Some properties of cardiac troponin T structure

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1. Troponin T is eluted in multiple peaks when the whole bovine cardiac troponin complex is subjected to DEAE-cellulose chromatography in the presence of 8 M-urea. The heterogeneity observed is due to the presence of two forms of troponin T, differing in their M_r values, amino acid content, degree of phosphorylation and aggregation. 2. Cardiac troponin T contains up to 0.8 mol of phosphate/mol of protein. Rabbit skeletal-muscle troponin T kinase phosphorylates the single site located in the *N*-terminal pentapeptide of cardiac troponin T. The composition of this peptide, (Ser, Asx, Glx, Glx)Val, is similar to that of skeletal-muscle troponin T. 3. The single thiol group of cardiac troponin T is located some 50–70 residues from the *N*-terminus. 4. The C-terminal sequence of cardiac troponin T is Trp-Lys, i.e. as is the case of skeletal-muscle troponin T.

The troponin–tropomyosin complex is involved in the regulation of skeletal- and cardiac-muscle contraction (Perry, 1979; Adelstein & Eisenberg, 1980). Troponin consists of three components, namely troponin C, troponin I and troponin T (Greaser & Gergely, 1973; Tsukui & Ebashi, 1973; Brekke & Greaser, 1976). Many properties of skeletal-muscle troponin have been investigated in detail. The primary structure of all skeletal-muscle troponin components has been determined (Wilkinson & Grand, 1975; Pearlstone *et al.*, 1976; Collins *et al.*, 1977). Cardiac troponin has been studied less thoroughly. The primary structure of bovine cardiac troponin C and that of rabbit cardiac troponin I were first described by Van Eerd & Takahashi (1976) and by Grand *et al.* (1976). A comparison of troponin I and troponin C from skeletal and cardiac muscles has revealed a pronounced homology in their structure (Van Eerd & Takahashi, 1976; Grand *et al.*, 1976). The hybrid complexes obtained by mixing of skeletal- and cardiac-muscle troponin components have been found to possess physiological activity (Tsukui & Ebashi, 1973). Thus it has been postulated that the regulatory mechanisms of cardiac and skeletal muscles are quite similar. At the same time the M_r values of cardiac troponin T components are higher than those of skeletal-muscle troponin components (Tsukui & Ebashi, 1973; Brekke & Greaser, 1976). The fine mechanisms of

regulation of heart contraction differ from those of skeletal muscle (Perry, 1979; Robertson *et al.*, 1982). In view of the fact that the primary structure of cardiac troponin T is still unknown, it seemed sensible to investigate some properties of cardiac troponin T structure.

Materials and methods

Preparative methods

Bovine cardiac troponin was isolated by the procedure of Tsukui & Ebashi (1973), with slight modifications (Barskaya & Gusev, 1981). Troponin was separated into constituent components by DEAE-cellulose chromatography in the presence of 8 M-urea (Perry & Cole, 1974). Troponin components were eluted by an NaCl gradient (0–0.2 M), generated by an Ultragrad gradient-mixing system (LKB Produkter, Bromma, Sweden) connected to a Uvicord III apparatus (LKB). The increment in ionic strength was halted when the absorbance of the effluent had increased to 0.1. To remove urea, the fractions containing troponin I and troponin T were subjected to chromatography on CM-cellulose equilibrated with 0.1 M-sodium acetate buffer, pH 4.6, containing 15 mM-2-mercaptoethanol (Wilkinson, 1974). Rabbit skeletal-muscle troponin and troponin components were isolated as described previously (Staprans *et al.*, 1972; Perry & Cole, 1974; Wilkinson, 1974; Dobrovolskij & Gusev, 1978). Troponin T kinase was isolated by the procedure of Gusev *et al.* (1980), giving an enzyme preparation

Abbreviations used: SDS, sodium dodecyl sulphate; Dns or dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

with a specific activity of 0.6–1.0 $\mu\text{mol}/\text{min}$ per mg of protein with skeletal-muscle troponin T as substrate. Cardiac troponin T was phosphorylated in a medium that contained 6 mM-potassium phosphate, 5 mM-Tris, 2.5 mM-MgCl₂ and 0.5 mM-dithiothreitol, pH 7.4, in the presence of 10 μM -ATP containing [γ -³²P]ATP (The Radiochemical Centre, Amersham, Bucks., U.K.). The concentration of troponin T was 2 mg/ml, and that of troponin T kinase was 2 $\mu\text{g}/\text{ml}$. The incubation was performed for 15 min at 30°C.

Isolation of N-terminal peptides of troponin T

To isolate the N-terminal peptide, the phosphorylated cardiac troponin T was hydrolysed with Pronase (Calbiochem, San Diego, CA, U.S.A.); the substrate/enzyme weight ratio was 25:1, and the digestion was at pH 8.0 for 60 min at 37°C. The hydrolysate was loaded on a Dowex 50 (X2; H⁺ form) column (1.5 cm $\times$ 15 cm), and elution was with water. Unlike all other peptides and amino acids obtained by the digestion, only the N-terminal peptide has a blocked amino group and is not retained on the ion-exchanger (Wilkinson, 1974; Gusev *et al.*, 1980).

The chemical cleavage of troponin T at its single thiol group was performed as described by Degani & Patchornik (1974) and Jacobson *et al.* (1973). Phosphorylated troponin T (15 mg) was dissolved in 2 ml of 40 mM-Tris/HCl buffer, pH 8.0, containing 6 M-urea, 0.1 mM-phenylmethanesulphonyl fluoride and 1 μg of pepstatin/ml. Then dithiothreitol was added to a final concentration of 0.3 mM. After 30 min incubation at 37°C, 5,5'-dithio-(2-nitrobenzoic acid) was added to the mixture to give a final concentration of 2.4 mM. Then 30 min later an aqueous solution of KCN was added to the mixture to give a final concentration of 24 mM. The mixture was then incubated for 12–16 h at 37°C and was subjected to chromatography on a Sephadex G-50 (superfine grade) column (1.6 cm $\times$ 90 cm) equilibrated with 10 mM-HCl. Normally 40–50% of troponin T was cleaved under these conditions.

Determination of the C-terminal sequence of cardiac troponin T

The C-terminal sequence of the protein was determined by the method of Wilkinson (1978). Troponin T (0.5 mg) was dissolved in 250 μl of 10 mM-HCl and precipitated with 50 μl of 100% (w/v) trichloroacetic acid. The precipitate was washed with 300 μl of 20% trichloroacetic acid and twice with 250 μl of acetone. The protein was dissolved in 100 μl of 0.2 M-N-methylmorpholine adjusted to pH 8.5 with acetic acid, and was digested at 37°C with carboxypeptidase A (Reanal, Budapest, Hungary) or with carboxypeptidase B (Worthington Biochemical Corp., Freehold, NJ,

U.S.A.) at a substrate/enzyme weight ratio of 30:1. The samples collected at different time intervals were freeze-dried and dansylated. The amino acids liberated by the carboxypeptidases were identified as their dansyl derivatives after t.l.c. on polyamide sheets (Hartley, 1970). The C-terminal amino acids of the cardiac troponin T N-terminal peptide were determined in a similar manner.

Gel filtration of different forms of troponin T

Gel filtration was performed on a column (1.6 cm $\times$ 90 cm) packed with Sephacryl S-200 or Sephadex G-100 equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, containing 8 M-urea and 15 mM-2-mercaptoethanol. The following proteins with known M_r values were used for the calibration of the column: skeletal-muscle phosphorylase b (M_r , 92 500), ovalbumin (M_r , 45 000), tropomyosin (M_r , 32 000), myoglobin (M_r , 18 500) and cytochrome c (M_r , 12 500).

Amino acid analysis

The peptides and proteins were hydrolysed in 6 M-HCl *in vacuo* (120°C, 24 h). The amino acid composition was determined either in an LKB-3201 or in a Liquimat III (Labotron) amino acid analyser.

Analytical methods

The phosphate content of troponin T was determined by the method of Hess & Derr (1975). The radioactivity of phosphorylated peptides and proteins was measured by the Čerenkov method in an SL-4000 Inter technique scintillation counter. Polyacrylamide-gel electrophoresis was performed by the procedures of Weber & Osborn (1969) and Laemmli (1970). The method of Schaub & Perry (1969) was used for electrophoresis in the presence of 6 M-urea. Protein concentration was determined by the method of Spector (1978).

Results

Chromatographic separation of cardiac troponin components

Ion-exchange chromatography of the whole cardiac troponin in the presence of 8 M-urea results in seven protein peaks (Fig. 1). Troponin I is not adsorbed on the column, and is eluted immediately after application of the protein on the column (fraction 0). Troponin C is tightly adsorbed on DEAE-cellulose, and is eluted with high salt concentration (fraction 6). Results from SDS/polyacrylamide-gel electrophoresis performed by the procedure of Weber & Osborn (1969) suggest that fractions 1–5 contain homogeneous troponin T. To achieve a higher resolution, the protein eluted in fractions 1–5 was subjected to SDS/polyacrylamide-gel electrophoresis by the procedure of

Fig. 1. *Chromatography of the whole cardiac troponin complex on DEAE-cellulose*

Cardiac troponin (1g) was dissolved in 100 ml of buffer A (50 mM-Tris/HCl buffer, pH 8.0, containing 8 M-urea, 2 mM-EDTA and 15 mM-2-mercaptoethanol) and applied to a DEAE-cellulose (Whatman DE-32) column (2.5 cm $\times$ 30 cm) equilibrated with buffer A. The protein was eluted by a gradient of 0.2 M-NaCl dissolved in buffer A (see the Materials and methods section). The fractions were collected every 10 min at a flow rate of 60 ml/h. —, A_{280} ; ●—●, NaCl gradient monitored by conductivity measurements on collected fractions. The horizontal bars indicate the pooled fractions.

Laemmli (1970). It was found that fractions 1 and 2 contain troponin T with an apparent M_r value of 38 000, whereas fractions 4 and 5 contain troponin T with a higher M_r value (approx. 39 500). Fraction 3 contains both forms of troponin T (Fig. 2). Since the fractions of troponin T obtained are differently adsorbed on DEAE-cellulose, it seemed probable that the troponin T forms differ in their net charges. To check this assumption we performed polyacrylamide-gel electrophoresis in the presence of 6 M-urea (Fig. 3). Although fractions 1, 2, 4 and 5 were homogeneous during SDS/polyacrylamide-gel electrophoresis, multiple protein peaks were observed when different troponin T fractions were subjected to urea/polyacrylamide-gel electrophoresis (Fig. 3). It is worthwhile mentioning that the fractions tightly adsorbed on DEAE-cellulose contain protein bands with a higher electrophoretic mobility. These bands are absent from the fractions of troponin T that are weakly adsorbed on DEAE-cellulose. Thus we may conclude that the troponin T fractions tightly adsorbed on DEAE-cellulose bear a higher negative charge.

The apparent heterogeneity of troponin T fractions observed during urea/polyacrylamide-gel electrophoresis could be due to at least two reasons, i.e. to a different mode and degree of protein aggregation, on the one hand, and to a real

difference in the net charge, on the other. It is known that troponin T is poorly soluble at neutral pH and low ionic strength and shows a tendency to aggregate (Hincke *et al.*, 1979; Horwitz *et al.*, 1979). To check the assumption that even in the presence of urea troponin T tends to aggregate, we performed gel filtration of different troponin T fractions on Sephacryl S-200 and Sephadex G-100. Under the conditions used (50 mM-Tris/HCl buffer, pH 8.0, containing 8 M-urea and 15 mM-2-mercaptoethanol) troponin T was eluted as a protein with apparent M_r 68 000–70 000. This suggests that even in the presence of urea troponin T can aggregate. The heterogeneity of troponin T fractions revealed by urea/polyacrylamide-gel electrophoresis could be due to a real difference in the net negative charge.

Comparison of amino acid compositions of the two troponin T forms

In order to decide whether the troponin T forms differ in their net charges, we determined the amino acid composition of two homogeneous forms of troponin T, one with apparent M_r 38 000 and the other with M_r 39 500. The amino acid compositions of both troponin T forms are very similar; normally the difference in the amino acid content does not exceed 4–6% (Table 1). The only significant difference is observed in the content of glutamic

Fig. 2. Densitometric tracing of SDS/polyacrylamide gels after electrophoresis of different fractions of troponin T by the procedure of Laemmli (1970)

The numbers of the scans correspond to the numbering of pooled fractions shown in Fig. 1. A 5–10 μ g portion of protein was loaded on each gel.

acid/glutamine ($P < 0.05$; Student's t test). The form of troponin T with a higher M_r value and enriched in glutamic acid/glutamine is more tightly adsorbed on DEAE-cellulose (fractions 4 and 5, Fig. 1) and has a greater electrophoretic mobility during urea/polyacrylamide-gel electrophoresis. It may thus be concluded that the elevated content of glutamic acid/glutamine in the form of troponin T with apparent M_r 39 500 is responsible for the increased net negative charge.

Fig. 3. Densitometric tracing of urea/polyacrylamide gels after electrophoresis of fractions of troponin T differing in their adsorption on DEAE-cellulose

The numbers of the curves correspond to the numbering of pooled fractions shown in Fig. 1. A 15–30 μ g portion of protein was loaded on each gel. The arrow indicates the position of Bromophenol Blue.

Phosphorylation of cardiac troponin T

It is known that skeletal-muscle troponin T can undergo phosphorylation (Cole & Perry, 1975). The negative charge of cardiac troponin T can depend on the degree of phosphorylation. To check this assumption, we determined the phosphate content in different fractions of troponin T. The degree of phosphorylation of the troponin T fractions tightly adsorbed on DEAE-cellulose is higher than that of the fractions that are weakly adsorbed on the anion-exchanger (Table 2). The degree of phosphorylation does not exceed 1 mol of phosphate/mol of protein, thus suggesting that there exists only one site in troponin T structure that can be phos-

Table 1. Comparison of the two forms of cardiac troponin T

The proteins were obtained by three independent isolations of troponin components, with the use of DEAE-cellulose chromatography. The total number of amino acid residues was taken to be 350 per molecule of both troponin forms. The results are presented as means $\pm$ S.D., with the numbers of experiments given in parentheses. For a comparison the data of Burtnick *et al.* (1976) are presented in the last column.

Amino acid	Composition (residues/molecule)		
	Cardiac troponin T ($M_r = 38000$) ($n = 4$)	Cardiac troponin T ($M_r = 39500$) ($n = 6$)	Data of Burtnick <i>et al.</i> (1976)
Asx	30.9 $\pm$ 1.1	31.3 $\pm$ 1.4	31.0
Thr	9.2 $\pm$ 0.5	8.6 $\pm$ 0.8	8.6
Ser	8.1 $\pm$ 0.5	7.8 $\pm$ 0.6	7.7
Glx	94.7 $\pm$ 1.9	98.9 $\pm$ 2.4	100.0
Pro	7.1 $\pm$ 3.8	6.5 $\pm$ 2.8	10.3
Gly	14.9 $\pm$ 0.3	14.2 $\pm$ 0.9	14.4
Ala	27.0 $\pm$ 0.9	28.2 $\pm$ 1.8	29.6
Val	13.5 $\pm$ 0.7	12.8 $\pm$ 1.1	13.7
Met	4.8 $\pm$ 0.7	4.4 $\pm$ 0.8	4.0
Ile	12.8 $\pm$ 1.0	12.4 $\pm$ 0.9	13.0
Leu	20.7 $\pm$ 1.7	20.4 $\pm$ 1.0	21.0
Tyr	6.3 $\pm$ 1.8	6.4 $\pm$ 1.7	5.1
Phe	9.9 $\pm$ 1.4	10.3 $\pm$ 1.2	8.6
His	6.7 $\pm$ 0.3	6.5 $\pm$ 0.5	4.9
Lys	44.0 $\pm$ 1.2	42.9 $\pm$ 2.2	38.9
Arg	38.3 $\pm$ 1.9	38.2 $\pm$ 1.4	38.3
Total	348.9	349.7	349.1

Table 2. Degree of phosphorylation of cardiac troponin T fractions obtained after ion-exchange chromatography on DEAE-cellulose

The results are presented as means $\pm$ S.D. for two analyses. The proteins were obtained by separation of two different cardiac troponin preparations. The numbering of fractions is as shown in Fig. 1.

Phosphorylation (mol of phosphate/mol of protein)

Fraction 2	Fraction 3	Fraction 4	Fraction 5
0.09 $\pm$ 0.03	0.26 $\pm$ 0.08	0.20 $\pm$ 0.06	0.81 $\pm$ 0.02

phorylated. To determine the phosphorylation site, we phosphorylated both forms of cardiac troponin T, with apparent M_r values of 38000 and 39500, with rabbit skeletal-muscle troponin T kinase in the presence of [γ - 32 P]ATP. The phosphorylation did not influence the mobility of troponin T during SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). The phosphorylated troponin T was digested with Pronase, and the digest was passed through a

Dowex 50 column. The radioactive material was not adsorbed and was eluted with water immediately after application of the sample on the column. This finding indicates that in both forms of troponin T the phosphorylation site is located in the blocked *N*-terminal peptide (Wilkinson, 1974, 1978; Gusev *et al.*, 1980). The amino acid composition of the phosphorylated *N*-terminal peptide of cardiac troponin T is (Ser,Asx,Glx₂,Val); the only amino acid liberated after carboxypeptidase A digestion of this peptide is valine. Thus we may conclude that the two forms of cardiac troponin T having different apparent M_r values do not differ in their *N*-terminal amino acid composition.

Localization of the cysteine residue in cardiac troponin T

The results from the amino acid analysis suggest that cardiac troponin T contains only one cysteine residue (Brekke & Greaser, 1976; Burtnick *et al.*, 1976). In order to determine the approximate localization of this residue, we performed chemical cleavage of the polypeptide chain of phosphorylated troponin T at the thiol group. The mixture of peptides thus obtained was subjected to SDS/polyacrylamide-gel electrophoresis. The chemical cleavage of troponin T at the cysteine resulted in two peptides, one of which, with apparent M_r about 30000–33000, did not contain the radioactive material, whereas the other one, a small peptide migrating with the dye (Bromophenol Blue), contained the total radioactivity of phosphorylated troponin T. Taking the apparent M_r of troponin T to be 40000 and keeping in mind that the site of phosphorylation is located in close proximity to the *N*-terminus, we may conclude that the cysteine residue is located some 50–70 residues from the *N*-terminus of troponin T.

Determination of the C-terminal sequence of troponin T

The C-terminal sequences have been determined for troponin T isolated from rabbit skeletal muscle (Pearlstone *et al.*, 1976) and chicken skeletal muscle (Wilkinson, 1978). In both these cases the C-terminal sequence is Trp-Lys. We made an attempt to determine the C-terminal sequence of cardiac troponin T, using carboxypeptidase B digestion. After a short-term incubation with carboxypeptidase B a spot of Dns-Lys was detected on the polyamide sheets. Prolonged incubation with carboxypeptidase B led to an appearance of spots of Dns-Lys and Dns-Trp. Qualitatively the same results were obtained when two forms of troponin T differing in their M_r values were subjected to carboxypeptidase digestion. Thus the C-terminal sequence of two forms of cardiac troponin T is

Trp-Lys, in which respect it is identical with all troponins T isolated from different skeletal muscles.

Discussion

It is known that various types of skeletal muscles contain different isoforms of troponin T (Clarke *et al.*, 1976; Wilkinson, 1978; Dhoot *et al.*, 1979). These isoforms differ in their M_r values or immunological properties and are probably the products of different genes. Even the preparations of rabbit skeletal-muscle troponin T that are homogeneous during SDS/polyacrylamide-gel electrophoresis produce eight or nine bands during isoelectrofocusing in the presence of urea (Wilkinson, 1974; Drabikowski *et al.*, 1974). It was supposed that this type of microheterogeneity is due to some kind of post-translational modification (Wilkinson, 1974; Clarke *et al.*, 1976).

Cardiac troponin T is represented by two proteins having apparent M_r values of 38 000 and 39 500. Although troponin T is very susceptible to proteolysis, it seems quite improbable that the form of cardiac troponin T with a lower M_r value is a proteolytic fragment of the form with a higher M_r value. The amino acid compositions of the N- and C-terminal peptides of the both forms of cardiac troponin T are identical. This practically excludes the possibility that one form of troponin T is a proteolytic fragment of the other form of the protein. Thus there exist two isoforms of cardiac troponin T that differ in their M_r values and amino acid compositions. The two isoforms of bovine cardiac troponin T resemble those of bovine skeletal-muscle troponin T, having apparent M_r values of 37 000 and 40 000, described by Clarke *et al.* (1976).

The separation of two forms of cardiac troponin T is complicated for several reasons. As already mentioned, the forms of troponin T that are homogeneous during SDS/polyacrylamide-gel electrophoresis (fractions 1, 2, 4 and 5) produce numerous bands during urea/polyacrylamide-gel electrophoresis. Gel filtration in the presence of 8M-urea revealed that cardiac troponin T migrates as a protein with M_r 68 000–70 000. These data indicate that even in the presence of high concentrations of urea troponin T tends to aggregate or is very asymmetric. Besides the tendency for aggregation, each form of cardiac troponin T exists in phosphorylated and dephosphorylated states.

Phosphorylation is a widespread mode of post-translational modification of proteins. The degree of phosphorylation of skeletal-muscle troponin T is 0.8 mol of phosphate/mol (Perry, 1979); that of cardiac troponin T was taken to be 0.3–0.4 mol of phosphate/mol (Cole & Perry, 1975). According to our data, the degree of phosphorylation of some forms of troponin T is equal to 0.8 mol of phosphate/

mol, which is comparable with that of skeletal-muscle troponin T. Specific troponin T kinases belonging to the group of casein kinases of the second type have been isolated from both skeletal and cardiac muscles (Kumon & Villar-Palasi, 1979; Gusev *et al.*, 1980; Dobrovol'skij *et al.*, 1981; Villar-Palasi & Kumon, 1981). The physiological role of troponin T phosphorylation is unknown. Nevertheless it seems essential that the primary structure vicinal to the site of phosphorylation is very similar for troponins T isolated from rabbit skeletal muscle (Pearlstone *et al.*, 1976), chicken skeletal muscle (Wilkinson, 1978) and bovine cardiac muscle (the present paper). The homology of N-terminal peptides of troponin T isolated from different sources may indicate that the N-terminal part of the molecule is involved in troponin interaction with other protein components of the thin filament.

The data presented suggest that, although cardiac troponin T differs from its skeletal-muscle counterpart in terms of M_r value, presence of a single cysteine residue and a higher net negative charge, both proteins share some common features. To these we may add the similarity of their C-terminal sequences and the ability to be phosphorylated in the immediate proximity to the N-terminal site.

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