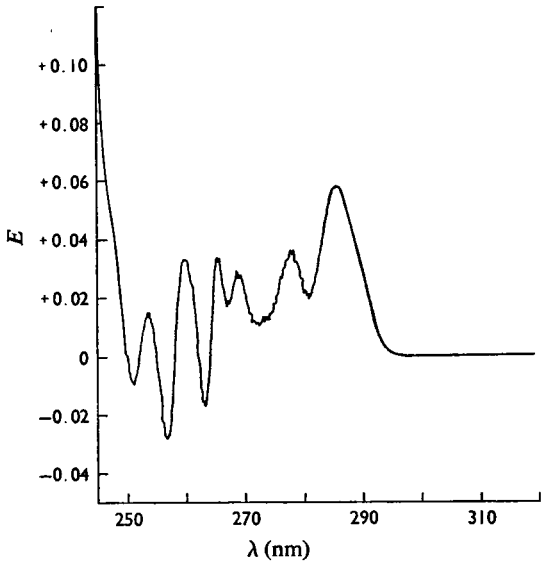


Fig. 3. *Difference spectrum of calcium-binding protein on addition of Ca^{2+} to the calcium-free protein*

In the reference cell (1 cm light-path) was calcium-binding protein (6mg/ml) freed of Ca^{2+} by treatment with Chelex (see the Methods section), in 100mM-KCl–10mM-Tris–7.5mM-HCl, pH7.6. The sample cell contained the same constituents with 0.1mM-$CaCl_2$ added.

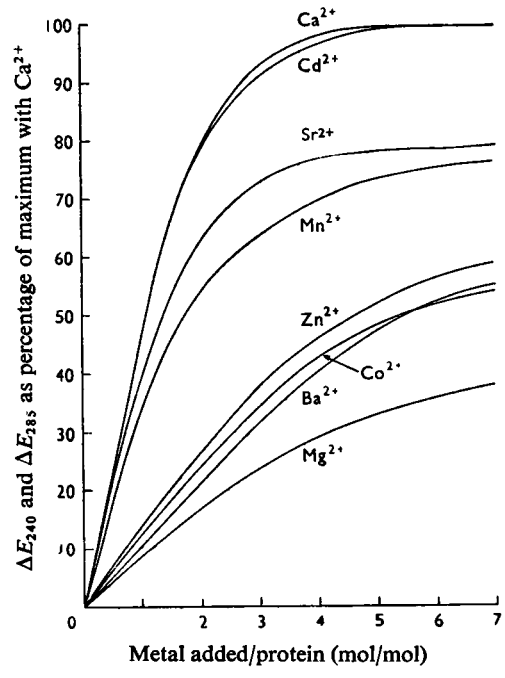


Fig. 4. *Change in absorption at 240 and 285 nm on addition of bivalent cations to metal-free calcium-binding protein*

Calcium-binding protein (1mg/ml) was in 100mM-KCl–10mM-Tris–7.5mM-HCl, pH7.6. Results are plotted as percentages of maximum change obtained when protein is fully saturated with Ca^{2+}. Curves were obtained by joining up points obtained by duplicate measurements of absorbance at different bivalent cation concentrations.

1967). Over the region 200–230nm the difference spectrum showed a sharp rise and no peak could be observed at the lower wavelengths, owing to the limitations of the spectrophotometer.

Measurement of change of absorption at the maxima with increasing Ca^{2+} concentration showed a saturation relationship. On addition of 2mol of Ca^{2+} per mol of calcium-binding protein the spectral changes were virtually complete, and little further change occurred on addition of excess of Ca^{2+} (Fig. 4).

When Ca^{2+} was replaced by Mg^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Sr^{2+} or Ba^{2+} difference spectra similar to that produced by Ca^{2+} were obtained, although generally higher metal ion/protein ratios were required to bring about the spectral change (Fig. 4). In many cases this did not appear to be complete compared with the effect obtained with Ca^{2+} even in the presence of excess of bivalent cation. Some correlation was apparent between the efficiency in replacing Ca^{2+}, as measured by the molar ratio of cation to protein required to complete the spectral change, and the similarity in ionic radius to that of Ca^{2+}. In these studies the protein was freed of Ca^{2+} either by passage through Chelex 100 or by treatment with 10mM-EGTA. In the latter case the Ca–EGTA and EGTA were removed by exhaustive dialysis against deionized water to prevent release of Ca^{2+} by displacement from the Ca–EGTA complex.

Complex-formation between the calcium-binding and inhibitory proteins

It has been reported (Schaub & Perry, 1969; Schaub *et al.*, 1972) that electrophoresis of the troponin complex and calcium-binding protein preparations at alkaline pH values on 8% polyacrylamide gel gives a band pattern which is dependent on the Ca^{2+} concentration. Further detailed investigation of this phenomenon with highly purified samples of calcium-binding and inhibitory proteins has shown that the mobility changes observed are due not only to changes in the calcium-binding protein but also to the formation of a complex between this protein and the inhibitory protein, which is dependent on the presence of Ca^{2+}. This complex has a mobility in 6M-urea–25mM-Tris–80mM-glycine, pH7.6, which is about 60% that of calcium-binding protein alone;

under these conditions the inhibitory protein in the absence of calcium-binding protein does not move towards the anode (Plate 1*b*). The presence of both proteins in the slower band obtained on the electrophoresis of a mixture of the inhibitory protein with excess of calcium-binding protein was proved by cutting out and further analysing the portion of the gel containing the unstained band. The protein in this slice was extracted into 1% sodium dodecyl sulphate–10mM-sodium phosphate buffer (pH7.0)–50mM-2-mercaptoethanol and electrophoresed on polyacrylamide gel containing 100mM-sodium phosphate buffer, pH7.0, and 0.1% sodium dodecyl sulphate (gel F, Plate 1*b*).

At pH8.6 complex-formation could be observed on electrophoresis in polyacrylamide gels containing up to 8M-urea. In all cases, however, the addition of 2mM-EGTA to the sample completely abolished the slower-migrating band, leaving the fast-moving band of calcium-binding protein and material staining at the origin that corresponded to the inhibitory protein.

When mixtures of equimolar amounts of the calcium-binding and inhibitory proteins are electrophoresed in the presence of 0.1mM-Ca^{2+} all the protein moved as the band corresponding to the complex. At all other molar ratios material was observed either at the origin, representing excess of inhibitory protein, or as a fast-migrating band corresponding to the calcium-binding protein. With higher ratios of the latter protein a faint slower-moving band, which may indicate the presence of a complex of different stoicheiometry, was sometimes observed (e.g. Plate 1*b*, gel E). Complex-formation occurred in urea over the pH range 7.0–8.6 and also could be observed at pH10.0 (50mM-glycine–32mM-NaOH) and at pH5.0 (50mM-succinic acid–53mM-NaOH). Formation of the complex was also observed when the urea present in the polyacrylamide gel was replaced by 40% (v/v) glycerol in the same pH range, 7.0–8.6. Under the latter conditions of electrophoresis the bands were more diffuse than was the case when urea was present. The addition of tropomyosin or the '37000 component' (troponin T) to the mixture of the inhibitory and calcium-binding proteins did not modify the complex-formation between the latter two proteins in concentrations from 0 to 8M-urea so far as could be judged by electrophoretic studies. The carbamoylmethylated calcium-binding protein showed similar mobility changes in the presence and absence of Ca^{2+} to those with the untreated protein. It also formed a complex with the inhibitory protein of similar electrophoretic mobility to that obtained with the native protein. If [^{14}C]carbamoylmethylated calcium-binding protein was added to the inhibitory protein in the presence of excess of untreated binding protein, the complex band contained no radioactivity, implying that the unmodified form of calcium-binding protein had displaced the carbamoylmethylated protein from the complex. This was presumably due to the higher affinity of the untreated calcium-binding protein for the inhibitory protein.

Sedimentation-equilibrium studies on the complex between the calcium-binding and inhibitory proteins were complicated by the tendency of the inhibitory protein to break down during the time required to attain equilibrium conditions, probably owing to the action of trace amounts of endogenous proteinase present in the preparation (cf. Wilkinson *et al.*, 1972). This problem was resolved by the inclusion of the proteinase inhibitor, pepstatin, in all solutions used (Hartshorne & Dreizen, 1972). A value of 0.715 for the partial specific volume was calculated from amino acid analysis for a 1:1 mixture of calcium-binding protein and inhibitory protein (Wilkinson *et al.*, 1972). By using this value an average molecular weight of 44000 was obtained from three runs carried out in 0.1M-KCl–0.1mM-$CaCl_2$–1mM-dithiothreitol–20mM-Tris–15mM-HCl, pH7.6 and containing 1mg of pepstatin/litre at a rotor speed of 23150rev./min. The inclusion of 2mM-EGTA in the buffer in place of 0.1mM-Ca^{2+} resulted in a fall in the estimated molecular weight to 22000, determined at the same rotor speed.

When a mixture of equimolar amounts of calcium-binding and inhibitory proteins was treated with the cross-linking reagent dimethyl suberimidate in the presence of 0.1mM-Ca^{2+} a cross-linked species having a molecular weight of 42000 was observed on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (Plate 2*a*). If the 0.1M-$CaCl_2$ was replaced by 2mM-EGTA little or no cross-linking occurred under otherwise identical conditions. When calcium-binding protein alone was treated with dimethyl suberimidate at equivalent protein concentrations no cross-linking was obtained. Inhibitory protein is relatively insoluble under the conditions used for the cross-linking reaction and a saturated solution precipitated during the reaction. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate showed the presence of high-molecular-weight complexes (>60000) when inhibitory protein was treated in this way. Higher-molecular-weight complexes were also formed at higher protein concentrations of the complex and with calcium-binding protein alone at $CaCl_2$ concentrations greater than or equal to 1mM.

On gel filtration of mixtures of calcium-binding and inhibitory proteins on Sephadex G-200 in the presence of 6M-urea–20mM-Tris–15mM-HCl (pH7.6)–0.1mM-$CaCl_2$ a discrete peak was eluted in advance of those corresponding to the individual proteins. Protein from the least retarded peak migrated as a single slow-moving band on polyacrylamide-gel electrophoresis in 6M-urea, pH8.6, and as two bands of approximately equal intensity on electrophoresis

in sodium dodecyl sulphate, pH7.0. Although it was estimated that the elution position of the peak due to the complex corresponded to a molecular weight in the range 30000–40000, an accurate value could not be determined under these conditions.

Calcium-binding protein and complex-formation in other muscle cell types

On polyacrylamide-gel electrophoresis of extracts of whole rabbit soleus and cardiac muscles in 6M-urea–25mM-Tris–80mM-glycine, pH8.6, in the presence of 2mM-EGTA the calcium-binding protein moved well in front of the other protein bands. The calcium-binding protein from these two tissues migrated with a mobility identical with that obtained from rabbit white muscle both in 6M-urea at pH8.6 and in sodium dodecyl sulphate at pH7.0. This similarity of electrophoretic behaviour of the calcium-binding protein from the different muscle types enabled the isotope-dilution technique using ^{14}C-labelled calcium-binding protein from white skeletal muscle to be used for estimations in cardiac and red skeletal muscle (see below).

Despite the similarity of the electrophoretic mobility of the calcium-binding proteins there was evidence that in the red and cardiac muscles the mobility of the complex between calcium-binding protein and the inhibitory protein was different from that observed in white skeletal muscle (Plate 2*b*). Cardiac calcium-binding protein and white-skeletal-muscle inhibitory protein combined to give a complex, the electrophoretic mobility of which in 6M-urea, pH8.6, was identical with that of the homologous complex from white skeletal muscle. This suggests that the inhibitory protein from red skeletal and cardiac muscles in the rabbit differs from that present in skeletal muscle.

Determination of amount of calcium-binding protein present in striated muscle

Because of its strong negative charge the calcium-binding protein migrates on polyacrylamide gel, at pH8.6, with a mobility greater than that of any other proteins of rabbit skeletal muscle. This fact together with the ease with which it can be isolated and purified by the preparative polyacrylamide-gel-electrophoresis method makes the calcium-binding protein particularly suitable for determination of the amount present in whole muscle by the isotope-dilution technique. Very consistent results were obtained with rabbit psoas muscle. With soleus and cardiac muscles the values obtained were lower than for the psoas and considerably more variable (Table 2). If actin is taken to make up 20% of the total myofibril protein, which itself represents 60% of the total protein of rabbit psoas, then the molar ratio of actin monomers of molecular weight 45000 to calcium-binding protein of molecular weight 18000, is 7.3:1. In cardiac and soleus muscle the amount of calcium-binding protein was similar but significantly lower per g wet wt. than in psoas. In view of the fact that the actin content is lower, owing to the lower contribution of myofibrils to the total cell protein, in cardiac muscle (Zak *et al.*, 1972) and probably also in soleus muscle it is likely that the ratio of actin monomers to molecules of calcium-binding protein is similar in all the muscle types studied.

Discussion

The results of the present studies involving sedimentation equilibrium, gel filtration and electrophoresis in sodium dodecyl sulphate in phosphate buffer confirm the molecular weight of 18500 reported earlier by our group (Schaub & Perry, 1971; Wilkinson *et al.*, 1972) and by Hartshorne & Pyun (1971). One reason for the higher values reported in some earlier studies (Murray & Kay, 1972; Greaser *et al.*, 1972; Ebashi *et al.*, 1972) may have been the presence of inhibitory protein of molecular weight 23000 in the preparations as a complex with the calcium-binding protein. Inhibitory protein can be a very consistent contaminant of calcium-binding-protein preparations unless the complex it forms with the latter is dissociated at some stage in the preparation by lowering the Ca^{2+} concentration to below 0.1 μM (Schaub *et al.*, 1972; Perry *et al.*, 1972). For the reasons stated above this impurity is not readily detected when the purity of preparations is monitored by electrophoresis in sodium dodecyl sulphate in Tris–borate buffer, pH7.0. The amino acid analysis data, however, suggest that the

Table 2. *Calcium-binding-protein content of rabbit muscles*

Results are calculated on the basis that actin, mol.wt. 45000, represents 20% of the total myofibrillar protein. For further details see the Methods section.

Muscle	Calcium-binding protein (% of protein N)	Myofibrils (% of protein N)	Actin/troponin C molar ratio
Psoas	0.67 ± 0.023 (7)	60	7.3
Soleus	0.46 (3)	45	8.0
Heart	0.53 ± 0.13 (5)	45	6.9

preparations studied by various investigators are very similar, with the possible exception of the glutamic acid and proline contents (Table 1). Earlier reports from this laboratory (Schaub & Perry, 1969; Schaub *et al.*, 1972) in which large changes in mobility on electrophoresis in urea, pH8.6, were obtained on addition of EGTA were presumably due to the presence of inhibitory protein in calcium-binding preparations.

The change in the u.v.-absorption spectrum, the decrease in elution rate on gel filtration and the increase in electrophoretic mobility at pH8.6 all suggest that a marked change in conformation occurs on binding of Ca^{2+} to the calcium-binding protein. In particular the changes in rate of elution on gel filtration and in the electrophoretic mobility, which suggest that the molecule becomes more compact when Ca^{2+} is bound, fit in well with the evidence for an increase in α-helical content reported by Murray & Kay (1972) to occur under these conditions.

The observation that addition of two calcium atoms per molecule is required for completion of the spectral changes implies that the calcium-binding protein can bind two calcium atoms per molecule. Some caution is perhaps necessary in drawing this conclusion, however, for the free Ca^{2+} concentration was not measured in these experiments. Although there is no general agreement as to the number of binding sites per molecule of calcium-binding protein (cf. Potter & Gergely, 1972) the results would imply that the high- and low-affinity sites reported by Hartshorne & Pyun (1971) need to be saturated to bring about completion of the conformational change.

The conformational changes that occur as a result of Ca^{2+} binding enable the calcium-binding protein to form a stable complex with the inhibitory protein. The possibility that the Ca^{2+} forms a bridge between the two proteins cannot be excluded, but from the relative stability of the complex to high ionic strength and from general considerations this mechanism appears unlikely. It would seem more probable that the binding with Ca^{2+} alters the conformation of the binding protein so that a highly specific interaction involving amino acid side chains, which is stable to high urea concentrations, can occur with the inhibitory protein.

The functional significance of the Ca^{2+}-regulated formation of the complex has yet to be determined. Although it might be expected to bring about neutralization of the inhibitory action of the inhibitory protein there is *prima facie* evidence suggesting that complex-formation involving Ca^{2+} is not required to neutralize the action of the inhibitory protein. Neutralization of inhibition of the Mg^{2+}-stimulated ATPase of actomyosin by the calcium-binding protein can be obtained in the presence of EGTA, under which conditions the complex is dissociated (Schaub *et al.*, 1972; Perry *et al.*, 1972). Nevertheless, although complex-formation and the conformational changes associated with Ca^{2+} binding are not essential for simple neutralization of the action of the inhibitory protein, they would be expected to play a role in the reversible regulation of the Mg^{2+}-stimulated adenosine triphosphatase by the complete regulatory system.

From the isotope-dilution experiments it can be concluded that in rabbit psoas muscle at least, and probably in other muscle types, the molar ratio of calcium-binding protein to actin monomers is about 7:1. The finding correlates well with the recent report that the 38.5nm spacing present in the X-ray-diffraction pattern obtained with glycerated rabbit psoas is enhanced 4–5-fold when muscle is treated with antibody to the calcium-binding protein (Rome *et al.*, 1973). This implies localization of the calcium-binding protein at this periodicity, which corresponds very closely to the distance along the I filament corresponding to seven actin monomers (Huxley, 1972).

Taken together, these results obtained by different techniques indicate in a precise way that, if the I filament is of uniform composition along its length, there is one molecule of calcium-binding protein for every seven monomers, i.e. two molecules per 38.5nm because of the double-stranded structure of the I filament, as was originally suggested by Ebashi *et al.*, (1969). On excitation two Ca^{2+} ions are bound to each molecule of calcium-binding protein associated with seven actin monomers. The conformational change induced in the calcium-binding protein in some way produces effects on the remaining components of the regulatory-protein system, which result in each of the seven actin monomers being able to interact with myosin. As a consequence ATP is hydrolysed at a high rate and contraction takes place.

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