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Primary structure of three minor isoforms of amphioxus sarcoplasmic calcium-binding proteins

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Previously we reported the amino acid sequences of 4 well-defined sarcoplasmic, high-affinity Ca^{2+}-binding proteins in the protochordate amphioxus, *Branchiostoma lanceolatum* [1]. Here we report on the complete amino acid sequence determination of 3 additional minor isoforms. The seven isoforms differ from each other in 9 positions of a contiguous 17-residue-long segment (positions 20–36) and can be classified in a α (ASCP I, III and IV) and a β lineage (ASCP II, V, VI and VII).

Amphioxus; Ca^{2+}-binding protein; Isoform

1. INTRODUCTION

The muscle of amphioxus contains abundant amounts of a soluble, 20 kDa, high-affinity Ca^{2+}-binding protein, ASCP, which seems to be involved in the buffering of Ca^{2+} and Mg^{2+} during muscular activity [2]. A property that is common to most Ca^{2+} buffering proteins is polymorphism: in amphioxus we previously described the primary structure of 4 isoforms [1] and observed that the difference is due to seven amino acid substitutions in a short segment of the protein. Besides these 4 isoforms, comprising over 95% of total SCP, the muscle contains a minor form eluting at high salt concentrations from a DEAE-52 column [1]. This paper reports that this fraction contains 3 isoforms, the complete amino acid sequences of which have been determined.

2. MATERIALS AND METHODS

The fraction containing the purified minor forms of ASCP (see section 3) was dissolved in 10 mM Tris-HCl buffer, pH 9.0, containing 4 M urea and digested with 2 μg of lysyl endopeptidase (Wake Pure Chemicals) at 37°C for 4 h. The resulting peptides were purified by reverse-phase HPLC with a linear gradient of acetonitrile. The N-terminal amino acid sequences of the intact proteins and the complete sequences of the purified lysyl endopeptidase peptides were determined with an automated sequencer (Applied BioSystem Model 477A on line with a Model 120A PTH-analyzer).

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3. RESULTS AND DISCUSSION

3.1. *Isolation of acidic forms of ASCP*

After DEAE-52 chromatography of amphioxus myogen as described previously [1] and analysis of the fractions for protein-bound Ca^{2+} [3] or with a specific polyclonal antibody against ASCP, isoforms were found in different peaks eluting at conductivities of 2–6 $m\Omega^{-1}$. The ASCP peak eluting at the highest salt concentration represents less than 5% of total ASCP. These fractions were pooled and further purified by chromatography on a Sephadex G-75 column (2 × 145 cm) equilibrated in 20 mM Tris-HCl, pH 7.5, 7.5 mM mercaptoethanol, 5 μM $CaCl_2$, 0.1 mg/l pepstatin A, 1 mg/l leupeptin and 70 mg/l phenylmethanesulfonyl fluoride. Electrophoresis in the presence of sodium dodecyl sulfate revealed that the acidic ASCP fraction eluted from this column as a very pure 20 kDa protein. Isoelectric focusing of these fractions yielded a more complex pattern with 2 major protein bands (pI ca. 4.8).

3.2. *Amino acid sequence of minor ASCP isoforms*

Of the 15 major lysyl endopeptidase peptides which were separated by HPLC, 12 displayed the same elution profile as found in the previous studies [1,3], which dealt with the sequence determination of the major ASCP isoforms. Their complete sequence determination with the automated sequencer revealed their identity with the lysyl endopeptidase peptides originating from the constant parts of ASCP isoforms I–IV. These peptides cover the whole protein sequence from residues 37–185, as well as the segment 1–7 (data not shown). In addition to these peptides of the constant region, 3 major lysyl endopeptidase peptides were purified by HPLC and

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A

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        X   Y   Z  -Y  -X    -Z
IV      Y N K N G S I Q W E D F E E M I K
I       Y N K D G S I Q W E D F E E M I K
III     M N K D G S I Q W E D F E E M I K
VII     M N H D G S I Q W E D F E D M M T
VI      M N H D G S I Q W N D F E D M M T
V       M N H D G A I Q W N D F E D M M T
II      M N H D G S I Q D N D F E D M M T
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B

	α			β			
	IV	I	III	VII	VI	V	II
IV	-						
I	1	-					
III	2	1	-				
VII	6	5	4	-			
VI	7	6	5	1	-		
V	8	7	6	2	1	-	
II	8	7	6	2	1	2	-

Fig. 1. Sequence similarities in the variable segments (from residue 20–36 in the complete sequences) of ASCP isoforms. (A) Alignment of the variable sequence of ASCP isoforms. Bold characters correspond to the variable positions. The upper line represents the Ca^{2+}-coordinating ligands of the first site. (B) Number of non-identical positions. The matrix representation of sequence differences suggests the existence of two lineages.

subsequently sequenced. Table I shows that they cover the ASCP segment 10–39, 12–39 and 8–39 of ASCP, respectively. They encompass a constant segment up to residue 19, the variable region 20–36 and the constant segment 37–39. The variable parts in the three major peptides code for three new isoforms of ASCP (Fig. 1) which have been named ASCP V, VI and VII.

In Fig. 1 the sequence of all the isoforms of ASCP were compared. The differences occur in 9 positions of a 17-residue-long segment. This segment comprises the first functional EF-hand, i.e. the Ca^{2+}-binding loop *plus* three α-helical turns at the C-terminal end of this loop. It should be noted that in SCP the C-terminal α-helix of this EF-hand takes an orientation (for *Nereis* SCP, see [4]; confirmed recently for ASCP II by Dr. W. Cook, personal communication) which is quite different from that in all other EF-hands crystallized thus far [5]. Of the 9 variable positions, four could have diverged by virtue of a single nucleotide substitution, but the others require 2 to even 3 nucleotide substitutions. Although the variable segment encompasses the first Ca^{2+}-binding site, the 9 variable positions systematically correspond to residues which are not involved in the coordination of Ca^{2+} and seem not to be critically important for the orientation of the C-terminal α-helix, suggesting that this EF-hand indeed binds Ca^{2+} in all the isoforms. Experimentally this was confirmed for the isoforms I–IV [1], but not yet for the isoforms V–VII, for lack of sufficient material. Fig. 1 also shows that two lineages

Table I

Amino acid sequence of the variable lysyl endopeptidase peptides of amphioxus SCP V, VI and VII

	ASCP V	ASCP VI	ASCP VII
1	I (324)	F (121)	Q (100)
2	K (482)	T (96)	K (108)
3	F (720)	F (130)	I (259)
4	T (431)	D (113)	K (146)
5	F (601)	F (111)	F (114)
6	D (218)	F (240)	T (119)
7	F (458)	L (54)	F (187)
8	F (483)	D (74)	D (71)
9	L (273)	M (62)	F (183)
10	D (185)	N (33)	F (202)
11	M (204)	H (18)	L (158)
12	N (67)	D (47)	D (54)
13	H (86)	G (36)	M (54)
14	D (147)	S (13)	N (52)
15	G (92)	I (20)	H (12)
16	A (94)	Q (12)	D (76)
17	I (75)	W (20)	G (55)
18	Q (46)	N (10)	S (17)
19	W (20)	D (10)	I (53)
20	N (37)	F (12)	Q (43)
21	D (41)	E (9)	W (22)
22	F (53)	D (15)	E (27)
23	E (19)	M (7)	D (27)
24	D (34)	M (7)	F (34)
25	M (29)	T (5)	E (25)
26	M (32)	R (12)	D (31)
27	T (18)	Y (4)	M (24)
28	R (34)	K (1)	M (26)
29	Y (14)		T (15)
30	K (2)		R (22)
31			Y (11)
32			K (6)

Yields are indicated in parenthesis.

of ASCP isoforms can be distinguished, which differ by at least 4 point mutations: the α lineage with ASCP I, III and IV and the β lineage with ASCP II, V, VI and VII. Within each lineage the different proteins show at the uppermost 2 mutations.

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