TROPONINS C FROM REPTILE AND FISH MUSCLES AND THEIR RELATION TO MUSCULAR PARVALBUMINS†

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1. Introduction

Parvalbumins are sarcoplasmic acidic proteins, with a MW around 12 000, which are abundant in the white muscles of fishes and amphibians. The amino acid sequence of eight parvalbumins has confirmed their homology and has allowed the building of a phylogenetic tree, incorporating two major lines of genetic descent, α and β (see ref. [1] for a review). Similarities between parvalbumins and TN-C*, the calcium-sensitizing factor of the myofibrillar troponin–tropomyosin regulatory system [2], have been noted on several occasions [3,4], leading to the suggestion that these two types of protein might be structurally and functionally related, and that they might share a common ancestry. As a first step towards the exploration of this hypothesis, this paper describes the isolation and properties of TN-C from two reptiles, the lowest class of vertebrates lacking parvalbumins, and from one fish, the hake, for which the single major parvalbumin is well known [5]. These TN-C's and parvalbumins are then compared, with the result that their functional dissimilarity becomes evident in spite of important sequence and structural isologies. The contraction of fish and reptile muscles thus appears to be regulated by interaction of Ca^{2+} ions with TN-C, in agreement with previous data [6].

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* *Abbreviations:* TN, troponin; TN-C, troponin C; TN-I, troponin I; TN-T, troponin T; HMM, heavy meromyosin; dansyl, 1-dimethylamino-naphthalene 5-sulfonyl; SDS, sodium dodecyl sulphate; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetate; TPCK, L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone.
Enzymes: ATPase, ATP phosphohydrolase (EC 3.6.1.3); trypsin (EC 3.4.4.4); pepsin (EC 3.4.4.1).

2. Materials and methods

2.1. *Materials*

African lizards *(Varanus exanthematicus exanthematicus)* and pythons *(Pytho sebae)* were provided by the Institut Fondamental d'Afrique Noire, Dakar. Hakes *(Merluccius merluccius)* were obtained from the local market. Pepsin-PM, 2800 U/mg, and trypsin-TPCK, 198 U/mg, were from Worthington. Protein standards for molecular weight determinations were obtained from Sigma. All other reagents were either sequanal grade, from Pierce, or analytical grade from Merck. Antisera anti-hake 4.36 parvalbumin and anti-coelacanth 5.0 parvalbumin were obtained after repeated injections to rabbits of protein emulsions in Freund's complete adjuvant.

2.2. *Troponin C preparation*

Troponin C was prepared essentially as described by Greaser and Gergely [7], with the following minor changes. Isoelectric precipitation of tropomyosin was performed only once. Troponin, dissolved in a 6 M urea, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 50 mM Tris (43 mM HCl) pH 8.0 buffer, with which the DEAE-Sephadex A-50 column was equilibrated, was pretreated [8] with EGTA (20 times molar excess over bound Ca^{2+}). TN components were eluted from the column by a linear KCl gradient in the starting buffer. Varan and python TN-C were eluted as a symmetrical peak at 0.35 M and 0.33 M chloride, respectively. Hake TN chromatography separates 3 TN-C-like peaks, called components 1, 2 and TN-C, eluted at 0.24, 0.28 and 0.31 M chloride, respectively.

2.3. *Analytical techniques*

Molecular weights were measured by SDS-10% polyacrylamide gel electrophoresis [9]. Protein concentrations were determined by the biuret reaction [10]. Protein-bound calcium was measured by atomic absorption spectrophotometry, using an apparatus Model 290 from Perkin–Elmer and 0.25% strontium diluent [11], after excess free Ca^{2+} ions had been separated from the protein-bound Ca^{2+} by filtration on Sephadex G-25.

N-Terminal analyses were performed with the dansyl chloride procedure [12], using either guanidine hydrochloride or SDS to unfold the peptide chains. Identification of DNS-amino acids, amino acid analyses, high voltage paper electrophoresis, paper chromatography, peptide column chromatography on Dowex 50 × 4 and 1 × 2, and peptide sequencing were performed as described previously [5]. Diagonal methods were used for the selective purification of the cysteine peptide [13], and for the study of the methionine [14] and lysine [15] peptides.

Immunological cross reactions were studied in 1% agarose gel double diffusion plates [16]. The increasing intensity of the cross reactions was noted from + to ++++. Unless otherwise stated, the patterns were of partial identity.

2.4. *Chemical and enzymatic cleavages*

CNBr cleavage [17] was performed in 90% trifluoroacetic acid, in which TN-C remained soluble. Peptic digestion [18] was conducted with an E/S ratio of 5%. Tryptic digestion of TN-C was extremely difficult to achieve with good yields. A two step procedure was used routinely, taking advantage of the conformational changes which occur in proteins after citraconylation [19]. A first limited digestion (E/S = 4%) was performed on TN-C, previously citraconylated in the presence of 6 M guanidine hydrochloride and then desalted. The resulting peptides were unblocked [19], and a second identical digestion carried out, at 37°C during 2 hr, in a 0.1 M *N*-ethylmorpholine-acetic acid buffer pH 8.0. Limited tryptic digestion was performed on maleylated TN-C as previously described [5].

2.5. *Effect of parvalbumin and TN-C's on rabbit acto-HMM ATPase*

ATPases were measured, at 25°C and pH 7.0, using the pH-stat [20]. Rabbit proteins (HMM, actin, tropomyosin, TN-I and TN-C) were purified, and all protein concentrations were determined by ultraviolet absorption at 278 or 280 nm [21]. The incubation mixture (7.5 ml) was 2 mM ATP, 1 mM Mg^{2+}, 30 mM KCl, 1 mM EGTA, 0.09 mg/ml tropomyosin, 0.029 mg/ml TN-I, 0.2 mg/ml actin, 0.5 mg/ml HMM. The ATPase rate of HMM alone was subtracted from rates given in table 4.

3. Results and discussion

3.1. *Isolation, properties and composition of troponins C*

The reptile TN-C's were isolated as homogeneous proteins, giving a single band on SDS-polyacrylamide gel, corresponding to a MW 20 000, identical to that obtained for rabbit TN-C with the same technique [2]. The yield from the 1 M KCl extract was 0.015 and 0.012 for the varan and the python muscles respectively. Hake TN-C (yield: 0.008, MW = 19 000) was eluted after two Ca^{2+}-binding components, Cpt 1 (yield: 0.009, MW = 14 000) and Cpt 2 (yield: 0.009, MW = 21 000).

Table 1
Properties of lower vertebrate calcium-binding myofibrillar proteins

	Reptiles		Hake		
	Varan TN-C	Python TN-C	Cpt 1	Cpt 2	TN-C
M.W. (approx.)	20 000	20 000	14 000	21 000	19 000
N-Terminus	Blocked	Blocked	Blocked	Blocked	Blocked
$A_{278}/A_{260\,nm}$	0.98	0.83	1.00	0.98	1.02
$A^{1\%}_{1\,cm,}$ 278 nm	1.85	2.23	2.85	1.78	2.16
$A^{1\%}_{1\,cm,}$ 260 nm	1.88	2.68	2.86	1.81	2.11
Ca^{2+}-binding (moles/mole)	1.9	2.8	2.0	2.2	3.1

Table 2
Amino acid composition of calcium-binding myofibrillar proteins*

Amino acid	Rabbit TN-C [2]	Varan TN-C	Python TN-C	Hake Cpt 1	Hake Cpt 2	Hake TN-C
Lys	10.6	10.2	10.1	3.9	8.6	11.8
His	1.2	0.5	1.2	0	0	0.3
Arg	7.5	8.0	8.7	4.9	8.2	7.2
Asx	25.4	22.7	23.2	14.8	28.8	34.5
Thr	6.1	7.3	5.6	7.1	7.0	3.1
Ser	7.3	5.3	5.7	5.7	8.2	7.9
Glx	38.0	35.0	35.7	29.3	39.8	30.6
Pro	2.2	2.0	2.1	1.8	1.6	1.8
Gly	13.8	12.1	12.4	10.6	13.9	10.1
Ala	14.0	11.1	13.2	8.0	12.0	9.8
Cys	1.0	0.2	tra.	0	0.3	0.5
Val	7.7	7.8	7.7	5.1	7.0	6.2
Met	10.0	8.9	8.7	5.5	8.3	7.4
Ile	9.9	7.9	7.8	5.4	8.6	8.4
Leu	9.8	10.1	10.9	11.1	16.9	15.0
Tyr	2.0	1.9	1.3	1.4	2.1	1.5
Phe	10.1	9.0	10.0	7.6	11.1	8.7
Trp	0	0	0	0	0	0

* Residues per mole. The MW are given in table 1. MW of rabbit TN-C was assumed to be 20 000 [2].

All these proteins exhibit very similar properties, except for their MW (table 1). The N-terminus is blocked; the ultraviolet spectrum, as that of rabbit TN-C [2], is characterized by a $A_{278}/A_{260\,nm}$ ratio close to 1, indicative of the lack of tryptophan, and of a high Phe: Tyr ratio (table 2), with the absorption maxima at 253, 259 and 265 nm of the phenylalanine chromophore. They all bind 2–3 Ca^{2+} atoms/mole.

The amino acid compositions (table 2) of reptile and rabbit TN-C's are very similar, in particular with respect to the presence of a single residue of His and Cys, and of 2 residues of Tyr (confirmed by alkaline vs neutral spectra) next to 9–10 Phe residues. In contrast to parvalbumins, the same high Arg and Met content is also found. The hake TN-C composition exhibits comparable characteristic features, but is less close to the rabbit TN-C composition (see e.g., the number of Leu, Gly and Ala residues). The possibility that hake Cpt 1, at least, was a result of proteolytic degradation [22] of TN-C is probably to be ruled out in view, among other things, of the threonine content of the two molecules.

3.2. *Primary structure*

Peptide maps of the reptile and fish TN-C digests confirmed their homology. Thus, preliminary experiments towards the examination of possible chemical isologies in TN-C and parvalbumins were conducted on varan TN-C, which was available in larger quantity.

As, at the onset of this work, the amino acid sequence of rabbit TN-C [23] was unknown, a preliminary study of the distribution of Met, Arg and Lys residues of varan TN-C was first undertaken in regard to the possibility that TN-C could correspond to an elongated parvalbumin, whereby it seemed plausible, from a study of the corresponding amino acid compositions, that the additional tail would be relatively rich in Arg and Met residues while being poor in Lys residues. Citraconylated CNBr peptides were found to be eluted, on Sephadex G-100, from $K_{av} = 0.3$ to $K_{av} = 1.0$ as a broad asymmetrical peak with a maximum at $K_{av} = 0.7$. Thus Met residues appeared to be distributed all along the chain, the CNBr cleavage giving rise to peptides of very different sizes, as has indeed been found for rabbit TN-C [24]. A limited tryptic digestion of fractions eluted from Sephadex G-100 showed also that Arg residues are not clustered in the low MW peptides, and that a –Met–Arg– sequence exists in varan TN-C, as is now known to be present in rabbit TN-C [23]. Diagonal electrophoretic techniques on a complete tryptic digest and on a limited tryptic digest of the maleylated protein, confirmed that Arg and Met residues on one hand, and Lys residues on the other hand, are not segregated in different parts of the peptide chain.

In addition, attempts were made to isolate, by anion and cation exchange chromatography of a tryptic digest of varan TN-C, an acidic peptide isologous to the 88-96 calcium-binding region of parvalbumins, and free Lys, which are usually, but not always, found in such digests of parvalbumins [1]. This search was unsuccessful. However, an acidic peptide, Asp–Gly–Asp–Glu–Lys, was then isolated which could perhaps be isologous to residues 92–96 (Asp–Gly–Asp–Asp–Lys) of the coelacanth 5.4 parvalbumin [1]. By this time, the complete amino acid sequence of rabbit TN-C [23] became available which abundantly substantiated this and other suspected isologies between this protein and parvalbumins, and further suggested that they all might have many structural features in common. It can finally be mentioned that, during the above operations, another acidic peptide, Glu–Asp–Ala–Lys, was found which is identical to the tryptic peptide 84–87 of rabbit TN-C; also, the single cysteine peptide (Ala–Glu–Cys) of varan TN-C was isolated, by the diagonal electrophoretic technique, from its peptic digest: it is identical to the 95–97 stretch of rabbit TN-C.

From all these data, lower vertebrate TN-C's appear to be homologous to each other and to rabbit TN-C, and there is little doubt that all these proteins share a common ancestry with parvalbumins.

3.3. *Immunological cross reactions*

Table 3 shows that native TN-C's from lower vertebrates do not cross react with either α or β parvalbumins [1], when they are made to diffuse with an anti-β serum (anti-hake 4.36 parvalbumin) and an anti-α serum (anti-coelacanth 5.0 parvalbumin), respectively; this situation prevails even within the same species, the hake. In contrast, most parvalbumins tested so far cross react with one or both antisera, with the exception of the frog 4.88 and the dogfish major parvalbumins, which perhaps belong to still other lines of genetic descent. It is thus probable that: a) the Ca^{2+}-binding sites, partially or completely shiel-

Table 3
Immunological cross reactions of parvalbumins and Troponins C*

Protein	Serum anti-hake 4.36 parvalbumin	Serum anti-coelacanth 5.0 parvalbumin
Coelacanth 5.4 palb.	±	++++ (c.i.)
Coelacanth 5.0 palb.	+	homologous
Hake 4.36 palb.	Homologous	–
Carp 4.47 palb.	+++	–
4.37 „	+++	–
4.25 „	+++	–
3.95 „	+++	–
Th. ray 4.45 palb.	+	+
Frog 4.88 palb.	–	–
4.50 „	++	–
Dogfish major palb.	–	–
Hake TN-C	–	–
Cpt 1	–	–
Cpt 2	–	–
Varan TN-C	–	–
Python TN-C	–	–

* Abbreviations: c.i., complete identity; palb., parvalbumin (identified by its isoelectric point).
The data refer to the following species: coelacanth, *Latimeria chalumnae;* carp, *Cyprinus carpio;* thornback ray, *Raja clavata;* frog, *Rana esculenta;* dogfish, *Squalus sucklii.*

ded from solvent, are not immunogenic; b) the outer surface of the TN-C molecule exhibits no common immunogenic patch with parvalbumins; a significant part of this surface is indeed involved in interactions with TN-I and TN-T [2], which parvalbumins, apparently, are not able to achieve (see below).

3.4. *Calcium-sensitizing properties*

Indeed, as shown in table 4, parvalbumin and TN-C's behaved quite differently in a rabbit in vitro acto-HMM assay system. Whereas varan, python, and hake TN-C's were able to reverse the inhibition of acto-HMM ATPase by TN-I, in a concentration range similar to that of rabbit TN-C, hake 4.36 parvalbumin was found to be totally inactive. The reversal by hake Cpts 1 and 2 was not significant, and the question of their origin and role remains open. Parvalbumins, like the low MW Cpt 1, therefore appear to be unable to interact specifically with TN-I, suggesting that a certain chain length is necessary for this purpose, and that they play in the cell a different role.

Table 4
Effect of calcium-binding proteins on rabbit acto-HMM ATPase

Protein	Final concentration (mg/ml)	ATPase (nmoles/min/mg HMM)
–	–	13
Rabbit TN-C	0.05	214
Varan TN-C	0.05	158
	0.1	240
Python TN-C	0.05	216
Hake TN-C	0.05	147
	0.1	224
Hake Cpt 1	0.05	37
	0.1	64
Hake Cpt 2	0.05	45
	0.1	85
Hake 4.36 parvalbumin	0.1	13

Regulation of muscular contraction by Ca^{2+} ions thus involves, in lower vertebrate muscles, a troponin–tropomyosin–actin system, quite similar to the mammalian system: the tropomyosin, TN-T and TN-I bands were actually easily recognized on SDS-polyacrylamide gels during the above purifications of TN-C, which yielded protein components structurally and functionally closely homologous to rabbit TN-C.

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