The complete amino acid sequence of bullfrog (*Rana catesbeiana*) parvalbumin pI4.97

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The primary structure of the parvalbumin (pI4.97) from bullfrog, *Rana catesbeiana*, skeletal muscle has been determined. It is composed of 110 amino acid residues and a free amino terminus, and has a molecular mass of 11919. The amino acid sequences which are thought to be functionally important sites are also conserved in the bullfrog parvalbumin. The calculated phylogenic tree indicates that this parvalbumin belongs to the α group of parvalbumins. The mutation rate of parvalbumin was fairly rapid in frogs compared to mammals. The subdivergence of frogs is also discussed.

Parvalbumin; Calcium binding protein; Muscle protein; Amino acid sequence; Molecular evolution; Frog phylogeny

1. INTRODUCTION

Parvalbumin (PA) is an acidic, low molecular weight calcium binding protein abundant in white muscle, especially in lower vertebrates [1]. The function of PA remains unknown with the possibility that it is involved in the relaxation process [2,3] and in labile heat production of muscle contraction [4]. Frog muscles have been frequently used in such physiological studies. The morphology of frogs has remained the same for about 300 million years and has required much effort by zoologists to classify [5,6]. The sequence comparison of frog PAs may be useful for muscle physiology and frog phylogeny. Parvalbumins from various sources are classified into two types, α and β, judged by the isoelectric points and recently, by the comparison of amino acid sequences [7]. α- and β-type PAs of *Rana esculenta* have been reported with the complete amino acid sequences [8,9]. Two forms of PA, pI4.78 and pI4.97, isolated from the skeletal muscle of bullfrog (*Rana catesbeiana*) have been studied on the physiological properties [10]. In this report, the primary structure of PA4.97 is described, and the function, molecular evolution and phylogeny of frog PA is discussed.

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Abbreviations: PA, parvalbumin: PA x, parvalbumin pIx.

2. MATERIALS AND METHODS

Two isotypes of bullfrog parvalbumin, PA4.78 and PA4.97, were purified from skeletal muscles [10]. The *S*-carboxymethylation of PA was performed according to Hirs [11]. Digestion of *S*-carboxymethylated PA 4.97 by *Achromobacter* lysyl endopeptidase (Wako) [12] was carried out at 400:1 (w/w) of substrate:enzyme in 0.05 M triethylamine acetate buffer (pH 9.5) at 30°C for 12 h. Disgetion of *S*-carboxymethylated PA4.97 by mouse submaxillary gland Arg-C endopeptidase (Boehringer) [13] was carried out at 100:1 (w/w) of substrate:enzyme in 1% $NaHCO_3$ (pH 8.0) at 37°C for 14 h. After the digestion, each enzymatic hydrolysate was lyophilized. Automated sequence analysis was performed with a JEOL JAS-47K sequence analyzer according to the manufacturer's manual using 0.1 M Quadrol buffer as a coupling buffer. Hydrazinolysis for the determination of the C-terminal amino acid was performed according to Narita et al. [14].

Separation of peptides was performed by reversed phase high-performance liquid chromatography with a Toyo Soda ODS-120T column (4.6 × 250 mm) using a linear gradient of 5–60% and 20–80% acetonitrile containing 0.1% trifluoroacetic acid for the digestions by lysyl and Arg-C endopeptidase, respectively. Phenylthiohydantoin amino acid derivatives were identified by the same apparatus under the isocratic elution condition (0.01 M ammonium acetate:methanol:acetonitrile, 15:9:1).

The mutation distance between amino acid sequences of PAs was calculated as the mininum number of nucleotide exchanges. Since several gaps were found in the aligned sequences, two calculating methods were used according to Fitch and Yasunobu [15] setting m_n value at 3. Phylogenic trees were constructed according to Fitch and Margoliash [16] using the calculated mutation distances.

3. RESULTS AND DISCUSSION

The complete amino acid sequence of bullfrog PA4.97 is shown in Fig. 1 together with the key peptides used for sequence determination. The PA4.97 consists of 110 amino acid residues having a molecular mass of 11 919. The number of residues is one more

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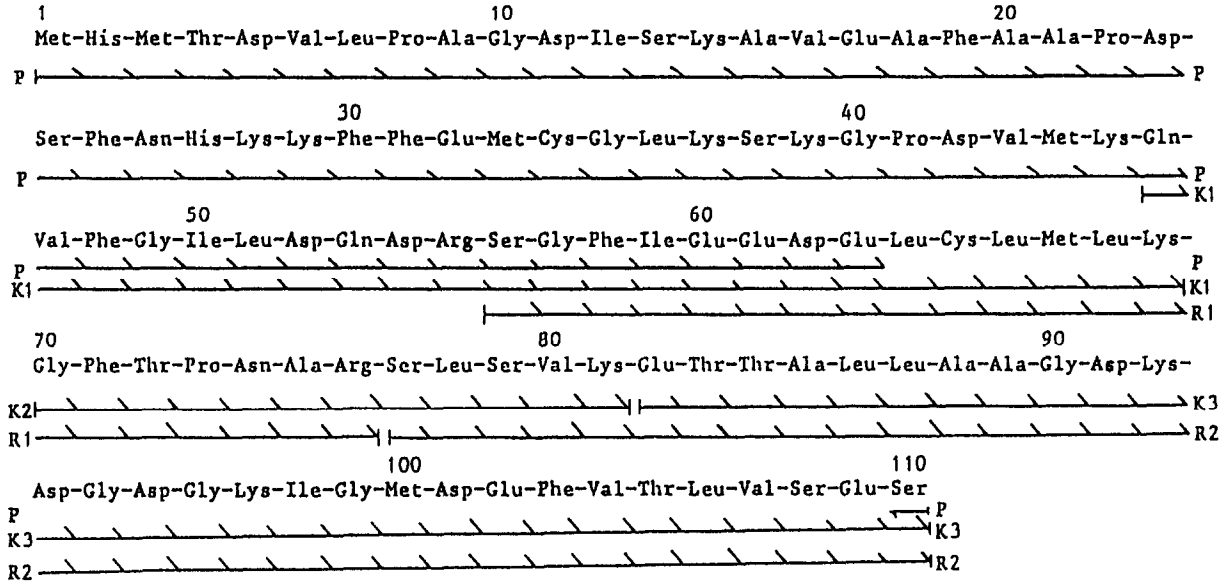

Fig. 1. Amino acid sequence of bullfrog parvalbumin pI4.97. P, *S*-carboxymethylated PA4.97; K1-K3, peptides obtained by lysyl endopeptidase digest; R1-R2, peptides obtained by Arg-C digest; (⟶) sequence analyzer run of the protein; (⟵) amino acid identified by hydrazinolysis. Only the overlapping key peptides are shown.

than that of *Rana esculenta* PA4.88 [8] or rabbit PA5.5 [17] and two more than that of *Rana esculenta* PA4.50 [9] or carp PA4.25 [18]. In addition, bullfrog, PA4.97 has a free, not acetylated, amino-terminus. This is a rare case in the PA family. Parvalbumins hitherto known as having a free amino-terminus are *R. esculenta* PA4.88 [8] and coelacanth PA5.44 [19]. Bullfrog muscle, however, contains another PA, PA4.78, whose amino terminal was blocked.

Fig. 2 shows the comparison of the amino acid sequence of bullfrog PA4.97 with the sequences of some other PAs. The functionally important sequences of PA are well conserved in PA4.97. First, the Arg-75 and Glu-81 sites, which are thought to form the salt bridge located in the interior of the molecule, exist in this PA. Second, the amino acid sequences of the 51–62 and 90–101 regions, each of which forms an octahedron to chelate a calcium ion [1], are also conserved.

The amino acid replacement of PA4.97 has two characteristics. First, the replacements frequently occur within the amino-terminal-half of the molecule. Second, these do not produce any serious changes in

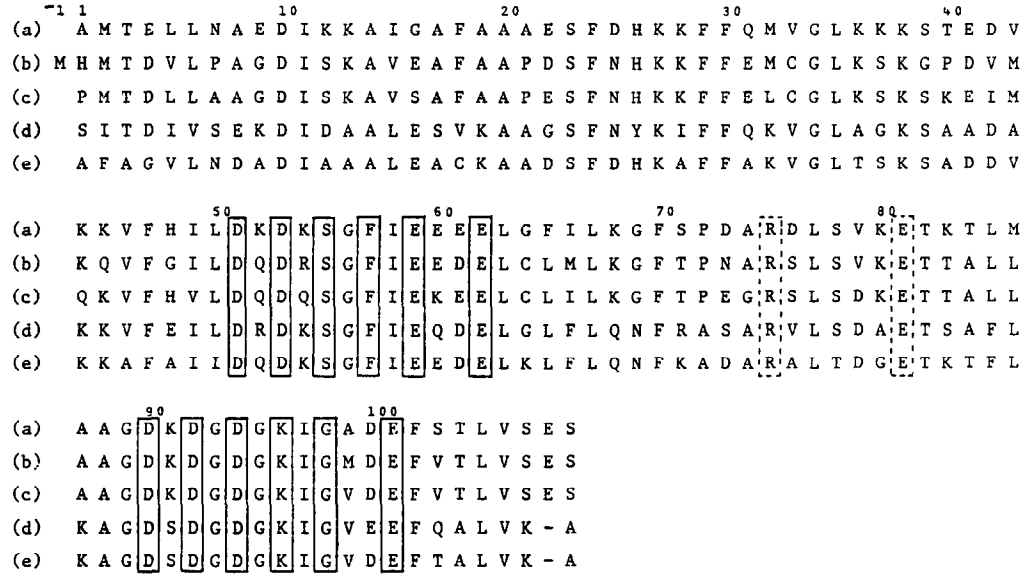

Fig. 2. Comparison of the amino acid sequence of bullfrog parvalbumin PI4.97, with the sequences of some other parvalbumins. (a) rabbit PA5.5 [17]; (b) bullfrog PA4.97 [this report]; (c) frog PA4.88 [8]; (d) frog PA4.50 [9]; (e) carp PA4.25 [18]. Numbering of residues is according to rabbit PA5.55. Residues enclosed within solid-line and dotted-line rectangles correspond to the calcium binding sites and to the site participating in the salt bridge, respectively.

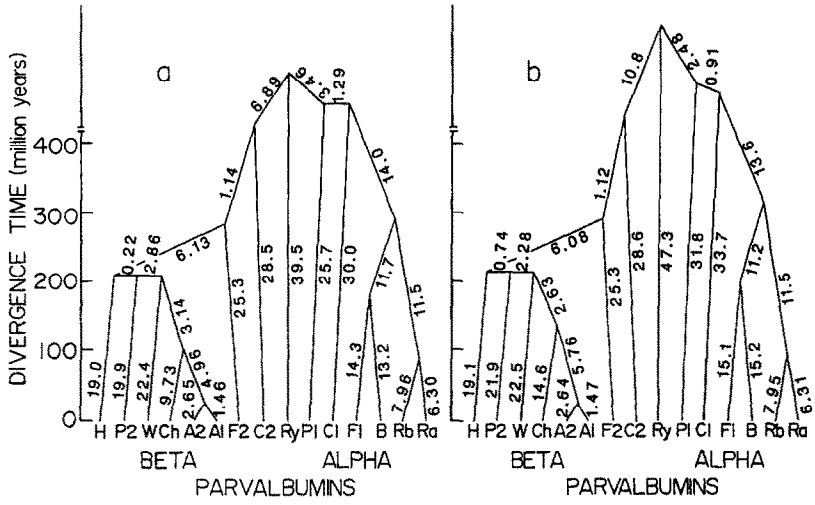

Fig. 3. Phylogenic trees for parvalbumins. Trees (a) and (b) were constructed according to the mutation distances of parvalbumins based on the values of d_u and d_n, respectively. The branch lengths show the calculated mutation distances. Since the divergence points calculated from the matrices were positioned based on fossil records [5], the branch lengths are not proportional to the values shown. Ra, Rb, Ry, B, F1, F2, C1, C2, Ch, P1, P2, A1, A2, W, and H denote rat PA, rabbit PA5.5, ray PA4.45, bullfrog PA4.97, frog PA4.88, frog PA4.50, coelacanth PA5.0, coelacanth PA4.5, chub PA, pike PA5.0, pike PA4.1, carp PA4.47, carp PA4.25, whiting PA4.50, and hake PA4.36, respectively [7].

the secondary structure in comparison with that of carp PA4.25, judging from the calculation by Chou and Fasman's method [20]. These results indicate that the molecular structure of the PA may resemble other PAs in the tertiary structure and the binding mode of calcium ions.

The sequence homology of bullfrog PA4.97 versus rabbit PA5.5 and carp PA4.25 is 69% and 55%, respectively. It is interesting to note that amino acid replacement occurs within the same *Rana* genus. The sequence homology of PA4.97 versus *R. esculenta* PA4.88 and PA4.50 [8,9] is 79% and 57%, respectively. These results show that PA4.97 belongs to the α group.

As shown in Fig. 3, two phylogenic trees were constructed based on the mutation distances calculated using d_u and d_n. PA4.97 was again shown to belong to the α group by both calculations. Each distance reconstructed from this tree was close to that obtained from the original mutation distance, with a standard deviation of 6.6% and 5.9% for the calculations using d_u and d_n, respectively. The trees seem to explain well the divergence of frogs. The present frogs are thought to have separated in the late Carboniferous Era (about 300 million years ago) [5]. Therefore, the subdivergence of frogs into *R. esculenta* with PA4.88 and *R. catesbeiana* with PA4.97 is indicated to have occurred about 200 million years ago. The phenotypic evolution of frogs has been 1/20 to 1/3 slower than that of mammals [6]. However, the average mutation rate of frog PAs is 1.37- or 1.41-fold higher, based on d_u or d_n, respectively, than that of mammalian PAs. The more rapid mutation rate of molecular evolution than that of phenotypic evolution by immunological comparison of albumins [6] has been also reported in frogs.

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