Investigation of cation-binding properties of cardiac troponin C peptides by circular-dichroism spectroscopy

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1. Bovine cardiac troponin C was cleaved at residues cysteine-35 and cysteine-84. Three peptides, *N*-terminal (residues 1–34), central (residues 35–83) and *C*-terminal (residues 84–161), of cardiac troponin C were obtained in a homogeneous state. 2. Saturation of troponin C or its *C*-terminal peptide with Ca^{2+} or Mg^{2+} is accompanied by an increase in the ellipticity at 222 nm in the c.d. spectrum. The half-maximal changes in the ellipticity of troponin C were observed at 32 nM- Ca^{2+} or 56 μM - Mg^{2+} . The corresponding values for the *C*-terminal peptide are 7.1 nM for Ca^{2+} and 4.5 μM for Mg^{2+} . 3. The ellipticity of the central peptide (residues 35–83) containing the second cation-binding site was decreased on saturation with Ca^{2+} . The half-maximal changes in the ellipticity occur at 80 μM - Ca^{2+} . 4. Study of the c.d. spectra suggests that the α -helices flanking the second cation-binding site of cardiac troponin C exist independently of Ca^{2+} . Saturation of the third and fourth sites with these cations is associated with a considerable increase in the α -helix content, probably due to the formation of an α -helix flanking the third site on the *N*-terminus.

The troponin–tropomyosin complex regulates the contractility of skeletal and cardiac muscles (Perry, 1979; Adelstein & Eisenberg, 1980). The interaction of actin with myosin occurs only after saturation of troponin C, one of the three troponin components, with Ca^{2+} . The cation-binding properties of skeletal troponin C are well established. There is a large body of evidence on the number and localization of the cation-binding sites in this protein (Potter & Gergely, 1975; Leavis *et al.*, 1978), as well as on their specificity for Ca^{2+} and Mg^{2+} (Potter *et al.*, 1977; Leavis *et al.*, 1978). The cation-binding properties of some synthetic peptides whose primary structure is similar to that of the cation-binding sites of skeletal troponin C (Reid *et al.*, 1980, 1981; Kannelis *et al.*, 1983) have been investigated. However, the properties of cardiac troponin C have been studied in less detail. The previously published data (Leavis & Kraft, 1978; Stull & Buss, 1978; Kohama, 1979) have recently been critically reviewed and supplemented by new evidence (Holroyde *et al.*, 1980; Potter *et al.*, 1982). The experimentally obtained data point to the existence of only one Ca^{2+} -specific cation-binding site as well as to the localization of two $\text{Ca}^{2+}/\text{Mg}^{2+}$ -binding sites in the *C*-terminal part of cardiac troponin C (Holroyde *et al.*, 1980; Barskaya & Gusev, 1982). Since the

properties of cardiac troponin C have not met with thorough attention of investigators, it seemed essential to investigate the cation-binding ability of the native protein and its derivative peptides. C.d. spectroscopy has proved to be a convenient tool for the study of the cation-binding properties of skeletal troponin C. This method permits us to obtain information about the changes in the structure of troponin C and its peptides on binding of divalent cations (Leavis *et al.*, 1978; Nagy *et al.*, 1978; Nagy & Gergely, 1979; Reid *et al.*, 1980, 1981; Brzecka *et al.*, 1983). At the same time the use of c.d. spectroscopy is reported only in some papers devoted to cardiac troponin C (Burtinick & Kay, 1977; Leavis & Kraft, 1978; Holroyde *et al.*, 1980), and has never been employed for the study of structure of cardiac troponin C peptides. The aim of the present work was to investigate the structure and cation-binding properties of native cardiac troponin C and its peptides by means of c.d. spectroscopy.

Materials and methods

The whole troponin complex and troponin C were isolated from bovine heart by previously described methods (Tsukui & Ebashi, 1973; Barskaya & Gusev, 1981). The procedures used for

the cleavage of troponin C at cysteine residues and for isolation of the *N*-terminal (residues 1–34) and *C*-terminal (residues 84–161) peptides formed were as described previously (Barskaya & Gusev, 1982). For isolation of the central peptide (residues 35–83), fractions V and VI obtained by gel filtration of troponin C hydrolysate on Sephadex G-50 (Fig. 1 in Barskaya & Gusev, 1982) were mixed and subjected to further purification, which included two gel-filtration steps on a Sephadex G-50 (superfine grade) column (1.6 cm × 90 cm) equilibrated with 0.01 M-HCl. The purity of these peptides was checked by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) and by amino acid analysis. The amino acid composition was determined, after a 24 h hydrolysis in 6 M-HCl at 120°C *in vacuo*, by using a Liquimat III (Labotron) amino acid analyser.

The c.d. spectra were measured on a mark V autodichrograph (Jobin Yvon) calibrated with a standard solution of 3 β -hydroxy-5 α -androstan-17-one in dioxan. This compound has a positive c.d. band at 304 nm with $\Delta\epsilon = +3.31 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The c.d. data for each wavelength, λ , were expressed as mean residue ellipticity:

$$[\theta]_{\lambda} = \frac{\theta_{\lambda} \cdot M}{10 \cdot l \cdot c} \text{ deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1} \quad (1)$$

where θ_{λ} is the measured ellipticity in degrees, l is the optical path-length in cm, c is the concentration of a protein or a peptide in g/ml, and M is taken as the mean residue weight, 115. Each measurement was repeated two to four times; the values obtained were averaged, the ellipticity of a buffer solution containing no protein was subtracted and the resulting values were used for calculation of the mean residue ellipticity. The c.d. spectra were measured in the range 210–240 nm; each spectrum was recorded two or three times at 1 nm steps and a time constant 5–10 s. The spectra obtained were averaged and recorded automatically. Concentrations of peptides and proteins were determined by the method of Lowry *et al.* (1951) or by that of Spector (1978), with bovine cardiac troponin C as a standard.

All measurements were performed in 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH buffer, pH 7.0, at room temperature, in a quartz cell with optical path-length 1 cm. To minimize Ca^{2+} contamination, the buffer solution was passed through a column of Chelex-100 in the K^{+} form. The constant concentration of bivalent ions in the incubation medium was maintained by the use of 0.5 mM- Ca^{2+} -EGTA, - Ca^{2+} -EDTA, - Ca^{2+} -nitrilotriacetate or - Mg^{2+} -EDTA. The concentrations of free non-chelated bivalent

cations were calculated from the values of association constants: $5.01 \times 10^6 \text{ M}^{-1}$ for the Ca^{2+} -EGTA complex, $2.14 \times 10^6 \text{ M}^{-1}$ for the Ca^{2+} -EDTA complex, $5.10 \times 10^2 \text{ M}^{-1}$ for the Ca^{2+} -nitrilotriacetate complex and $2.39 \times 10^5 \text{ M}^{-1}$ for the Mg^{2+} -EDTA complex.

There exist quite a few different methods for prediction of the secondary structure of peptides from the data of c.d. spectroscopy. The c.d. spectroscopy in the far-u.v. region (210–240 nm) provides reliable information about the α -helix content in the peptide (Siegel *et al.*, 1980; Hennessey & Johnson, 1981), and therefore we used the c.d. spectra recorded in that range. Three different approaches were used for calculation of the α -helix content. Two of them are based on a simple linear dependence of the mean residue ellipticity at 222 nm [θ_{222}] and the α -helix content (Chen & Yang, 1971; Lux *et al.*, 1972):

$$C = \frac{[\theta_{222}] + A}{B} \quad (2)$$

where C is the α -helix content, [θ_{222}] is the absolute value of the mean residue ellipticity and A and B are constants. The values of these constants were determined either from the data of Lux *et al.* (1972) or by the approximation of the data of Reid *et al.* (1981) for skeletal troponin C and its peptide. The third method consists in a comparison of the mean residue ellipticity of the protein under study at 13 different wavelengths, ranging from 210 to 240 nm, to the c.d. spectra of 16 proteins with a known ternary structure. The α -helix content in the unknown protein can be determined by the computer-assisted method introduced by Siegel *et al.* (1980). In the present paper we give the values of the α -helix content as calculated from eqn. (2) [the mean value determined by the methods of Lux *et al.* (1972) and Reid *et al.* (1981)] or obtained by the method of Siegel *et al.* (1980).

Results

Isolation of cardiac troponin C peptides

A complete cleavage of troponin C at two cysteine residues (35 and 84) results in three peptides. The *N*-terminal peptide (residues 1–34) does not contain intact cation-binding sites. The *C*-terminal peptide (residues 84–161) contains two intact cation-binding sites. The central peptide (residues 35–83) contains a *C*-terminal α -helix of the first cation-binding site and the second cation-binding site flanked on each side by an α -helix. The procedure for the isolation of the *N*- and *C*-terminal peptides described previously by Barskaya & Gusev (1982) includes gel filtration of

Table 1. Amino acid composition of the central peptide containing the second cation-binding site of cardiac troponin C

Amino acid	Composition (residues/molecule)	
	Peptide 5-2-2	Central peptide (residues 35-83)*
Asx	5.6	6
Thr	2.8	3
Ser	1.9	2
Glx	9.0	9
Pro	1.9	2
Gly	3.7	4
Ala	0.6	0
Val	3.6	5
Met	2.4	5
Ile	1.9	2
Leu	3.2	4
Phe	1.9	2
Lys	2.1	2
Arg	1.6	2

* Based on the sequence determined by van Eerd & Takahashi (1976).

troponin C hydrolysate on a Sephadex G-50 column. The same method is suitable for the isolation of the central peptide of troponin C, although the yield of this peptide was lower than that of the *N*- and *C*-terminal peptides. The isolated central peptide was homogeneous according to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The amino acid composition of the peptide isolated (5-2-2) was similar to that of the peptide containing residues 35-83 of cardiac troponin C (van Eerd & Takahashi, 1976) (Table 1).

Investigation of cation-binding properties of native troponin C and its peptides

It is well known that the binding of Ca^{2+} and Mg^{2+} to skeletal-muscle troponin C or its peptides results in significant changes in their ellipticity (Potter *et al.*, 1977; Leavis *et al.*, 1978; Nagy *et al.*, 1978; Nagy & Gergely, 1979; Reid *et al.*, 1980, 1981). Hence c.d. spectroscopy seems to be the method of choice in the study of the cation-binding properties of cardiac troponin C and its peptides. Native troponin C and its peptides were titrated with Ca^{2+} and Mg^{2+} , and the ellipticity at 222 nm was measured simultaneously. When Ca^{2+} concentration was increased from pCa9 to pCa5, a biphasic increase in troponin C ellipticity was observed (Fig. 1). Using eqn. (2) or the method of Siegel *et al.* (1980), we assumed that in the absence of Ca^{2+} 31% of the amino acids (50 residues) of troponin C are involved in the formation of the α -helix (Table 2). Ca^{2+} induces significant structural

Fig. 1. Dependence of the ellipticity at 222 nm on Ca^{2+} concentration for native cardiac troponin C (●), its *C*-terminal peptide (■) and its central peptide (▲)

Vertical bars designate standard deviations, the absence of the bar indicating that the standard deviation is smaller than the size of the symbol used.

changes; about 50% of the amino acids (80 residues) participate in the α -helix formation in the presence of this cation (Table 2). A half-maximal increase in ellipticity at the first stage occurred at 30 nM- Ca^{2+} (Table 3). At the second stage, with only a limited increase in the 222 nm ellipticity, the half-maximal changes were observed at 3.2 μM - Ca^{2+} (Fig. 1). These results agree well with those obtained by Potter *et al.* (1977) and Johnson *et al.* (1980), and suggest that the most appreciable changes in the ellipticity of cardiac and skeletal-muscle troponin C take place after Ca^{2+} interaction with the high-affinity binding sites ($K_{\text{ass.}} = 3 \times 10^7 \text{ M}^{-1}$). The additional minor changes in the ellipticity at high Ca^{2+} concentrations may be due to the interaction of Ca^{2+} with the low-affinity sites or to small changes in the overall structure of troponin C resulting from Ca^{2+} binding to the high-affinity sites (Nagy & Gergely, 1979; Reid *et al.*, 1981). A monophasic increase in troponin C ellipticity is observed when the protein is titrated with Mg^{2+} , with the half-maximal change taking place at 56 μM - Mg^{2+} . The increase in the ellipticity of troponin C induced by Mg^{2+} is 80-90% of that induced by Ca^{2+} .

The ellipticity of the *C*-terminal peptide of cardiac troponin C markedly increases as Ca^{2+} saturation rises (Fig. 1 and Table 2). The increase in ellipticity is paralleled by an increase in the α -

Table 2. Calculation of the number of amino acid residues involved in α -helix formation in native cardiac troponin C and its peptides

Protein or peptide	No. of residues	$[\theta_{222}]$	No. of amino acid residues involved in α -helix formation	
			Calc. from eqn. (2)	Calc. by method of Siegel <i>et al.</i> (1980)
Cardiac troponin C	161			
+ Ca ²⁺		-14 100	78 $\pm$ 9	82 $\pm$ 5
- Ca ²⁺		-8900	51 $\pm$ 3	49 $\pm$ 6
Skeletal-muscle troponin C	159			
+ Ca ²⁺		-14 800*	87†	
- Ca ²⁺		-9600*	57†	
C-Terminal peptide of cardiac troponin C (residues 84-161)	78			
+ Ca ²⁺		-11 600	32 $\pm$ 3	33 $\pm$ 1
- Ca ²⁺		-6500	19 $\pm$ 1	13 $\pm$ 2
C-Terminal peptide of skeletal-muscle troponin C/TR-2 (residues 89-159)	71			
+ Ca ²⁺		-12 900*	34†	
- Ca ²⁺		-5900*	16†	
Central peptide of cardiac troponin C (residues 35-83)	49			
+ Ca ²⁺		-7000	13 $\pm$ 1	10 $\pm$ 1
- Ca ²⁺		-9200	16 $\pm$ 1	14 $\pm$ 1
CB 8 peptide of skeletal-muscle troponin C (residues 46-77)	32			
+ Ca ²⁺		-2670*	4 $\pm$ 1	
- Ca ²⁺		-2170*	3 $\pm$ 1	

* According to the data of Leavis *et al.* (1978).† According to the data of Reid *et al.* (1981).Table 3. Cation-binding parameters of peptides and native cardiac troponin C
Abbreviation: N.D., not determined.

	Native troponin C	C-Terminal peptide (residues 84-161)	Central peptide (residues 35-83)
Concn. of Ca ²⁺ at which the half-maximal changes in $[\theta_{222}]$ are observed	32 nM	7.1 nM	79 μ M
Hill coefficient	2.0	0.6	1.2
Concn. of Mg ²⁺ at which the half-maximal changes in $[\theta_{222}]$ are observed	56 μ M	4.5 μ M	N.D.
Hill coefficient	1.7	1.4	N.D.

helix content from 17-24% (13-19 amino acid residues) up to 41-42% (32-33 amino acid residues). These data agree well with the corresponding values for skeletal-muscle troponin C C-terminal peptide. Saturation of this peptide with Ca²⁺ results in a more than 2-fold increase in the ellipticity at 222 nm, which corresponds to an increase in the α -helix content from 22 to 48% (Leavis *et al.*, 1978; Reid *et al.*, 1981). The half-maximal changes in the ellipticity of the C-terminal peptide of cardiac troponin C are observed at 7 nM-Ca²⁺ and 4.5 μ M-Mg²⁺ (Table 3). It is worthwhile mentioning that the half-maximal increase in the tyrosine fluorescence of the C-terminal peptide is observed at 5 nM-Ca²⁺ (Bars-

kaya & Gusev, 1982). Thus the apparent binding constant for the C-terminal peptide interaction with Ca²⁺ is 3-4-fold and that with Mg²⁺ 8-10-fold higher than those for native cardiac troponin C.

The data in Fig. 1 demonstrate that even in the absence of Ca²⁺ the central peptide of cardiac troponin C (residues 35-83) has rather a high α -helix content. In the absence of Ca²⁺ the α -helix content in this peptide is 32% (16 residues), which is higher than that in the C-terminal peptide (24%). Saturation of this peptide with Ca²⁺ results in a decrease of the 222 nm ellipticity and a concomitant decrease of the α -helix content (Fig. 1 and Table 2). The half-maximal changes in the ellipticity were observed at 79 μ M-Ca²⁺ (Table 3).

Hence the apparent dissociation constant of Ca^{2+} for the central peptide is equal to $80\ \mu\text{M}$ and is comparable with that for the CNBr-cleavage peptide CB 8 ($125\ \mu\text{M}$) containing the second Ca^{2+} -binding site of skeletal-muscle troponin C (Leavis *et al.*, 1978). Summing up, we shall assume that, in contrast with native troponin C or its C-terminal peptide, saturation of the second Ca^{2+} -binding site with Ca^{2+} leads to a destruction of the α -helix. This property of the central peptide of cardiac troponin C makes it different from all other peptides of skeletal-muscle troponin C and from most of the Ca^{2+} -binding proteins described previously (Leavis *et al.*, 1978; Nagy & Gergely, 1978; Reid *et al.*, 1981; Brzecka *et al.*, 1983). The only exception reported in the literature is brain S-100b protein, saturation of which with Ca^{2+} results in a 12% decrease in its 222 nm ellipticity (Mani *et al.*, 1982).

Discussion

A comparison of the structure of different Ca^{2+} -binding proteins with that of parvalbumin leads to the conclusion that each Ca^{2+} -binding site is flanked by two α -helices (Kretsinger, 1980). In the absence of Ca^{2+} not all of the predicted α -helices are completely formed. Studies on the C-terminal peptide of skeletal-muscle troponin C suggest that in the absence of Ca^{2+} two eight-to-ten-residue helices flank the third and the fourth Ca^{2+} -binding sites at their C-terminus, whereas in the presence of Ca^{2+} two additional helices are formed at the N-termini of both cation-binding sites (Nagy *et al.*, 1978; Nagy & Gergely, 1979; Reid *et al.*, 1981).

Supposing that the structure of the C-terminal peptides of cardiac and skeletal-muscle troponin C are similar, we may suggest that in the absence of Ca^{2+} the C-terminal peptide of cardiac troponin C contains two or three α -helices, each containing six to eight amino acid residues. Using the method of Chou & Fasman (1974), we can predict the location and length of the α -helical sites. There are three sites in the C-terminal peptide, in which the probability of α -helix formation is the greatest. The first α -helix (residues 114–123) is located at the C-terminus of the third cation-binding site, and the second and third ones flank the fourth cation-binding site in the N-terminus (residues 133–138) and C-terminus (residues 152–158). Besides the three above-mentioned sites, there is the fourth site (residues 94–104) with a fairly high probability of α -helix formation. Bearing in mind that the probability of helix formation at this site is lower than that for the first three sites, we can speculate that the N-terminal helix of the third cation-binding site is formed only after saturation of this site with Ca^{2+} . Thus, according to our predictions, on saturation of the C-terminal peptide of cardiac

troponin C with Ca^{2+} , the number of residues involved in α -helix formation increases from 22 to 33. This agrees well with the data on the changes in the ellipticity of the C-terminal peptide (Table 2).

It is worthwhile mentioning that the binding constants for Ca^{2+} and Mg^{2+} measured for the C-terminal peptide are much higher than those for native troponin C. Earlier the same phenomenon was observed when other methods were employed for the study of the C-terminal peptides of both skeletal-muscle and cardiac troponin C (Leavis *et al.*, 1978; Barskaya & Gusev, 1982). Therefore the deletion of the N-terminal part of troponin C may in some way facilitate the interaction between Ca^{2+} and the cation-binding sites located in the C-terminal part of troponin C.

The association constant for Ca^{2+} binding to the central peptide (residues 35–83) is roughly 20-fold lower than that for the Ca^{2+} -specific site of cardiac troponin C (Potter *et al.*, 1982). The same results were reported by Leavis *et al.* (1978), who studied the cation-binding properties of skeletal-muscle troponin C peptides (CB 8 and TR 1), containing Ca^{2+} -specific sites, as well as by Kannelis *et al.* (1983) for a synthetic peptide whose primary structure is similar to that of the second cation-binding site of skeletal-muscle troponin C. These data are consistent with those of Reid & Hodges (1980), suggesting that the third and fourth sites in native troponin C are the first ones to be saturated with Ca^{2+} . Saturation of these sites with Ca^{2+} brings about significant conformational changes and facilitates the interaction of Ca^{2+} -specific sites with Ca^{2+} .

As mentioned above, Ca^{2+} decrease the ellipticity of the central peptide (residues 35–83) of cardiac troponin C. The interaction of Ca^{2+} with homologous peptides of skeletal-muscle troponin C either results in a slight (approx. 23%) increase in the ellipticity of peptide CB 8, or does not influence that of peptide TR 1 (Leavis *et al.*, 1978). To sum up, we may assume that Ca^{2+} affects the conformation of the peptides containing the first and second cation-binding sites to a much weaker degree than that of the peptides containing the third and fourth cation-binding sites.

According to both Nagy & Gergely (1979) and Evans *et al.* (1980) independently on Ca^{2+} concentration the first and the second cation-binding sites of skeletal-muscle troponin C are flanked on both sides by completely formed α -helices. If the structures of the N-terminal parts of cardiac and skeletal-muscle troponin C are similar, we might expect that the central peptide of cardiac troponin C (residues 35–83) will contain three α -helical sites. Using the method of Chou & Fasman (1978), we may predict that residues 40–45, 55–60 and 76–82 are involved in α -helix formation. The Ca^{2+} -

induced decrease in 222 nm ellipticity of the central peptide might be due to the 'melting' of one of the three α -helical regions, presumably residues 40–45. We cannot exclude the possibility, also, that the decrease in the ellipticity of the central peptide is a result of Ca^{2+} -induced translocation of 2-iminothiazolidine, formed in the place of cysteine-35, towards the Ca^{2+} -binding loop of the second Ca^{2+} -binding site. According to Reid & Hodges (1980), the saturation of the second Ca^{2+} -binding site with Ca^{2+} results in replacement of threonine-71 by cysteine-35 in the Ca^{2+} -binding loop of the second Ca^{2+} -binding site. Taking into account that the bulky 2-iminothiazolidine ring is formed in the place of cysteine-35, we may finally suggest that the above-mentioned translocation can significantly influence the structure of the short-chain central peptide of cardiac troponin C.

A comparison of the secondary structure of cardiac and skeletal-muscle troponin C has revealed many common features. The Ca^{2+} -induced increase in the α -helix content in both proteins is mainly due to the spiralization taking place in the vicinity of the third and fourth sites in the C-terminal part of troponin C. The α -helix content in the N-terminal part of troponin C only slightly depends on Ca^{2+} . A possible reason for this is that the formation of α -helices flanking the first and second cation-binding sites does not depend on Ca^{2+} .

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