Comparative calcium binding and conformational studies of turkey and rabbit skeletal troponin C

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Troponin C from turkey skeletal muscle has been compared with its chicken counterpart in terms of amino acid composition and fragmentation patterns and with rabbit TN-C by Ca^{2+} binding and conformational response to Ca^{2+} as monitored by CD and fluorescence. Cyanogen bromide and tryptic digestion mixtures of chicken and turkey TN-C have been separated by reversed-phase HPLC. The similarity of the elution profiles, along with the almost identical amino acid compositional data, suggest that the sequences are essentially equivalent. Both turkey and rabbit TN-C bound 2 mol Ca^{2+}/mol protein at pH 5.3, while at pH 6.8, this figure was raised to 4 mol/mol protein. Circular dichroism and fluorescence measurements indicated that the conformations of the two proteins responded in a very similar manner to the presence of Ca^{2+}.

Calcium binding *Circular dichroism* *Fluorescence* *HPLC* *Troponin C*

1. INTRODUCTION

The X-ray structures of TN-C from turkey and chicken skeletal muscle have recently been described at a resolution of 2.8 and 3.0 Å, respectively [1,2]. The announcement of these structures has generated a great deal of interest, for in both cases, although the crystals were grown from solutions containing enough Ca^{2+} or Mn^{2+} to fill all 4 Ca^{2+}-binding sites, only the 2 high-affinity sites of the C-terminal domain (III and IV) were found to be occupied by metal. Since the regulatory (N-terminal) domains are metal-free, they adopt a different conformation from the metal-filled domain.

With reference to the solved crystal structures, a query arises as to whether they are indicative of some peculiar property of avian TN-C that has not become apparent, since most Ca^{2+}-binding and conformational studies on TN-C have been done using the protein from rabbit skeletal muscle. To shed some light on this question, we have undertaken an examination of the Ca^{2+}-binding and conformational properties of turkey skeletal TN-C under several solvent conditions, including those approximating the achievement of crystallization, and compared the results directly with the rabbit protein. In addition, we have compared the CNBr and complete tryptic fragmentation patterns of chicken and turkey TN-C by analytical reversed-phase HPLC with a view to exploring sequence identity.

The results show that at pH 5.3 both turkey and rabbit skeletal TN-C bind 2 mol Ca^{2+}/mol protein, whereas at pH 6.8, this figure is raised to 4 mol/mol protein. In 1.7 M ammonium sulfate, an approximation to the concentration used in crystallization and at pH 6.8, although a considerable amount of helical structure had been induced, the proteins still bound 4 mol Ca^{2+}/mol protein. The close parallelism in amino acid com-

Abbreviations: CNBr, cyanogen bromide; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); $[\theta]_{221\,nm}$, mean residue ellipticity at 221 nm; TN-C, Ca^{2+}-binding subunit of troponin complex

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positional data and reversed-phase HPLC chromatograms for turkey and chicken TN-Cs suggest that their sequences are, in all probability, very similar.

2. MATERIALS AND METHODS

2.1. *Protein preparations*

TN-C was prepared from acetone powders of chicken and turkey breast muscle, rabbit back muscle, and bovine cardiac muscle by established procedures from this laboratory [3,4] and was homogeneous when examined by SDS-PAGE. Concentrations were estimated by UV absorption after clarification (Beckman model L8-70M, or Brinkmann air centrifuge) using extinction coefficients of 0.175 (277 nm), 0.120 (259 nm), 0.132 (259 nm) and 0.23 (276 nm) for 1 mg/ml solutions of rabbit, chicken, turkey skeletal and bovine cardiac TN-C, respectively. For conversion to the apo state the TN-C samples were given the 'heated-chelex' treatment described in [5] in which ~20 mg of salt-free protein, in the Ca^{2+}-saturated form, was dissolved in 10 mM NH_4HCO_3 at pH 9, introduced into a drained pre-equilibrated resin bed of Chelex 100 (Bio-Rad Labs), heated to 60°C for 45 min, eluted from the column directly into an acid-washed polyethylene bottle and immediately lyophilized. All solutions used in this study employed water purified in a Milli-Q assembly (Millipore).

2.2. *Reversed-phase HPLC*

CNBr and tryptic peptides of chicken and turkey TN-C were separated on a Synchropak RP-P®, C-8 column (4.1 × 250 mm). Programmed analytical chromatographic runs were performed on a Varian Vista series 5500 liquid chromatograph interfaced with a Varian CDS 402 data system. The detector was a Varian UV-200 operated at 220 nm. Peptides were dissolved in 0.1% trifluoroacetic acid, rendered free of particulates by 10 min hard centrifugation, injected onto the column, and eluted with a linear gradient using a solvent of 0.1% trifluoroacetic acid, 70% acetonitrile.

2.3. *Protein fragmentation*

CNBr digests were prepared with a 200-fold molar excess of reagent over methionine residues in 70% formic acid for 20 h at room temperature. CNBr and formic acid were removed by dilution with water, lyophilization, neutralization with NH_4OH and lyophilization. Complete tryptic digestions were carried out in 50 mM NH_4HCO_3, 2 mM EDTA, pH 8.4, at 37°C for 4 h using 2% (w/w) TPCK-treated trypsin (Sigma). The digests were lyophilized.

2.4. *Ca^{2+}-binding assay*

Consultation of technical literature available from Amicon Corp. as well as an early publication on membrane ultrafiltration suggested the possibility of using the relatively new Centricon 10™ microconcentrators from Amicon as ultrafiltration devices to perform rapid-flow dialysis as a means to monitor Ca^{2+} binding to the TN-C samples [6]. Since it has been well documented that the binding of Ca^{2+} to TN-C and the associated conformational changes occur on the millisecond time scale [7] we are confident that the incubation period and subsequent centrifugation time are sufficient to ensure equilibrium. Free Ca^{2+} in the supernate (in this case the solution centrifuged across the dialysis membrane) was analyzed employing the metallochromic indicator arsenazo III (Sigma) according to ([8] and references cited therein). Samples of apo TN-C(s) were dissolved in and dialyzed against either 100 mM NaCl, 50 mM Na acetate, pH 5.3, or 100 mM NaCl, 25 mM Pipes, 0.5 mM DTT, pH 6.8 (1.7 M $(NH_4)_2SO_4$). 40–50 nmol protein was placed in a prewashed Centricon 10 microconcentrator along with a known amount of Ca^{2+} (5–10-fold molar excess), incubated for 15 min and the solution centrifuged in a Beckman J-21 centrifuge with a J-14 rotor at 4000 rpm for 30 min at 20°C. The supernate was assayed for Ca^{2+} with arsenazo III using a 20 μM solution of dye in 100 mM Pipes, pH 6.8, and monitoring the Ca^{2+}-induced absorbance changes at 650 nm on a Cary model 118C spectrophotometer in polystyrene cells of 1 cm path length. Standard curves (known amount of Ca^{2+} added vs ΔA_{650}) were done at the start and conclusion of an experiment to check the reproducibility of the dye. A minimum of 4 assays were done on each supernate sample. From the amount of free Ca^{2+} found in the supernate, along with the total Ca^{2+} added and the amount of protein used, it was possible to calculate the amount of Ca^{2+} bound to the protein.

2.5. *Circular dichroism (CD)*

CD studies of TN-Cs in the absence and presence of Ca^{2+} were done on a Jasco J500C spectropolarimeter interfaced with a DP500N data processor. The instrument was routinely standardized with d_{10}-camphorsulfonic acid and pantoyl lactone.

2.6. *Amino acid analysis*

Amino acid analyses were performed on a Durrum D-500 automated amino acid analyzer. Samples were hydrolyzed in constant boiling 6 N HCl for 24 or 48 h. Phenol (1%) was included to minimize degradation of tyrosine residues.

2.7. *Fluorescence*

Fluorescence emission and excitation spectra were measured on a Perkin-Elmer MPF 44B recording spectrofluorometer operating in the ratio mode and equipped with a thermostatted cell assembly maintained at 20°C. For rabbit skeletal TN-C, where tyrosine emission was monitored at 304 nm after excitation at 276 nm, both emission and excitation slit band widths were usually set at 5 nm. With turkey TN-C, where the much weaker phenylalanine fluorescence was measured at 283 nm after excitation at 257 nm, the excitation slit band width was 5 nm, while the emission one was set at 10 nm. Solution concentrations were adjusted so that the absorbance at the excitation wavelength was always less than 0.05, thus eliminating the need to make any corrections for the inner filter effect.

3. RESULTS AND DISCUSSION

The amino acid composition of turkey skeletal TN-C is shown in table 1 along with comparable data for the rabbit and chicken analogues. The compositions of the avian TN-Cs are very similar, the turkey protein having 2 extra Glx residues, 1 less alanine and 1 less phenylalanine. It is not unreasonable to assume that the sequences would also show great similarity. This is strongly suggested by the results of reversed-phase HPLC on CNBr and tryptic digests of the 2 proteins. As shown in fig.1, the elution profiles are rather similar for the comparable digests from both proteins, the differences being in line with the very limited substitutions noted already.

Table 1

Amino acid compositions of several TN-Cs isolated from various species

Amino acids	No. of residues/molecule		
	Rabbit[a]	Chicken[b]	Turkey[c]
Asx	22	25	24.6
Thr	6	7	7.1
Ser	7	6	6.0
Glx	31	30	32.2
Gly	13	13	13.2
Ala	13	13	11.7
Val	7	6	6.0
Met	10	11	10.8
Ile	10	11	9.9
Leu	9	10	10.7
Tyr	2	0	0
Phe	10	11	10.0
His	1	1	0.9
Lys	9	10	10.4
Arg	7	6	6.1
Cys	1	1	1.0
Pro	1	1	1.0

[a] Sequence data of Collins et al. [9]
[b] Sequence data of Wilkinson [10]
[c] Average values of 24 and 48 h hydrolysis times; this study

The results of the Ca^{2+}-binding studies are shown in table 2. At pH 6.8 turkey TN-C apparently binds Ca^{2+} in a similar manner to the rabbit protein, namely 4 mol/mol protein, within the limits of error of the measurements. Bovine cardiac TN-C, under the same conditions, binds only 3 mol Ca^{2+} in line with the known sequence deficiencies in binding site I [11]. In the acetate buffer at pH 5.3 all 3 proteins bind only 2 mol Ca^{2+}/mol protein even though they were incubated with Ca^{2+} concentrations up to a 10-fold molar excess. Ammonium sulfate (1.7 M) (Schwartzmann Ultrapure which had been treated with Chelex 100 to remove traces of Ca^{2+} and other metal contamination) has been incorporated into the buffer system at pH 6.8 to mimic somewhat the salt concentration used for crystallization; we could not of course repeat the measurements at pH 5.3, in view of protein precipitation. As noted in fig.1, the high concentration of $(NH_4)_2SO_4$ had essentially no effect on the Ca^{2+}-binding capacities of the TN-Cs.

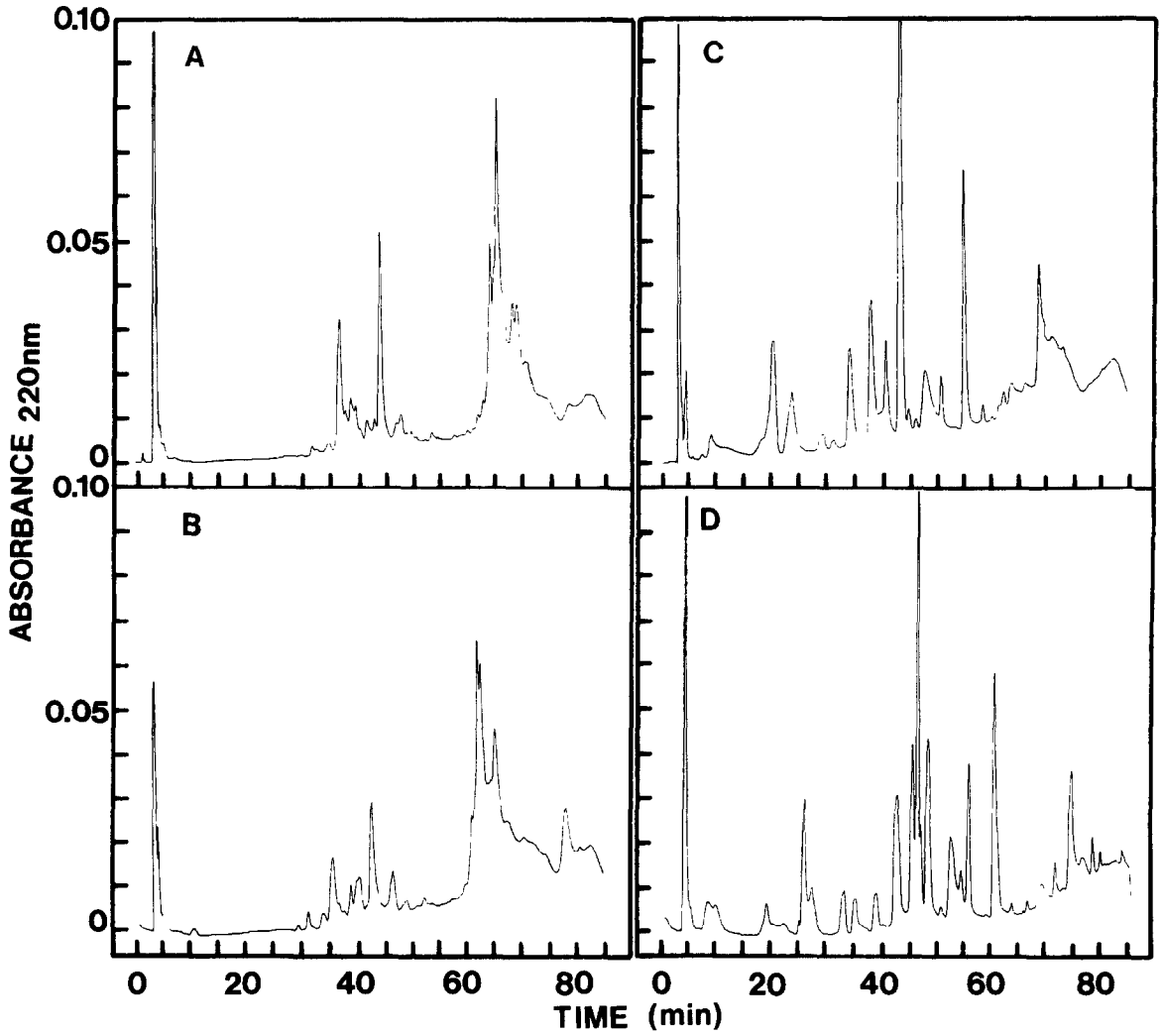

Fig.1. Reversed-phase HPLC of CNBr (A,B) and tryptic (C,D) peptides from turkey and chicken skeletal TN-Cs, respectively. Conditions: AB gradient; at pH 2.0, solvent A consisted of 0.1% trifluoroacetic acid-water and solvent B of 0.1% trifluoroacetic acid-70% acetonitrile-water. Gradient conditions: 0–5 min, 100% A; 5–70 min, 0→65% B; i.e., 1% B/min at a flow rate of 1 ml/min. The detector was set at 0.1 A/mV with the recorder set at 1 mV full scale. The elution profiles shown are representative runs from a C-8 Synchropak RP-P C-8 column, 250 × 4.1 mm i.d., 6.5 μm particle size, 300 Å pore size, carbon loading ~10%.

At pH 6.8 in 100 mM NaCl, 25 mM Pipes, the far-UV ellipticity of turkey and rabbit TN-C as measured by CD studies underwent a 30% increase in the presence of Ca^{2+}. For example, for turkey TN-C, $[\theta]_{221\,nm}$ was $-12075°$ initially and in the presence of sufficient Ca^{2+} to saturate all 4 Ca^{2+}-binding sites, $[\theta]_{221\,nm}$ rose to $-16000°$. For rabbit skeletal TN-C, the values under comparable conditions were -12260 and $-16000°$. If 2 mM EGTA was incorporated into the starting buffer, and the measurements repeated, the full 50–55% change in $[\theta]_{221\,nm}$ was evoked in both proteins [12]. Now the initial value of $[\theta]_{221\,nm}$ was lowered to $\sim -10000°$ for both proteins. The final values in the presence of Ca^{2+} were essentially unchanged. Repeating these CD measurements in the

Table 2

Amount of Ca^{2+} bound to several TN-Cs under different solvent conditions

pH of measurement	mol Ca^{2+} bound/mol TN-C		
	Rabbit	Turkey	Beef cardiac
6.8	3.5 ± 0.3	3.7 ± 0.3	3.0 ± 0.3
6.8[a]	3.5 ± 0.3	3.7 ± 0.3	–
5.3	1.6 ± 0.2	1.9 ± 0.2	2.2 ± 0.2

[a] This solvent includes 1.7 M ammonium sulfate

The binding of Ca^{2+} to these TN-Cs was followed by a modification of the equilibrium dialysis procedure with the amount of free Ca^{2+} determined with a metallochromic indicator. Details are given in the text

acetate buffer at pH 5.3 revealed that the Ca^{2+}-induced change in $[\theta]_{221\,nm}$ was only 7% ($[\theta]_{221\,nm}$ went from $\sim -14900°$ in the absence of Ca^{2+} to $\sim -16000°$ in the presence of this cation) in agreement with earlier CD studies which showed that as the pH was lowered the ensuing binding of protons produced a change in protein conformation similar to that induced by Ca^{2+} [13]. Perhaps the most interesting aspect of this part of the work were the results of CD measurements on the TN-Cs in 1.7 M ammonium sulfate at pH 6.8. Although not affecting the number of moles of Ca^{2+} bound at this pH, this high concentration of salt produced a considerable increase in α-helical content such that subsequent addition of excess Ca^{2+} invoked only a further 4% increase in structure; $[\theta]_{221\,nm}$ was raised to $\sim -15360°$ in this solvent and only underwent $\sim -650°$ increase in the presence of Ca^{2+}.

The fluorescence emission spectrum of apo rabbit skeletal TN-C has been well documented and need not be reproduced here. Upon excitation at 276 nm the emission maximum typical of tyrosine was noted near 304 nm. The emission spectrum of turkey TN-C which lacks tyrosine residues, was much weaker and upon excitation at 257 nm displayed a maximum due to phenylalanine emission at 283 nm. The effect of Ca^{2+} on the tyrosine fluorescence of rabbit TN-C and the phenylalanine fluorescence of turkey TN-C is shown in fig.2. Rabbit TN-C exhibited the well known 50% increase in fluorescence that has been well

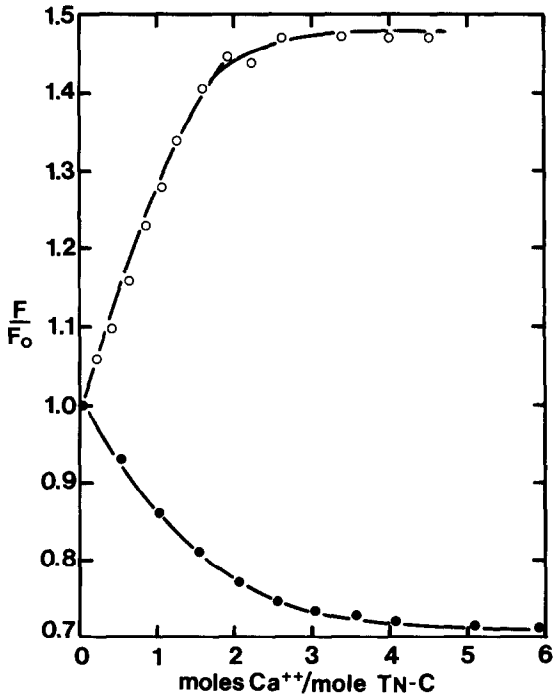

Fig.2. Fluorescence titrations of turkey TN-C (●) and rabbit skeletal TN-C (○) with Ca^{2+}. The ratio of the fluorescence with added Ca^{2+} (F) and that without added Ca^{2+} (F_0) was plotted against the mole ratio of Ca^{2+} to protein. For rabbit TN-C, excitation was at 276 nm and the tyrosine fluorescence emission monitored at 304 nm. For turkey TN-C, excitation was at 257 nm and the phenylalanine fluorescence emission measured at 283 nm. Temperature was 20°C. The solvent was 100 mM NaCl, 25 mM Pipes, 0.5 mM DTT, pH 6.8. The rabbit TN-C concentration was 2.3×10^{-5} M, while that of turkey TN-C was 1.73×10^{-5} M.

documented [14]. The fluorescence of turkey TN-C, on the other hand, was quenched by about 30% by the action of this cation. This response is akin to the Ca^{2+}-induced quenching of the tyrosine fluorescence of pike skeletal TN-C noted already [3]. It is noteworthy that the titrations of turkey and rabbit TN-C are complete upon addition of 2 mol Ca^{2+} per mol protein. In other words, although the direction of the fluorescence changes as well as the fluorophores are different, the conformational sensitivity of the 2 systems is similar in their overall response to this cation.

Both the salt effect of ammonium sulfate and the low pH used for crystallization undoubtedly lead to a helical structure which, in the N-terminal

domain at least, precludes binding of Ca^{2+} at the low-affinity sites. Finally, our direct binding studies at both pH 6.8 and 5.3 would seem to rule out the possibility of turkey TN-C binding a different amount of Ca^{2+} than the rabbit protein and the CD and fluorescence conformational studies have indicated a similar conformational response to this cation for both homologues. It is noteworthy in this regard to point out that in the crystal structure of the related protein calmodulin, all 4 Ca^{2+}-binding sites are filled with metal ions even though the conditions for crystal growth were similar to those reported for TN-C [15]. This difference is presumably related to the fact that in calmodulin the binding affinities for all 4 sites are much more similar than in the case of TN-C and hence complete filling of the 4 sites is more readily achieved.

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