THE AMINO ACID SEQUENCE OF THE TROPONIN C-LIKE PROTEIN (MODULATOR PROTEIN) FROM BOVINE UTERUS

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1. Introduction

Recent studies have shown that a troponin C-like protein is present in vertebrate smooth muscle [1–5]. This protein can form Ca^{2+}-dependent complexes with troponin I from fast skeletal muscle in a similar manner to those obtained with skeletal muscle troponin C [2,3]. It can be isolated from rabbit uterus using an affinity chromatographic procedure which depends on this property of complexing with skeletal muscle troponin I [1] but a more convenient, relatively simple, method of isolating the protein from smooth muscle and other tissues in good yields has recently been described [3]. The troponin C-like protein from smooth muscle possesses other properties similar to those of skeletal muscle troponin C. These are the abilities to inhibit the phosphorylation of troponin I by 3′,5′-cyclic AMP-dependent protein kinase and to neutralise the inhibitory activity of skeletal troponin I on the Mg^{2+}-stimulated ATPase of desensitized skeletal actomyosin [2]. Unlike skeletal muscle troponin C, however, the smooth muscle protein activates cyclic nucleotide phosphodiesterase and contains the unusual amino acid, trimethyl lysine [3]. These latter properties are a feature of the Ca^{2+}-binding protein of bovine brain, the brain modulator protein, which Amphlett et al. [6] have demonstrated can substitute in the skeletal troponin complex in the place of troponin C and will restore full calcium sensitivity to desensitized actomyosin in the presence of troponin I and tropomyosin alone.

Cheung et al. [7] have shown that a protein similar to the brain modulator protein is present in many of the tissues of mammals, and Waisman et al. [8] have demonstrated its presence in more primitive species such as the snail (Ampullavia) and the star fish (Asterias). Sequence studies indicate that the primary structures of the Ca^{2+}-binding proteins present in bovine brain [9] and in rate testes [10] are very similar and that both proteins are closely related, but not identical, to troponin C from rabbit skeletal muscle [11] and bovine cardiac muscle [12].

In the present paper we report the amino acid sequence of the troponin C-like protein present in bovine uterus. Apart from an ambiguity which still exists in the N-terminal tripeptide and the position of certain amide groups the sequence differs only in two residues from the modulator protein of bovine brain.

2. Materials and methods

Troponin C-like protein was prepared from bovine uterus using the organic solvent method described elsewhere [3].

Troponin C-like protein (50 mg) in which methionine residues had been labelled with iodo-[^{14}C] acetic acid was digested with trypsin. The resulting digest, containing peptides that covered most of the molecule, was fractionated by chromatography on a column of Sephadex G50 (2.2 cm × 110 cm) that was equilibrated and eluted with 50 mM NH_4HCO_3, pH 7.9. The largest tryptic peptide, consisting of residues 38–74 was further purified on a DEAE cellulose column (2 cm × 10 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.0, and eluted with a gradient of NaCl (0 → 0.75 M). The smaller peptides were purified by high voltage paper electrophoresis.

Tryptic peptides were sequenced directly by the dansyl-Edman technique [13] or further digested with thermolysin or V8 protease.

The troponin C-like protein (65 mg) was digested with CNBr and the peptides chromatographed on a Sephadex G50 column (2.2 cm × 110 cm) equilibrated and eluted with 20 mM NH_4HCO_3, pH 7.9. The CNBr fragments were further purified by chromatography on a DEAE cellulose column (2 cm × 10 cm) equilibrated in 20 mM Na phosphate buffer, pH 7.0 eluted with a gradient of NaCl (0 → 0.75 M) or by high voltage paper electrophoresis. The purified fragments were sub-digested with trypsin, thermolysin or V8 protease and the resulting peptides purified by high voltage electrophoresis. The conditions for radioactive labelling, digestion with proteolytic enzymes and with CNBr, amino acid analysis, high voltage electrophoresis and amide group assignment were as described by Grand and Wilkinson [14].

3. Results and discussion

Figure 1 shows the elution profile obtained after chromatography of the CNBr digest of the troponin C-like protein from bovine uterus on Sephadex G50. In all seven peptides designated CNB1 to CNB7 were isolated. Of these, peptide CNB1 was a partial cleavage product consisting of residues 77–124 (fig.2). This peptide was present in the peak corresponding to fraction 1 in pure form. The major component of fraction 2 was the N terminal peptide CHB2, residues 1–36, which was further purified by chromatography on DEAE cellulose (see section 2). After freeze-drying the material in fraction 3 was redissolved in 3 ml water and the pH reduced to 2 by the addition of 10 mM phosphoric acid. The CNBr peptide, CNB3, residues 125–145, precipitated in pure form and was collected by centrifugation. The supernatant was chromatographed on DEAE cellulose and the peptide, CNB4, residues 52–71, obtained as a single peak. Fraction 4 consisted mainly of peptide CNB5, residues 37–51, which was purified by high voltage paper electrophoresis, fraction 5 was found to contain peptide CNB6, residues 73–76 and peptide CNB7, residues 146–149, both of which were purified in a similar manner. Free homoserine was also present in this fraction. The compositions of the CNBr peptides are given in table 1.

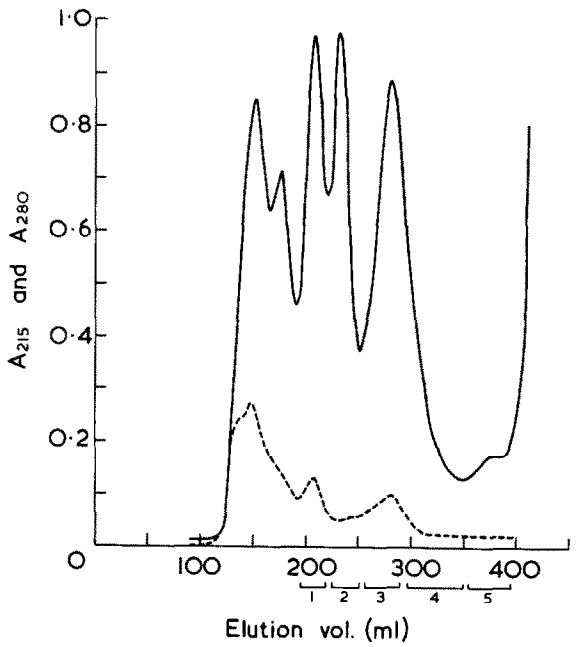

Fig.1. Chromatography of CNBr digest of uterus troponin C-like protein. The digest (65 mg in 4 ml of 6 M guanidine 0.4 M Tris/HCl, pH 8.2) was applied to a column (2.2 × 110 cm) of Sephadex G50 and equilibrated with 20 mM NH_4HCO_3, pH 7.9 and eluted with the same buffer. (– – – – –), A_{280}; (———), A_{215}. Horizontal bars indicate the fractions pooled.

The CNBr fragments were aligned by sequencing the radioactive tryptic peptides isolated after labelling the methionine residues with iodo-[^{14}C]acetic acid. Cleavage with trypsin occurred at the expected points in the sequence (fig.2) except that there was no cleavage between residues 94 and 95, probably due to the proximity of the two aspartic acid residues to the lysine. Cleavage did not occur after the trimethyl lysine residue. Watterson et al. [15] have found that trypsin splits the bovine brain modulator protein C-terminal to the Met–Met sequences (residues 72 and 145); this was not, however, observed with the smooth muscle protein despite the identity of the sequence in this region, possibly due to the modification of the methionine residues with iodoacetic acid.

The sequence has been established unequivocally except for the N-terminal tripeptide and the amide assignments between residues 49 and 50, 58 and 60, and 137 and 139 (each of these pairs has one charged

Table 1
Composition of peptides isolated from CNBr digest of bovine uterus troponin c-like protein

	Peptides isolated from CNBr digest													
	CNB1 (77–124)		CNB2 (1–36)		CNB3 (125–144)		CNB4 (52–71)		CNB5 (37–51)		CNB6 (73–76)		CNB7 (146–148)	
	Actual	Found	Actual	Found	Actual	Found	Actual	Found	Actual	Found	Actual	Found	Actual	Found
Asp	8	6.7	4	4.3	4	4.1	5	4.8	2	2.2	–	–	–	–
Thr	3	4.0	5	4.3	–	–	2	1.1	1	1.1	–	–	1	1.0
Ser	2	2.5	1	1.3	–	–	–	–	1	1.0	–	–	–	–
Glu	9	9.2	7	7.9	5	5.3	2	4.3	4	3.7	–	–	–	–
Pro	–	–	–	–	–	–	1	0.8	1	1.0	–	–	–	–
Gly	3	3.8	3	3.0	2	2.1	2	2.2	1	1.3	–	–	–	–
Ala	3	3.9	3	3.0	1	1.5	1	1.2	1	1.2	1	0.9	1	1.2
Val	3	3.0	1	1.4	2	1.8	1	1.6	–	–	–	–	–	–
Ile	2	2.0	2	1.8	2	1.6	2	1.8	–	–	–	–	–	–
Leu	3	3.3	3	2.5	–	–	1	0.6	2	1.8	–	–	–	–
Tyr	1	0.7	–	–	1	0.8	–	–	–	–	–	–	–	–
Phe	2	2.4	3	2.7	1	1.1	2	1.7	–	–	–	–	–	–
His	1	0.6	–	–	–	–	–	–	–	–	–	–	–	–
Lys	2	2.0	3	2.5	–	–	–	–	–	–	1	1.0	1	0.7
Arg	3	2.0	–	–	1	0.8	–	–	1	0.8	1	0.9	–	–
Hse	1	+	1	+	1	+	1	+	1	+	1	1.2	–	–
Tml[a]	1	+	–	–	–	–	–	–	–	–	–	–	–	–

[a] Tml, trimethyl lysine

Glu figures include homoserine. Free homoserine was also isolated (see text). Numbers in parenthesis are residue numbers of CNBr peptides

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                                CNB2                                    10
                              X-(Ala,Asp,Gln) Leu Thr Glu Glu Gln Ile Ala Glu Phe Lys
Ac Asp Thr Gln Gln Ala Glu Ala Arg Ser Tyr     Ser         Met

                              20            (Asn)                     30
   Glu Ala Phe Ser Leu Phe Asp Lys Asp Gly Asp Gly Thr Ile Thr Thr Lys Glu Leu Gly
   Ala         Asp Met        Ala         Gly     Asp     Ser Val

               CNB5           40                                      50      CNB4
   Thr Val Met Arg Ser Leu Gly Gln Asn Pro Thr Glu Ala Glu Leu Gln Asp Met Ile Asn
                     Met           Thr         Lys Glu         Asp Ala Ile     Glu

                              60                                      70          CNB6
   Glu Val Asp Ala Asp Gly Asn Gly Thr Ile Asp Phe Pro Glu Phe Leu Thr Met Met Ala
               Glu         Ser                       Glu             Val         Val

               CNB1           80                                                  90
   Arg Lys Met Lys Asp Thr Asp  -  -    -  Ser Glu Glu Glu Ile Arg Glu Ala Phe Arg
       Gln         Glu Asp Ala Lys Gly Lys                 Leu Ala     Cys

                         (Asn)        100                                       110
   Val Phe Asp Lys Asp Gly Asp Gly Tyr Ile Ser Ala Ala Glu Leu Arg His Val Met Thr
   Ile         Arg Asn Ala                 Asp     Glu         Ala Glu Ile Phe Arg

                                       120                 CNB3                130
   Asn Leu Gly Glu Tml Leu Thr Asp Glu Glu Val Asp Glu Met Ile Arg Glu Ala Asn Ile
   Ala Ser         His Val                 Ile Glu Ser Leu Met Lys Asp Gly Asp Lys

                                       140                     CNB7
   Asp Gly Asp Gly Glu Val Asn Tyr Glu Glu Phe Val Gln Met Met Thr Ala Lys  -
   Asn Asn         Arg Ile Asp Phe Asp         Leu Lys         Glu Gly Val Gln
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Fig.2. A comparison of the amino acid sequences of bovine uterus troponin C-like protein and troponin C from rabbit skeletal muscle [11]. The uterus protein is given on the top line, residues which differ in skeletal muscle troponin C are shown underneath. The numbering of the residues refers to the uterus protein. (X), indicates an unknown blocking group: (–), a deletion and Tml, trimethyl lysine. The residues in parenthesis are those which have been shown to be different in bovine brain modulator protein [15].

and one amide group). Limited evidence suggests, however, that these residues are identical in the uterus and brain proteins. Apart from these ambiguities the amino acid sequence of bovine uterus troponin C-like protein, shown in fig.2, differs from the brain modulator protein only in the assignment of the amide groups on residues 24 and 97. Watterson et al. [15] have shown residues 24 and 97 in the brain protein to be asparagines whilst in the uterus protein these are both charged aspartic acid residues. The troponin C-like protein from bovine uterus shows a greater number of sequence differences when compared with the rat testes Ca^{2+}-binding protein [10]. These are amide assignments at residues 3, 42, 53, 111, 137 and 143 and the substitution of an asparagine for alanine at residue 57 and alanine for asparagine at residue 60.

In view of the properties of the smooth muscle troponin C-like protein and the virtual identity of its sequence with that of the brain protein we propose to call it bovine uterus modulator protein. The protein contains 148 amino acids and has a molecular weight of 16 722 daltons. Bovine uterus modulator protein has a blocked N-terminus and although the nature of the blocking group has not been determined by analogy with bovine brain modulator protein, the N-terminus is probably acetylated. The protein carries an overall negative charge of –25 at pH 7, compared to –29 for rabbit skeletal muscle troponin C. No particular difficulties were experienced in sequencing except for the N-terminal tripeptide. Digestion with CNBr resulted in the expected peptides except that only very limited cleavage occurred between residues 109 and 110. The presence of Met–Met sequences at

residues 71, 72 and residues 144, 145 was indicated by amino acid analysis, the ^{14}C content of the bridge peptides after labelling with [1-^{14}C]iodoacetic acid and by the isolation of free homoserine after CNBr treatment.

The sequence presented in this paper confirms the view that the Ca^{2+} binding protein of bovine uterus is much more closely related to brain modulator protein than troponin C. To give maximum homology between the sequences of bovine uterus modulator protein and rabbit fast skeletal muscle troponin C three deletions must be introduced into the former sequence, between residues 80 and 81 (fig.2). Of the 151 residues that are comparable, there are 73 substitutions giving a difference of 48%. If conservative replacements (i.e. those arising by single nucleotide substitution giving rise to a similar amino acid) are considered, 47 differences are present giving a 31% difference.

Weeks and Perry [16] have demonstrated that the CNBr peptide CB9 of skeletal muscle troponin C consisting of residues 83–134 is a site of interaction with troponin I. The amino acid sequence of the uterus troponin C-like protein corresponding to this region (residues 77–125) shows a considerable number of substitutions (29 out of 52 residues). Nevertheless the corresponding site in bovine brain modulator protein interacts in a similar way to troponin C of fast skeletal muscle (T. C. Vanaman and S. V. Perry, unpublished observations).

On the basis of amino acid analysis and CNBr digest pattern of polyacrylamide gels, previous studies [3] have indicated that the troponin C-like proteins of bovine aorta, chicken gizzard and rabbit liver are also very similar to the bovine uterus and brain modulator proteins. The differences in the bovine brain and uterus and rat testis proteins are relatively minor and indeed when the sequences are confirmed in other laboratories some of them may disappear. Taken at their face value they reflect very small differences at the gene level and imply that modulator protein may well be as conservative in amino acid sequence as actin.

The role of the modulator protein in smooth muscle has yet to be determined. Evidence exists for its activation of the myosin light chain kinase [4,17]. This is unlikely to be its only role as it is probably present in about 100 × molar excess over the kinase (judged by the kinase level of smooth muscle [18] and the amount of modulator protein present [3]). Also it is complexed in smooth muscle with proteins that are of different molecular weight to the myosin light chain kinase (R. J. A. Grand and S. V. Perry, unpublished observations). Presumably it could play a role in the regulation of phosphodiesterase [19], adenyl cyclase [20] and the membrane (Ca^{2+}–Mg^{2+})-ATPase [21] for which experimental evidence already exists. If a troponin I-like protein is present in smooth muscle the uterus modulator protein would be able to play a part in a system for regulation of the actomyosin ATPase in response to changing Ca^{2+} concentration. All of its properties studied to date suggest that its role will be multifunctional.

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