Circular-Dichroic Studies on the Conformational Behaviour of Troponin and Tropomyosin from Bovine Cardiac Muscle

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Bovine cardiac troponin is similar to rabbit skeletal troponin with respect to secondary structure, amino acid composition and molecular weight of the subunits, but differs slightly with respect to biological activity and surface charges of the subunits. Previous circular-dichroic studies of the subunits and recombination of subunits have indicated significant Ca^{2+} -induced delocalized conformational changes. Present studies of the native troponin complex are not in accord with such changes. Furthermore the formation of the troponin-tropomyosin complex *in vitro* results in no delocalized conformational changes, nor does it sensitize troponin to Ca^{2+} -induced changes. It is suggested that the troponin complex cannot be dissociated into subunits without significant and irreversible conformational perturbation.

Ca^{2+} ions regulate muscle contraction in both skeletal and cardiac muscle systems through their interaction with the regulatory proteins troponin and tropomyosin. Troponin binds Ca^{2+} , and in association with tropomyosin plays an essential role in the regulatory mechanism. Troponin is a complex of three subunits, troponin C (TN-C), troponin I (TN-I) and troponin T (TN-T), with each subunit having a specific function. Troponin C is the Ca^{2+} -binding subunit, troponin I is the subunit that inhibits the Ca^{2+} -activated ATPase of actomyosin and troponin T is the tropomyosin-binding subunit. The mechanism by which Ca^{2+} -binding to troponin initiates muscle contraction is not yet fully understood. Previous studies (Bremel & Weber, 1972; Ebashi *et al.*, 1972; Greaser *et al.*, 1972) suggested that in the absence of Ca^{2+} the regulatory proteins inhibit contraction by sterically blocking the myosin-binding sites on actin, and that one troponin-tropomyosin complex exerts its control over seven actin molecules by co-operative interaction. The binding of Ca^{2+} to troponin C represses the inhibition and activates the actin-myosin interaction.

An important question is, how do the Ca^{2+} -induced signals disseminate from the binding sites at troponin C to tropomyosin? Since troponin C does not bind directly to tropomyosin, the transmission of such signals must be via troponin T and/or troponin I. Two models are worth considering:

(1) at one extreme, a mechanism involving 'delocalized' conformational changes that transmits deformation energy from the active sites at troponin C to troponin I, troponin T, tropomyosin and F-actin and (2) in the other extreme, a mechanism involving 'localized' conformational changes that alter local surface charges in the binding interfaces between some components in the system.

Previously we have demonstrated the usefulness of far- and near-u.v. c.d. studies for differentiating between delocalized and localized conformational changes in proteins (Cassim & Lin, 1975). Therefore similar c.d. studies of troponin and the troponin-tropomyosin complex in the absence and presence of Ca^{2+} should resolve the question of delocalized conformational changes resulting from Ca^{2+} perturbation. However, current c.d. studies of troponin have emphasized the effects of Ca^{2+} on the subunits and the reconstituted complexes only (Murray & Kay, 1972; McCubbin *et al.*, 1974; Mani *et al.*, 1974, 1975; Burtnick & Kay, 1976; Burtnick *et al.*, 1976; Hincke *et al.*, 1977). Although the observed Ca^{2+} -induced changes in the far-u.v. spectra of such studies have been interpreted as large Ca^{2+} -induced conformational changes originating in the troponin C moiety and delocalizing over the entire troponin complex, it is not clear that these changes are of biological significance. Separation of the troponin into subunits could significantly alter the subunit especially with regard to conformational stability. In view of this, the present study was initiated to determine if the previously reported Ca^{2+} -induced delocalized confor-

Abbreviations used: ATPase, adenosine triphosphatase; SDS, sodium dodecyl sulphate.

mational changes are an intrinsic property of the troponin molecule.

A procedure for preparing and purifying bovine cardiac troponin (as a complex) suitable for biochemical and spectral studies is described. The conformation (as characterized by c.d. and absorption spectroscopy), amino acid composition and molecular weight of the subunits of cardiac troponin are very similar to those of skeletal troponin. However, there are slight differences in the biological activity and surface charges of the subunits between the two troponins. The effects of Ca^{2+} , other bivalent cations and protein denaturants on the near- and far-u.v. c.d. of troponin and troponin-tropomyosin complex were studied. No significant Ca^{2+} -induced spectral changes were observed, although the spectrum was sensitive to perturbation of protein structure by small amounts of denaturants. Furthermore the c.d. spectrum of the troponin-tropomyosin complex was approximately the sum of the c.d. spectra of the constituents. Clearly, delocalized conformational changes can be ruled out as a reasonable possibility. Therefore it is suggested that conformational models based on subunit studies should be reconsidered.

Experimental

Preparation of muscle proteins

Fresh ox hearts were immersed in crushed ice immediately after excision. All the subsequent steps in the preparation of the muscle proteins were performed at 4°C. Papillary muscle, fat and debris were removed and muscle tissues from both ventricles were used. All the solutions used for the following preparations contained 15mM-2-mercaptoethanol, except the solution used in the final step of each preparation.

Cardiac troponin. Crude troponin was isolated by the method of Tsukui & Ebashi (1973), with some essential modifications as follows. After myosin was removed from the minced muscle, the remaining residue was blended with 3 vol. of 30mM- NaHCO_3 for 30s in a Waring blender. The homogenate was centrifuged at 1000g for 15min, and the supernatant was discarded. This procedure was repeated several times until the supernatant was clear after centrifugation. Troponin was extracted from the thoroughly washed homogenate by $(\text{NH}_4)_2\text{SO}_4$ fractionation between 37 and 45% saturation. The troponin extract was dissolved in 1.5mM- NaHCO_3 and exhaustively dialysed against this medium with several changes of the dialysing solution. The dialysis residue was centrifuged at 100000g for 1h. The supernatant thus obtained was the 'crude' troponin.

Cardiac tropomyosin. Tropomyosin was prepared directly from the supernatant of native tropomyosin

extract after troponin was removed at 45% $(\text{NH}_4)_2\text{SO}_4$ saturation. Tropomyosin was obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation at between 50 and 60% saturation. Tropomyosin was further purified by two isoelectric precipitations at pH4.5 in 1M-KCl and $(\text{NH}_4)_2\text{SO}_4$ fractionation at between 50 and 60% saturation.

Cardiac actomyosin. Actomyosin was prepared by the method of Stowring *et al.* (1966) with a minor modification in which the mince was blended with 3 vol. of 30mM- NaHCO_3 and thoroughly washed with the same solution several times before the extraction of actomyosin. The actomyosin obtained was the natural actomyosin which contained troponin and tropomyosin, and was Ca^{2+} -sensitive as assayed by superprecipitation and ATPase tests. The actomyosin was desensitized by repeatedly washing the actomyosin gel with 2mM-Tris/HCl buffer at pH8.0 and 4°C until superprecipitation or ATPase test indicated that the actomyosin was insensitive to Ca^{2+} . Synthetic actomyosin was prepared by combining purified F-actin with purified myosin from rabbit skeletal muscle in a weight ratio of 10:1. Myosin was prepared by the method of Szent-Györgyi (1951). F-actin was extracted from an acetone-dried powder (Straub, 1942) and purified by the method of Mommaerts (1952). Synthetic actomyosin was repeatedly washed with deionized distilled water to assure complete removal of Ca^{2+} -sensitivity.

Purification of troponin

Bovine cardiac troponin was purified by a simple procedure using precipitation at low pH. Concentrated KCl, EDTA and sodium phosphate solutions were added to the crude troponin dissolved in 1.5mM- NaHCO_3 and 15mM-2-mercaptoethanol. The final concentration of each component was as follows: troponin, 8–10mg/ml, KCl, 0.8–1.2M, EDTA, 1mM, and sodium phosphate, 15mM. The pH of the solution was adjusted to 4.7 by the addition of 0.1M-HCl. The precipitated troponin was collected by centrifugation at 17000g for 20min. The sediment was dissolved in 0.5M-KCl and 40mM-sodium phosphate buffer, and the pH was adjusted to 7.5 with 1M-NaOH. The solution was gently stirred for 1h. The undissolved troponin was removed by centrifugation at 17000g for 15min. The supernatant was dialysed against 1.5mM- NaHCO_3 with several changes of the dialysing solution. After 24h, the dialysis residue was centrifuged at 100000g for 1h. The supernatant thus obtained was the 'acid-purified' troponin. Troponin was further purified by either DEAE-cellulose or DEAE-Sephadex A-50 column chromatography.

Biological activity of troponin

Activity of troponin was assayed by its ability to restore the Ca^{2+} -sensitivity of desensitized

actomyosin in the presence of tropomyosin as determined by the criterion of superprecipitation or ATPase activity. Superprecipitation of actomyosin was assayed by a procedure similar to that of Ebashi (1961). The time to the onset of superprecipitation was measured as the time at which the half-value of the final absorbance was reached. The ATPase activity of actomyosin was measured with a Radiometer pH-stat (Radiometer, Copenhagen, Denmark). Actomyosin was considered to be desensitized if the ATPase activity or the time required for the onset of the superprecipitation was the same in the presence of Ca^{2+} or EGTA. Troponin and tropomyosin were added to the desensitized actomyosin, and the ATPase activity and superprecipitation of the reconstituted actomyosin in the presence of Ca^{2+} and EGTA were assayed for biological activity of troponin.

Amino acid composition

Amino acid composition was analysed on a Beckman 116 Analyzer (Beckman Instruments, Palo Alto, CA, U.S.A.) by the single-column methodology of Durrum (Spackman *et al.*, 1958).

Electrophoresis

Disc polyacrylamide-gel electrophoresis in the presence of SDS was performed as described by Weber *et al.* (1972). Protein samples were prepared by the standard procedure. Molecular weights of the protein in various bands were obtained by a plot of $\log(\text{molecular weight})$ versus the mobility, with haemoglobin (mol.wt. 16000), trypsin (mol.wt. 23300), chymotrypsin (mol.wt. 25700), pepsinogen (mol.wt. 40400), ovalbumin (mol.wt. 43000) and serum albumin (mol.wt. 68000) as standard markers.

Protein concentrations

The concentrations of stock protein solutions were determined by the micro Kjeldahl method assuming the nitrogen content of all proteins to be 16%.

Optical measurements

C.d. spectra were recorded with a Cary 60 spectropolarimeter with a 6003 c.d. accessory (Varian, Palo Alto, CA, U.S.A.). Instrument calibration, optical-cell calibration, temperature control, experimental procedures and spectropolarimeter constants have been given elsewhere (Becher & Cassim, 1976). C.d. is expressed as mean residue molar ellipticity $[\theta]$ in units of $\text{degree} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. The mean residue weight of the proteins was assumed to be 115. As a standard procedure the spectrum of each sample in a given condition was recorded at least

twice. The reproducibility of c.d. measurement (expressed as the standard deviation) varied from ± 0.7 to ± 0.9 $\text{degree} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ in the near-u.v. region, and was ± 50 , ± 75 and ± 150 $\text{degree} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ at 222, 209 and 190 nm respectively in the far-u.v. region.

Absorption spectra were measured in a Cary 118C low-u.v. spectrophotometer equipped with a scattered transmission accessory (Varian, Palo Alto, CA, U.S.A.). Details of spectrophotometric procedures have been given elsewhere (Becher & Cassim, 1976). Absorbance is expressed as the absorption coefficient for 1 mg/ml of solution for 1 cm path length in units of absorbance $\text{cm}^2 \cdot \text{mg}^{-1}$.

Calculation of apparent α -helical content

The far-u.v.-absorption method of Rosenheck & Doty (1961) was used to estimate the apparent α -helical content for troponin and tropomyosin. The molar residue absorption coefficients of poly-L-glutamic acid were used as the reference: namely, 42000, 3300 and 2150 for the α -helix state, and 7000, 6550 and 3500 for the disordered state at 190, 197 and 205 nm respectively (Gratzer, 1967). Correction for the absorbance of the side-chain chromophores was calculated from the protein amino acid composition and the published side-chain absorbances (Gratzer, 1967). Molecular weights of 66500 and 82000 and total number of amino acid residues of 578 and 713 were used for tropomyosin and troponin respectively.

The α -helical content was also estimated from the mean residue molar ellipticities at three wavelengths, 222, 209 and 191 nm, where c.d. extrema are evident. The mean residue molar ellipticities of poly-L-glutamic acid -40400, -38500 and 91300 for the α -helix state and 4900, -2200 and -36000 for the disordered state at 222 nm, 209 nm and 191 nm respectively were used as 'standard' ellipticities (J. Cassim, unpublished results).

Reagents

SDS (Bio-Rad Laboratories, Richmond, CA, U.S.A.) was recrystallized several times in ethanol and was transparent over the entire spectral region studied. Urea was the ultra-pure grade from Schwartz/Mann (Orangeburg, NY, U.S.A.). Double-glass-distilled deionized water was used exclusively in all solutions and for rinsing glassware. All other reagents and chemicals were A.C.S.-certified reagent grade and were used without further purification.

Results and Discussion

Purification of bovine cardiac troponin

Skeletal-muscle tropomyosin is precipitated at pH4.6 in the presence of 1 M-KCl, whereas skeletal-

muscle troponin remains in the solution (Staprans & Watanabe, 1970). On the other hand, cardiac troponin was co-precipitated with cardiac tropomyosin at pH values below 4.7, even in the presence of 0.8–1.2M-KCl. This necessitated the separation of the troponin from the complex before the acidic precipitation method for purifying troponin could be used. Skeletal-muscle troponin, at pH 1.0 and in the presence of 1.0M-KCl, can be separated into troponin C and a complex of troponin I and troponin T (Hartshorne & Mueller, 1968). Unlike skeletal-muscle troponin, all the subunits of bovine cardiac troponin were precipitated at pH 1.0 in 1.0M-KCl, indicating that the troponin I and troponin T components of bovine cardiac troponin have surface charges different from those of their counterparts in rabbit skeletal-muscle troponin.

The elution profile of troponin during DEAE-cellulose column chromatography shows a small peak unretained by the column (approx. 5% of the total protein applied), presumably corresponding to the γ -component found in skeletal-muscle troponin (Staprans & Watanabe, 1970). The retained troponin was eluted as a main peak (65% recovery) with 0.22M-KCl, unlike skeletal-muscle troponin, which is eluted with 0.15M-KCl (Staprans & Watanabe, 1970). In addition to the observation that the surface charges of the subunits of cardiac troponin are different from their counterparts in skeletal-muscle troponin, the difference in chromatographic behaviour also suggests that the surface charge of the cardiac troponin complex may also be quite different from that in the skeletal-muscle troponin complex. The elution profile of troponin on DEAE-Sephadex was very similar to the one on DEAE-cellulose, but it was eluted at a higher KCl concentration (0.37M) than the former. A gel electrophoretogram of the purified troponin shows only three bands corresponding to its three subunits.

Biological activity of cardiac troponin

The biological activity of bovine cardiac troponin was demonstrated by its ability to restore the Ca^{2+} -sensitivity of actomyosin when the troponin was added to the desensitized actomyosin or synthetic actomyosin in the presence of tropomyosin. The activity of troponin assayed by the ATPase method is shown in Table 1 and that assayed by the superprecipitation test with desensitized cardiac actomyosin and synthetic skeletal-muscle actomyosin is shown in Figs. 1 and 2 respectively. The activity of cardiac troponin was better demonstrated by using synthetic skeletal-muscle actomyosin. It is evident that in the absence of Ca^{2+} cardiac troponin together with the presence of tropomyosin inhibited the ATPase activity and the superprecipitation of actomyosin, whereas in the presence of Ca^{2+} , this

Table 1. Mg^{2+} -ATPase assays of troponin activity
For the cardiac actomyosin system, the assay conditions were as in the legend to Fig. 1, and for the skeletal-muscle actomyosin system, they were as in the legend to Fig. 2.

	ATPase activity (μmol of P_i /min per mg of actomyosin)			
	Ca^{2+}		EGTA	
(a) Cardiac actomyosin system				
Natural actomyosin	0.074		0.029	
Desensitized actomyosin	0.057		0.059	
Reconstituted actomyosin	0.071		0.035	
	ATPase activity (μmol of P_i /min per mg of actomyosin)			
(b) Skeletal-muscle actomyosin system	pCa 8	pCa 7	pCa 6	pCa 5
Synthetic actomyosin	0.414	0.414	0.414	0.414
Reconstituted actomyosin	0.046	0.497	0.718	0.911

inhibition is repressed. Troponin or tropomyosin alone had very little effect on ATPase activity and superprecipitation of synthetic actomyosin. Therefore both proteins are required for conferring Ca^{2+} -sensitivity on actomyosin.

Amino acid composition

The amino acid composition of bovine cardiac troponin (Table 2) is not significantly different from that of skeletal-muscle troponin. However, the tyrosine content in cardiac troponin is approx. 40% higher than that in skeletal-muscle troponin (this also results in 30% higher absorbance at 277nm).

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis of the troponin purified by column chromatography indicated only three bands. The molecular weights of the three bands (average from eight preparations) were 37500 ± 1000 , 26500 ± 500 and 18000 ± 300 . They presumably correspond to the T, I and C subunits of troponin respectively. These values are similar to those reported for the subunits of skeletal-muscle troponin, 37000–40000, 22000–24000 and 17800–18000 for troponin T, troponin I and troponin C respectively (Ebashi *et al.*, 1972). Assuming that the three subunits combine in an equimolar ratio in cardiac troponin as in skeletal-muscle troponin, the molecular weight of the cardiac troponin complex is estimated to be 82000 ± 1800 .

Conformation of the regulatory proteins of cardiac muscle

The conformation of cardiac troponin was studied by near- and far-u.v. c.d. and absorption spectra. The near-u.v. c.d. spectrum of troponin (Fig. 3)

Fig. 1. Superprecipitation of actomyosin from bovine cardiac muscle in the presence of Ca^{2+} or EGTA

----, In the presence of 0.25 mM-EGTA; —, in the presence of 0.25 mM- CaCl_2 ; ····, without the addition of EGTA or CaCl_2 (control). (a) Natural actomyosin; (b) desensitized actomyosin; (c) reconstituted actomyosin. Final concentrations in the media in all assays were: 1 mM- MgCl_2 , 1 mM-ATP, 0.05 M-KCl and 10 mM-Tris/HCl, pH 7.0. Protein concentrations were: (a) 0.58 mg of actomyosin/ml; (b) 0.56 mg of actomyosin/ml; (c) 0.59 mg of actomyosin/ml, 0.086 mg of tropomyosin/ml and 0.092 mg of troponin/ml.

consists of four negative extrema at 282, 277, 269 and 262 nm and a shoulder at 288 nm which correlate with the features of the fine structure of the absorption spectrum (not shown). These four extrema have also been found in the c.d. spectrum of skeletal-muscle troponin (Staprans & Watanabe, 1970). However, in the spectrum of skeletal-muscle troponin there is, in addition, a positive extremum at 295 nm, but no indications of a shoulder at 288 nm. The task of assigning electronic transitions to the various fine features of the near-u.v. spectra of proteins is, as yet, a formidable one. The most that one can do at the present is to attribute the spectral features below 270 nm to the phenylalanine residues and those above 270 nm to the tyrosine and tryptophan residues.

Far-u.v.-absorption-spectral studies indicated an absorption maximum at 188.5 nm with an absorption coefficient of $88.1 \text{ (cm}^2 \cdot \text{mg}^{-1}\text{)}$ for troponin and maximum at 188.5 nm with an absorption coefficient of 55.4 for tropomyosin. Derivative spectra (not shown) showed the presence of two inflections at approx. 222 and 207 nm for tropomyosin, in contrast

with only one at 225 nm for troponin. The Davydov splitting of the amide ($\pi-\pi^*$) NV_1 transition moments due to excitonic coupling, which gives rise to bands at 188.5 and 207 nm and the hypochromism in the far-u.v.-absorption spectrum of tropomyosin, are in accord with theories of electronic transitions for a protein with a high degree of α -helical secondary structure (Moffit, 1956; Tinoco *et al.*, 1962).

The α -helical contents of cardiac troponin and tropomyosin were estimated by two independent methods, ellipticities at 222, 209 and 191 nm and hypochromism at 190, 197 and 205 nm (Rosenheck & Doty, 1961). The former method yielded $42.8 \pm 6.8\%$ and $100 \pm 2.2\%$ for cardiac troponin and tropomyosin respectively. The latter method (see Table 4) yields values that are internally consistent only at 190 and 197 nm. The value based on hypochromism at 205 nm is significantly deviated from the other values in both cases. The average α -helical contents calculated from the internally consistent values are $41.5 \pm 2.5\%$ and $95.5 \pm 1.5\%$

Fig. 2. Effect of bovine cardiac troponin and tropomyosin on the superprecipitation of synthetic actomyosin from rabbit skeletal muscle in the presence of Ca^{2+} or EGTA

(a) Synthetic actomyosin; (b) reconstituted actomyosin. Final concentrations in the assays were: 1 mM- MgCl_2 , 1 mM-ATP, 0.05 M-KCl and 10 mM-Tris/HCl, pH 7.0. Protein concentrations were: (a) 0.34 mg of actomyosin/ml; (b) 0.34 mg of actomyosin/ml, 0.053 mg of troponin/ml and 0.043 mg of tropomyosin/ml. Concentration of all EGTA/Ca buffers was 1 mM. Concentrations of free Ca^{2+} in the assays were: 10^{-8} M (pCa 8, $\cdots$); 10^{-7} M (pCa 7, ----); 10^{-6} M (pCa 6, - - - -); 10^{-5} M (pCa 5, —).

for troponin and tropomyosin respectively. These values are very similar to the ones previously obtained for the skeletal-muscle regulatory proteins (Staprans & Watanabe, 1970).

Perturbation studies of troponin

The effects of Ca^{2+} and EGTA on the far-u.v. c.d. spectrum of cardiac troponin were studied. The addition of 1–3 mM- CaCl_2 increased the intensity at extrema, 222 and 209 nm, by no more than 2% relative to the value in the presence of 1 mM-EGTA (unbound Ca^{2+} less than 10 nM). The small ellipticity change in the far-u.v. c.d. was found in troponin from both crude and purified preparations and was independent of the method of purification used.

Therefore this change cannot be attributed to protein modification during purification or to the presence of contaminants in troponin. This change was perfectly reproducible, as indicated by repeated measurements with several troponin preparations. The possibility that the small ellipticity change was attributable to light-scattering artifacts has also been minimized by discarding samples that gave greater than 0.003 absorbance at 400 nm. On the other hand, the addition of Ca^{2+} did not cause any observable change in ellipticity or position of the extrema in the near-u.v. c.d. spectrum of troponin within the limits of spectral reproducibility.

The effects of bivalent cations other than Ca^{2+} on the far-u.v. c.d. of troponin were also studied. As found for Ca^{2+} , the addition of Mg^{2+} , Sr^{2+} and

Table 2. *Amino acid compositions of bovine cardiac troponin and rabbit skeletal-muscle troponin from various preparations*

The results for rabbit skeletal-muscle troponin are unpublished results of T.-I Lin & J. Y. Cassim.

Amino acid residue	Amino acid composition (mol/10 ⁵ g of protein)	
	Bovine cardiac troponin	Rabbit skeletal-muscle troponin
Lys	84.3	99.6
His	14.8	16.4
Arg	74.5	66.7
Asx	89.5	87.2
Thr	34.8	25.0
Ser	35.5	36.9
Glx	180.2	173.6
Pro	22.3	26.0
Gly	53.5	44.3
Ala	71.3	76.8
Val	43.4	38.6
Met	23.8	34.5
Ile	35.9	33.5
Leu	66.5	70.7
Tyr	17.2	12.6
Phe	29.3	25.9
Trp	6.1	—

Ba²⁺ all caused a small change. Ellipticity changes at 222nm induced by these cations are summarized in Table 5. Ionic strengths over a wide range, up to 0.5M-KCl, have no appreciable effect on the c.d. spectrum of troponin in either the far- or the near-u.v. regions. The far-u.v. ellipticity changes induced by the addition of Ca²⁺ were also insensitive to ionic strength. However, the magnitudes of the changes were slightly higher at the lower ionic strengths.

The effects of two commonly used protein denaturants, urea and SDS, were studied in both far- and near-u.v. regions to determine the sensitivity of the c.d. of troponin to conformational changes. Additions of SDS up to 0.05% decreased the ellipticity of troponin at the characteristic extrema, 222 and 207nm, by 18 and 5% respectively, suggesting the loss of a small amount of helical structure in the protein. The effect of SDS on the near-u.v. c.d. of troponin is shown in Fig. 3. The addition of SDS up to 0.05% caused large changes in the ellipticities without altering the positions of the extrema. Urea also perturbed the near- and the far-u.v. spectra of troponin, but not as effectively as did SDS. A concentration of urea higher than 0.8M was needed to cause an observable change in the spectra.

Fig. 3. *Effect of SDS concentration on the near-u.v. c.d. spectrum of aqueous bovine cardiac troponin solution*
Protein concentration was 2.24mg/ml. Concentrations of SDS were: (A) 0%; (B) 0.01%; (C) 0.02%; (D) 0.03%; (E) 0.04%; (F) 0.05%.

Table 3. *Far-u.v. c.d. parameters of the regulatory proteins from bovine cardiac and rabbit skeletal muscle*

The parameters of the cardiac proteins were measured in 0.05M-KCl/6mM-Tris/HCl, pH7.0, and those of the skeletal-muscle proteins were measured in 0.1M-KCl/0.01M-phosphate (pH7.0)/0.2mM-dithiothreitol/0.2mM-EGTA. The latter results are taken from Staprans & Watanabe (1970).

	$10^{-4} \times [\theta]$			Cross-over (nm)
	222 nm	209 nm	191 nm	
Cardiac troponin	-1.50	-1.52	2.62	199
Skeletal-muscle troponin	-1.52	-1.58	2.59	201
Cardiac tropomyosin	-4.15	-3.75	—	201
Skeletal-muscle tropomyosin	-3.78	-3.55	7.25	202

Table 4. *Mean residue molar absorption coefficients and apparent α -helical contents of troponin and tropomyosin of bovine cardiac muscle*

	Mean residue molar absorption coefficient*		Percentage of α -helix
	Protein	Side chain†	
At 190 nm			
Troponin	10022	4125	39
Tropomyosin	6292	1919	94
At 197 nm			
Troponin	7551	2433	44
Tropomyosin	4610	1227	97
At 205 nm			
Troponin	3656	1108	71
Tropomyosin	2419	456	114 †

* Assuming 115 for the mean residue molecular weight of the protein.

† Based on values for aqueous solution.

Evidently the addition of Ca^{2+} to cardiac troponin resulted in virtually no change in the c.d. spectrum of the protein, although the Ca^{2+} concentrations were well above the physiological values. The significance of the small change observed only in the far-u.v. spectrum is doubtful. If this change is completely attributable to changes in the secondary structure of the protein, it would represent at the most an alteration of only a few amino acid residues from a disordered to a helical state. Furthermore this change was not Ca^{2+} specific.

The most important finding is the invariance of the near-u.v. c.d. of troponin to Ca^{2+} perturbation, even though c.d. in this spectral region seems to be very sensitive to structural perturbations as shown by the SDS and urea additions. In previous communications we have discussed in detail the significance of a similar lack of spectral sensitivity to protein

Table 5. *Effect of bivalent cations on the c.d. at 222 nm of bovine cardiac troponin*

Constituents of the perturbation media were: 0.328 mg of troponin/ml, 0.1M-KCl, 0.1M-Tris/HCl buffer, pH7.4. Concentration of all perturbants was 3mM. Percentage change is relative to $[\theta]_{222}$ in the presence of EGTA.

Perturbant	$10^{-4} \times [\theta]_{222}$	Percentage change
EGTA	1.500	0.00
$\text{MgCl}_2 + \text{EGTA}$	1.504	0.24
CaCl_2	1.533	2.16
MgCl_2	1.540	2.64
$\text{CaCl}_2 + \text{MgCl}_2$	1.543	2.88
SrCl_2	1.543	2.88
BaCl_2	1.613	4.09

perturbation (Cassim & Lin, 1975; Rafferty *et al.*, 1977). Obviously, any perturbation of a protein will result in a conformational change of some kind. The important question is the magnitude and extent of the resulting conformational distortion. The invariance of the near-u.v. c.d. of troponin to Ca^{2+} addition strongly suggests that the Ca^{2+} -induced conformational changes are subtle localized ones, in which the resulting conformational distortion is restricted to the region of inception (Ca^{2+} -binding sites in troponin C) in contrast with the delocalized one in which distortion would be transmitted over great distances from the point of inception (through and between subunits). This conclusion is based on simple reasoning. If the conformational distortion could be transmitted over appreciably large distances, then the local environments and interactions of sufficient numbers of aromatic amino acid residues should be altered to produce a measurable change in the near-u.v. c.d. Furthermore, in view of the virtual invariance of the c.d. spectrum, the possibilities of compensating delocalized conformational changes resulting in spectral changes that cancel out over the entire spectral range can also be considered to be highly unlikely (Cassim & Lin, 1975).

The findings of the present studies are contrary to the previously published ones based on studies of the subunit and the reconstituted complexes of the subunits of cardiac as well as skeletal troponin (Murray & Kay, 1972; McCubbin *et al.*, 1974; Mani *et al.*, 1974, 1975; Burnnick *et al.*, 1976; Burnnick & Kay, 1976; Hincke *et al.*, 1977). Kay and his collaborators have reported a large Ca^{2+} -induced change in the far-u.v. c.d. of troponin C, which not only persists but is enhanced by being complexed with the other subunits and tropomyosin. They interpret these spectral changes as resulting from a Ca^{2+} -induced conformational change which originates in the troponin subunit and is transmitted throughout the troponin complex to the actomyosin

complex via the tropomyosin molecule. They suggest that this Ca^{2+} -induced delocalized conformational change may be the essential event that controls muscle relaxation. The present findings are not in accord with such a mechanism of muscle regulation. One reason for the differences observed in spectral responses to Ca^{2+} in the two types of studies may be that the procedures required to isolate subunits can result in irreversible changes in conformation and/or conformational stability of the subunits.

C.d. studies of the troponin-tropomyosin complex

The interaction of troponin and tropomyosin was examined by measuring their c.d. in both near- and far-u.v. regions. Troponin and tropomyosin were mixed in equal molar ratio, assuming mol.wts. of 82000 and 66500 respectively. The far-u.v. c.d. spectra of troponin, tropomyosin and the complex are shown in Fig. 4 and compared with the calculated spectrum obtained by adding the individual c.d. spectra of troponin and tropomyosin and dividing by two. The calculated spectrum coincides with the measured spectrum over the entire far-u.v. spectral region, indicating a perfect additivity of the spectrum of the complex from the spectra of the constituents. The measured spectrum of the complex intersected the isoelliptic point of the spectra of troponin and tropomyosin at 202.4nm, indicating the presence of two optical species. The near-u.v. c.d. spectra of the complex and its constituents are

shown in Fig. 5. The measured c.d. spectrum of the complex differed slightly from the spectrum calculated from the spectra of the constituents, most noticeably at 282nm and the shoulder between 269 and 277nm. This small difference can be attributable to the fact that a few aromatic side chains of the constituent molecules which are located on or near the surface will change from a hydrophilic environment to a hydrophobic one in the complex. Similar changes have been reported for the skeletal-muscle troponin-tropomyosin complex (Staprans & Watanabe, 1970).

The effect of Ca^{2+} on the far-u.v. c.d. spectrum of the troponin-tropomyosin was similar to the one observed for troponin alone. The addition of 1–3 mM- CaCl_2 induced no more than 2 % increase in ellipticity at both extrema, 222 and 209nm, relative to the ellipticity in the presence of 1mM-EGTA. Furthermore the addition of up to 2.5mM- CaCl_2 to the complex did not cause any observable change in the ellipticities nor the positions of the extrema in the near-u.v., again imitating the effects of Ca^{2+} on troponin alone. The effects of EGTA and Ca^{2+} on the near- and the far-u.v. c.d. spectra of cardiac tropomyosin were also studied (even though it is known that Ca^{2+} does not bind to tropomyosin) in order to determine if the addition of Ca^{2+} to muscle protein solutions causes optical artifacts. In both spectral regions of the c.d., the spectrum of cardiac tropomyosin in the presence of either Ca^{2+} or EGTA was indistinguishable.

Fig. 4. Far-u.v. c.d. spectra for bovine cardiac troponin (·····), tropomyosin (-----), the complex of equimolar troponin and tropomyosin (●-●) and a calculated spectrum for the complex (—) in 0.05M-KCl/6mM-Tris/HCl at pH7.0

Concentrations of proteins were: troponin, 0.247 mg/ml; tropomyosin, 0.201 mg/ml; 0.1235 mg of troponin/ml and 0.1005 mg of tropomyosin/ml in the complex. C.d. is expressed as the measured ellipticity. Spectral display: 0.1 degrees on full scale. Optical path length: 1.011 mm.

Fig. 5. Near-u.v. c.d. spectra for bovine cardiac troponin (----), tropomyosin (-.-.-), the complex of equimolar troponin and tropomyosin (....), and a calculated spectrum for the complex (—) in 0.1 M-KCl/10 mM-Tris/HCl at pH7.0. Concentrations of proteins were: troponin, 3.44 mg/ml; tropomyosin, 2.85 mg/ml; 1.72 mg of troponin/ml and 1.43 mg of tropomyosin/ml in the complex. C.d. is expressed as the measured ellipticity. Spectral display, 0.02 degrees on full scale. Optical path length, 1 cm.

These results indicate that there are virtually no conformational changes in the troponin or the tropomyosin molecules attributable to complex-formation, excluding the very remote possibility of compensating conformational changes. Furthermore the conformational response of troponin to Ca^{2+} is not observably altered by complex-formation. It is concluded that the conformational behaviour of troponin or tropomyosin is not significantly altered by the isolation procedure necessary to separate them from the native troponin-tropomyosin complex, as seems to be the case in separation of the subunits of troponin from the native protein.

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