THE CALCIUM-BINDING PROPERTIES OF BOVINE CARDIAC TROPONIN C

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1. Introduction

Troponin in vertebrate skeletal-muscle performs its regulatory function by being able to recognize changes in the free Ca^{2+}-concentration in the surrounding sarcoplasm. Previous studies performed in this laboratory concerning bovine cardiac troponin, its components and their interactions [1–5] suggested that regulation of cardiac-muscle contraction could be explained, qualitatively, by a model similar to that proposed for the skeletal-muscle system [6–8].

A thorough review and re-evaluation of the calcium-binding parameters of skeletal-muscle troponin and its calcium-binding subunit, troponin C (TN-C), was performed recently by Potter and Gergely [9]. They reported that both skeletal troponin and TN-C could bind, maximally, four Ca^{2+}-ions/molecule.

Collins et al. [10], by an analysis of the skeletal TN-C amino acid sequence, had been able to predict correctly that TN-C could bind four Ca^{2+}-ions. The basis for this prediction was the presence of four stretches of sequence which displayed extensive homology with the calcium-binding regions of carp parvalbumin [11]. When Van Eerd and Takahashi [12] published the amino acid sequence of bovine cardiac TN-C, they attempted a similar prediction. Only three regions in the cardiac TN-C sequence bore significant homology to the parvalbumin calcium-binding sites. These three stretches of sequence corresponded to three of the four proposed calcium-binding regions of skeletal TN-C. Therefore, it was suggested that each cardiac TN-C molecule could bind three Ca^{2+}-ions.

This report details the results of experiments designed to investigate the parameters governing the interaction between Ca^{2+} and cardiac TN-C.

Abbreviations: ATPase adenosinetriphosphatase, $[Ca^{2+}]_f$ the concentration of free Ca^{2+}-ions in solution, CD circular dichroism, EGTA ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetra-acetic acid, *f* fraction titrated, *i* the number of moles of Ca^{2+} which bind to a mole of TN-C, K_{app} apparent association constant, TN-C troponin C, $\delta\theta$ the change in ellipticity value elicited at a certain wavelength by the addition of a particular amount of calcium to a solution containing TN-C, $\Delta\theta$ the maximum change in ellipticity value which can be elicited at a certain wavelength by the addition of calcium to a solution containing TN-C

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2. Materials and methods

2.1. *Bovine cardiac TN-C*

Troponin was extracted from beef hearts using the procedure of Tsukui and Ebashi [13]. TN-C was purified as detailed previously [1].

Concentrations of solutions of TN-C were determined spectrophotometrically using an extinction coefficient at 278 nm for a 1% solution of TN-C of 3.4 [1]. The molecular weight of cardiac TN-C was taken to be 18 459, as determined from its amino acid sequence [12].

2.2. *Calcium ion concentration*

The level of free Ca^{2+}-ions in solution was adjusted by means of an EGTA-containing buffer, employing principles discussed by Perrin and Dempsey [14]. From Schwarzenbach et al. [15], the stepwise equilibrium constants for the deprotonation of EGTA, $pK_{a1} = 2.0$, $pK_{a2} = 2.65$, $pK_{a3} = 8.85$ and $pK_{a4} =$

9.46, and the association constants for Ca^{2+}, and Mg^{2+}, $pK_{Ca} = 10.97$ and $pK_{Mg} = 5.21$, were obtained.

At pH 7.5, the pH employed throughout this investigation, the apparent association constants for EGTA with Ca^{2+} was calculated to be 4.4×10^7 M^{-1}. For EGTA with Mg^{2+}, it was 90 M^{-1}. Therefore, the binding of Mg^{2+} to EGTA was neglected in calculations of free Ca^{2+}-concentrations.

2.3. *Circular dichroism measurements*

CD measurements were performed with a Cary 6001 CD attachment to a Cary 60 recording spectropolarimeter as described by Oikawa et al. [16].

TN-C was dissolved in 0.15 M KCl, 50 mM Tris–HCl, pH 7.5, 1 mM EGTA and dialysed 24 h at 4°C against the same solvent prior to recording its CD spectrum in the peptide absorption-range. After the addition of an aliquot of $CaCl_2$, the spectrum was recorded again. This process was repeated until no further change in the spectrum could be detected. Similar sets of spectra were collected with TN-C equilibrated against the same solvent, but containing 2 mM $MgCl_2$.

From these data, the number of Ca^{2+}-ions bound per TN-C molecule, i, and an apparent binding constant, K_{app}, could be estimated as described by Willick and Kay [17] for the evaluation of the same parameters for Mg^{2+} binding to tRNA. Such calculations assume that the structural transition observed by CD involves only 2 states. Either TN-C has Ca^{2+} bound and has undergone the complete conformational change or no Ca^{2+} is bound and the conformational change has not occurred. The fraction of TN-C molecules in the calcium-bound state, f, can be determined as the ratio of the change in ellipticity value at a particular free Ca^{2+}-concentration at a particular wavelength, $\delta\theta$, to the maximum change which can be elicited in the ellipticity value at that wavelength, $\delta\theta$:

$$f = \frac{\delta\theta}{\Delta\theta} = \frac{K_{app}\,[Ca^{2+}]_f^i}{1 + K_{app}\,[Ca^{2+}]_f^i}$$

In this study, $\delta\theta$ and $\Delta\theta$ were measured at 221 nm, the position of one of the minima in the TN-C CD spectrum.

2.4. *Gel-filtration*

Hummel and Dreyer [18] first used gel-filtration as a rapid and accurate method to study protein-binding phenomena. Using this technique, with the modifications of Voordouw and Roche [9], the number of Ca^{2+}-ions bound/molecule of TN-C, and an apparent association constant, were determined.

To determine the maximal number of Ca^{2+}-ions which would bind to each cardiac TN-C molecule, a 1.0×30 cm column of Sephadex G-25 fine was equilibrated against 0.15 M KCl, 50 mM Tris–HCl, pH 7.5 and 1×10^{-4} M $CaCl_2$. Three to four mg of lyophilized TN-C were dissolved in 0.6 ml of the same buffer, but containing 5×10^{-4} M $CaCl_2$ and dialysed overnight at 4°C. The TN-C solution then was allowed to equilibrate to 22°C and was applied to the column. A constant elution rate of 20 ml/h was maintained by an LKB peristaltic pump and 2.5 min fractions were collected. Each fraction was analysed for protein-content by measuring its absorbance at 278 nm, and for calcium-content using a Unicam SP90A Series 2 atomic absorption spectrophotometer, with instrumental settings adjusted as described in Unicam Atomic Absorption Method Ca-2 [20].

To determine the dependence of Ca^{2+}-binding to Ca^{2+}-concentration, the column equilibration buffer was altered to contain 5×10^{-5} M EGTA and a specific concentration of $CaCl_2$ which would produce sub-maximal Ca^{2+}-binding to TN-C. In these experiments, the TN-C was applied to the column after it had been dialysed against 0.15 M KCl, 50 mM Tris–HCl, pH 7.5, 5×10^{-5} M EGTA and 1×10^{-4} M $CaCl_2$. Again, integration of the amount of Ca^{2+} eluting in the void volume peak and division of this sum by the total amount of protein in the peak provided the number of Ca^{2+}-ions bound to each TN-C molecule under the experimental conditions. Estimates for the parameters i and K_{app} were obtained by fitting the experimental data to a curve computed from the equation [9]:

$$Ca^{2+}/\text{TN-C} = \frac{i K_{app}\,[Ca^{2+}]_f}{1 + K_{app}\,[Ca^{2+}]_f}$$

For each experiment, a new standard curve for the determination of calcium was prepared. The column equilibration buffer was used in each case as the zero readout or baseline value to which additions of $CaCl_2$

yielded standard solutions covering the range of calcium concentration from 0–1 X 10^{-4} M, in increments of 1 X 10^{-5} M, above the baseline level.

As in the case of the CD titration studies, an analogous set of Ca^{2+}-binding experiments was performed in the presence of 2 mM $MgCl_2$ to observe how Mg^{2+}-ion affected the Ca^{2+}-binding parameters of cardiac TN-C.

To avoid contamination of solutions with Ca^{2+} from laboratory glassware, Nalgene beakers, test tubes, volumetric flasks, solution bottles and pipets were employed throughout the study.

3. Results

3.1. *CD titration studies*

The results of additions of $CaCl_2$ to a cardiac TN-C solution in the absence of Mg^{2+}-ions are presented in the form of a titration-curve in fig.1. The fraction of completion of the CD spectral change, *f*, is plotted against the negative logarithm of the free Ca^{2+}-concentration in solution, pCa_f. The data were collected from 6 sets of titrations on three different preparations of TN-C. The solid curve is a theoretical titration-curve calculated assuming TN-C possesses a single calcium-binding site and an apparent association constant for Ca^{2+} of 7 X 10^6 M^{-1}.

In the absence of added Ca^{2+}, it was shown that 2 mM $MgCl_2$ produced a change in the CD spectrum approximately one-half the magnitude of that produced by saturating levels of Ca^{2+} [1]. Therefore, the titration data for additions of $CaCl_2$ to TN-C solutions in the presence of 2 mM $MgCl_2$ are presented on a scale of from 50–100% complete (fig.2). The results represent six sets of titrations on three different preparations of TN-C.

Apparently, Mg^{2+}-ions compete with Ca^{2+} for occupation of the site responsible for the observed conformational change, with the result that higher concentrations of Ca^{2+} are required to attain the maximal CD spectral-change. This results in a reduced apparent association constant for the interaction of TN-C with Ca^{2+}. The solid line in fig.2 represents a theoretical curve calculated assuming an apparent association constant of 5 X 10^4 M^{-1} and that 0.6 Ca^{2+}-ions bind to each TN-C molecule. The fit of this curve to the experimental data is not excellent, but the

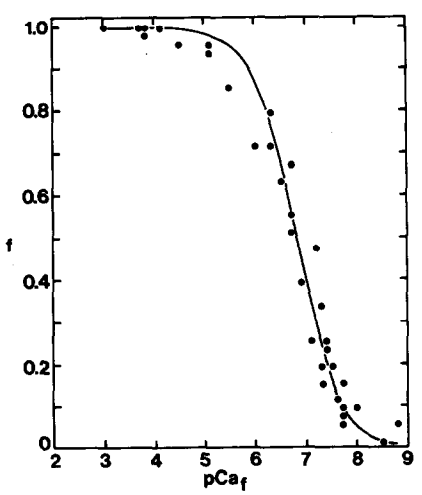

Fig.1. CD calcium titration of the conformational change in TN-C ('minus Mg^{2+}'). The protein was dissolved in 0.15 M KCl, 50 mM Tris–HCl, pH 7.5, 1 mM EGTA. $CaCl_2$ was added incrementally and $[\theta]_{221}$ recorded for each addition. *f* is the fraction of completion of the conformational change and $pCa_f = -\log[Ca^{2+}]_f$. The solid line represents a calculated titration curve assuming that one Ca^{2+}-binding site exists on TN-C and the apparent association constant for the interaction is 7 X 10^6 M^{-1}.

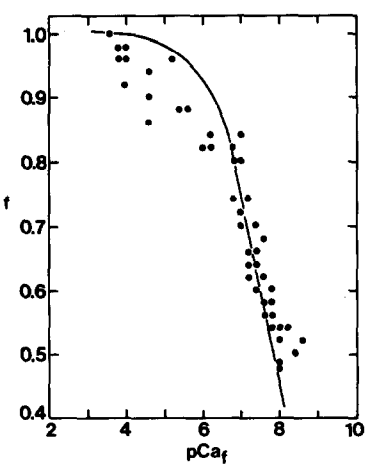

Fig.2. CD calcium titration of the conformational change in TN-C ('plus Mg^{2+}'). The experimental conditions were as described in fig.1 except that 2 mM $MgCl_2$ was present in all buffers. The solid line represents a calculated titration curve assuming that 0.6 Ca^{2+} binds to each TN-C and the apparent association constant for the interaction is 5 X 10^4 M^{-1}.

results provide a rough quantitation of the reduction of the association constant for the interaction between TN-C and Ca^{2+}. The fractional value for the number of Ca^{2+}-ions which bind to each TN-C molecule implies that there is not a complete displacement of the Mg^{2+}-ions from the site on TN-C to which divalent cations bind and induce the observed conformational change. Presumably higher free-calcium levels would displace a greater percentage of Mg^{2+}-ions.

Incremental additions of $MgCl_2$ to TN-C solutions in the absence of Ca^{2+} produced increases in observed ellipticity values over a broad concentration range. The data were not suitable for a binding-parameter analysis as performed with Ca^{2+}. From the Mg^{2+}-concentration required to produce one-half of the maximal observed ellipticity change at 221 nm, the apparent association constant for TN-C with Mg^{2+} was estimated to be between 10^3 and 10^4 M^{-1}.

Addition of $MgCl_2$ to TN-C in the presence of excess free Ca^{2+} produced no further change in its CD spectrum. Therefore, Mg^{2+}, if it does bind at a site other than the one responsible for the conformational change already described, does not induce a further structural change which is detectable in CD spectra.

3.2. *Gel-filtration studies*

Van Eerd and Takahashi [12] had predicted that three Ca^{2+}-ions could be bound to each cardiac TN-C molecule. Our CD titration results demonstrated that the binding of a single Ca^{2+}-ion could elicit the full conformational change observed in CD spectra. The possibility remained that, in total, more than one Ca^{2+}-ion could bind to each TN-C, but that only the one responsible for the altered CD properties had been detected.

To test this hypothesis, the gel-filtration technique of Hummel and Dreyer [18], as adapted by Voordouw and Roche [19], was employed to study the binding-parameters for the interaction of TN-C with Ca^{2+}.

As a basis for comparison, the maximal binding of Ca^{2+} to rabbit skeletal TN-C (kindly donated by Dr W. D. McCubbin) was determined. Assuming an extinction coefficient at 280 nm for a 1% solution of skeletal TN-C of 1.93 [21], each mole of TN-C was found to bind 3.9 ± 0.2 mol Ca^{2+}, in excellent agreement with the value of four found by Potter and Gergely [9].

An identical set of experiments was performed

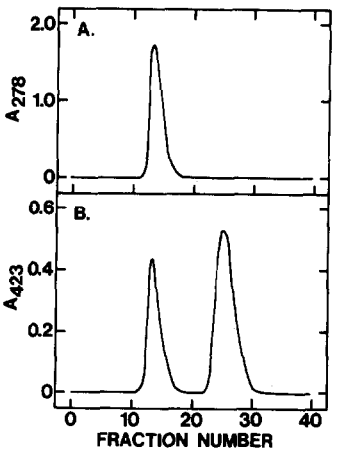

Fig.3. Elution of TN-C and calcium from a Sephadex G-25 column. TN-C (~ 1 mg/ml) was applied to a 1.0 × 30 cm column in 0.6 ml 0.15 M KCl, 50 mM Tris–HCl, pH 7.5, 5×10^{-4} M $CaCl_2$ and eluted with the column equilibration buffer, 0.15 M KCl, 50 mM Tris–HCl, pH 7.5, 1×10^{-4} M $CaCl_2$. (A) Protein-elution profile monitored spectrophotometrically at 278 nm. (B) Calcium-elution profile monitored using an atomic absorption spectrophotometer.

employing cardiac TN-C. Eluant from the gel-filtration column (fig.3) was monitored for protein-content and calcium-concentration. A single protein peak eluted at the column void volume. Two calcium peaks emerged, the first co-eluting with the protein and the second appearing later in the profile. The mole ratio of Ca^{2+} to TN-C in the first peak was determined to be 3.2 ± 0.2. When 2 mM $MgCl_2$ was incorporated into the column equilibration buffer, this value was found to be 2.9 ± 0.2. Cardiac TN-C is capable, therefore, of binding three Ca^{2+}-ions, on a mol/mol basis.

Incorporation of EGTA into the column equilibration buffer allowed investigation of calcium-binding to TN-C as a function of free Ca^{2+}-concentration (fig.4). The best fit (judged by eye) to the data collected in the absence of Mg^{2+} (fig.4, open squares) was obtained by setting $i = 3.2$ and $K_{app} = 7 \times 10^6$ M^{-1}. For data collected in the presence of 2 mM $MgCl_2$ (fig.4, closed squares) the best fit was generated by setting $i = 2.9$ and K_{app} 1×10^6 M^{-1}.

The results demonstrate that Mg^{2+}-ions in solution compete with Ca^{2+}-ions for binding sites on TN-C. An increased Mg^{2+}-concentration increases the level of Ca^{2+} necessary to occupy the sites. Once a sufficiently high

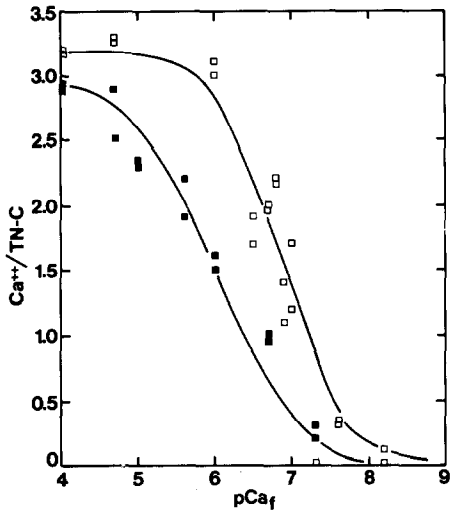

Fig.4. Determination of the moles calcium bound/mole TN-C at various concentrations of free calcium-ion. Open squares represent data collected in the absence of $MgCl_2$. The solid curve through these data (open squares) represents a titration curve calculated assuming 3.2 calcium-ions bind/TN-C and the association constant for the interaction is 7×10^6 M^{-1}. Closed squares represent data collected in the presence of 2 mM $MgCl_2$. The solid curve through these data (closed squares) was calculated assuming 2.9 Ca^{2+} bind to each TN-C and the association constant for the interaction is 1×10^6 M^{-1}.

Ca^{2+}-concentration has been attained, essentially all the Mg^{2+} can be displaced from the binding sites.

Our results differ from those reported by Drabikowski et al. [22] and by Brekke and Greaser [23]. Drabikowski et al. found only 1.64 Ca^{2+} bound/cardiac TN-C. However, the methodology they employed only measured 2.16 Ca^{2+} bound/skeletal TN-C. Brekke and Greaser, similarly, found 2.08 Ca^{2+} bound/cardiac TN-C, but only 2.25 bound/skeletal TN-C. These latter values may be low estimates for the maximal binding capacities of TN-C due to the experimental conditions employed. TN-C was dialysed against 0.5 M KCl, 2 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5 and 1×10^{-6} M $CaCl_2$. Clearly, in the presence of 2 mM $MgCl_2$, a free Ca^{2+}-concentration of 1×10^{-6} M would not saturate the binding sites on cardiac TN-C (figure 4). At $pCa_f = 6$, our results suggest that about 1.5–1.7 mol Ca^{2+} would be bound to a mole of cardiac TN-C. The somewhat higher value found by Brekke and Greaser may reflect that, although working at micromolar levels of Ca^{2+}, they did not use a metal-ion buffer system to control precisely free Ca^{2+}-concentrations.

4. Conclusions

Analytical gel-filtration studies revealed that cardiac TN-C can bind three Ca^{2+}-ions/molecule. Only one of these is responsible for the observed alterations of the CD properties of the protein in the presence of Ca^{2+}. The remaining two occupy sites which are 'invisible' to circularly polarized light.

The data do not indicate that different classes of sites, with respect to magnitude of association constant, exist on cardiac TN-C, as reported for skeletal TN-C [9]. They do suggest that the sarcoplasmic level of free Mg^{2+}-ions does affect the binding parameters for the interaction of TN-C with Ca^{2+}. Although the precise concentration of free Mg^{2+} within cells is unknown [24], a high estimate (2 mM Mg^{2+}) results in a reduction of the association constant from 7×10^6 M^{-1} to about 1×10^6 M^{-1}. The sensitivity of TN-C to a Ca^{2+}-induced conformational change would still fall within the range of Ca^{2+}-concentrations experienced in the sarcoplasm, 10^{-5} M in actively contracting-muscle to 10^{-8} M in resting-muscle.

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References

[1] Burtnick, L. D., McCubbin, W. D. and Kay, C. M. (1975) Can. J. Biochem. 53, 15–20.
[2] Burtnick, L. D., McCubbin, W. D. and Kay, C. M. (1975) Can. J. Biochem. 53, 1207–1213.
[3] Burtnick, L. D., McCubbin, W. D. and Kay, C. M. (1976) Can. J. Biochem. 54, 546–552.
[4] Burtnick, L. D. and Kay, C. M. (1976) FEBS Lett. 65, 234–237.
[5] Brittain, H. G., Richardson, F. S., Martin, R. B., Burtnick, L. D. and Kay, C. M. (1976) Biochem. Biophys. Res. Commun. 68, 1013–1019.

[6] Hitchcock, S. E., Huxley, H. E. and Szent-Györgi, A. G. (1973) J. Mol. Biol. 80, 825–836.
[7] Margossian, S. S. and Cohen, C. (1973) J. Mol. Biol. 81, 409–413.
[8] Potter, J. D. and Gergely, J. (1974) Biochemistry 13, 2697–2703.
[9] Potter, J. D. and Gergely, J. (1975) J. Biol. Chem. 250, 4628–4633.
[10] Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G. and Jackman, N. (1973) FEBS Lett. 36, 268–272.
[11] Kretsinger, R. H. and Nockolds, C. E. (1973) J. Biol. Chem. 248, 3313–3326.
[12] Van Eerd, J.-P. and Takahashi, K. (1975) Biochem. Biophys. Res. Commun. 64, 122–127.
[13] Tsukui, R. and Ebashi, S. (1973) J. Biochem. 73, 1119–1121.
[14] Perrin, D. D. and Dempsey, B. (1974) in: Buffers for pH and Metal-Ion Control, Chapman and Hall, Ltd, London.
[15] Schwarzenbach, G., Senn, H. and Anderegg, G. (1957) Helv. Chim. Acta 40, 1886–1900.
[16] Oikawa, K., Kay, C. M. and McCubbin, W. D. (1968) Biochim. Biophys. Acta 168, 164–167.
[17] Willick, G. E. and Kay, C. M. (1971) Biochemistry 10, 2216–2222.
[18] Hummel, J. P. and Dreyer, W. J. (1962) Biochim. Biophys. Acta 63, 530–532.
[19] Voordouw, G. and Roche, R. S. (1974) Biochemistry 13, 5017–5021.
[20] Unicam Methods Sheets (1966) Method Ca-2, Pye-Unicam Ltd, Cambridge, England.
[21] Greaser, M. L. and Gergely, J. (1971) J. Biol. Chem. 246, 4226–4233.
[22] Drabikowski, W., Dabrowska, R. and Barylko, B. (1975) in: Recent Advances in Studies on Cardiac Structure and Metabolism (Fleckenstein, A. and Dhalla, N. S. eds) vol. 5, pp. 245–252, University Park Press, Baltimore.
[23] Brekke, C. J. and Greaser, M. L. (1976) J. Biol. Chem. 251, 866–871.
[24] Polimeni, P. I. and Page, E. (1973) Circ. Res. 33, 367–374.