Parvalbumins from the lungfish (Propterus dolloi).

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Résumé.

Cinq parvalbumines ont été purifiées à partir des muscles blancs du Propterus dolloi ; les propriétés physico-chimiques de ces composants sont analogues à celles de cette famille de protéine. Elles se divisent en deux groupes distincts caractérisés par leur composition en acides aminés, leur résidu C-terminal, leur carte peptidique et leur réactivité immunochimique.

Les muscles rouges de ce poisson y compris le muscle cardiaque contiennent des parvalbumines en quantité plus faible de même que le cerveau et les reins.

Summary.

Five parvalbumins have been isolated from the white muscles of the lungfish. They can be divided into two subfamilies showing typical amino acid compositions, C-terminal amino acid residues, peptide maps and immuno-reactivity. The red muscles including the cardiac muscle also contain parvalbumins in amounts roughly inversely related to the concentration of myoglobin in the muscle.

Parvalbumins have also been detected in the brain and kidney.

Key words: Parvalbumin, Lungfish, fish muscle, Propterus dolloi.

to elucidate their physiological and biochemical characteristics.

Various studies (for a review see [2]) have been devoted to the Coelacanth; among them some reports are dealing with the aspect of molecular evolution such as the recent comprehensive study carried out on the parvalbumins of coelacanth muscles [3, 4, 5].

The actual surviving Dipni group two families: the Ceratodidae with one surviving australian species the Barramunda (Neoceratodus forsteri) and the Lepidosirenidae, gathering five species, one from South America (Lepidosiren paradoxa) and four from Africa: Propterus annectens, P. aethiopicus, P. dolloi roughly from central Africa and P. amphibiæus from western tropical Africa [6]. All have an elongated body, eel like, and paired fins of flammentious structure. The most remarkable anatomical characteristic of these Dipni are the existence of bilobed and elongated lungs lying dorsally. Gills are reduced and inadequate for respiration. These species are therefore highly adapted to drought and can survive several years in the absence of water by producing a water impermeable cocoon of dried slime surrounded by a thick layer of dessicated mud. During this period, called estivation, the fish does not ingest food or

Abbreviations:

Ethylenediaminetetra-acetic acid : EDTA.
Polyacrylamide gel electrophoresis : PAGE.
Trichloro acetic acid : TCA.
water nor excretes waste products except respiration gases. This particularly also explains why most of the physiological and biochemical studies concerning these fishes, since the pioneer work of Smith [7], have been mainly polarized on the metabolism of the estivating fish [8] as for example on problems like respiration [9-10] and nitrogen metabolism [11, 12]. Other works concern molecular evolution of proteins such as structural studies on the activation peptide of trypsinogen [13] and on the thyroglobulins [14].

The aim of our study is to add new informations about the relatedness which can be found at the protein level between a lungfish representative, *Protopterus dolloi*, its close cousin the coelacanth and the amphibians represented by frog. We have chosen a family of Ca²⁺ binding proteins, the parvalbumins largely distributed in the animal kingdom [15] which are present in high amount in the white muscles of lower vertebrates in which they seem to act as soluble relaxing factor [16-18].

The present paper is a rather general survey concerning the parvalbumins of the *Protopterus dolloi*; it will be followed by other papers on the amino acid sequences of the lungfish parvalbumins which will be compared to the already published primary structures of the parvalbumins from frog [19] and from coelacanth [4, 5]. This hopefully will provide at the molecular level, new evidence of the phylogenetic relatedness of these species through Amicon UM10 membranes and then dialyzed 4 h against 0.015 M HCl-piperazine buffer pH 5.7.

The final solution was chromatographed on a DEAE-cellulose column (Whatman DE32). Proteine fractions were concentrated by ultrafiltration and desalted on a Biogel P₂ column in 0.05 M NH₄HCO₃.

**Distribution of parvalbumins in various muscles and tissues.**

A weighed amount of about 1g of the frozen material was ground with 500 mg of sand in 1.5 ml of the extracting buffer. The homogenate was centrifuged at 25000 g and the supernatants were examined by polyacrylamide gel electrophoresis, double immunodiffusion and immunoelectrophoresis.

**Analytical methods.**

Starch gel electrophoreses were made according to Scopec [20].

Polyacrylamide electrophoreses were run in slab gel (12 x 19 cm) using 7.5 per cent polyacrylamide, 2M urea and 0.02 M Tris-0.12 M Glycine (pH 6.0). The gels were stained 15 min in the following mixture : 75 ml methanol, 186 ml H₂O, 30 g TCA and 0.1 per cent of Coomassie blue R250 and destained in an acetic acid : methanol : water 1:2:7 mixture. Alternatively the gels were stained by D-periodate and stained also by a specific creatine kinase test [28].

The amino acid composition of the performic acid oxidized proteins and the tryptic peptide maps were obtained as described previously [22] but in the case of enzyme digestion the proteins were first denatured by a 2 h treatment in 6 M Guanidinium chloride, 0.05 M EDTA followed by desalting on a Biogel P₃ column in 0.05 M NH₄HCO₃ and lyophilisation.

The presence of a blocked N-terminal amino acid residue was ascertained by a dinitrophenylation procedure [29]. The identification of the acetyl group was made by hydrolysis of the protein (0.3 μmol) with p-toluene sulfonic acid [24] 12 h at 107°C followed by gas chromatography analysis of the liberated acid [25].

The C-terminal amino acid residues were identified by hydrolysis of about 0.2 μmol of the performic acid oxidized proteins [28].

Ca²⁺ measurements were made using an atomic absorption spectrometer Perkin-Elmer Model 303. The isoelectric points of the proteins were determined by a 2 h isoelectrofocussation on Ampholine PAG plate, pH 3.5-9, using an LKB power supply 2103 and a constant power of 30 W.

The ultra-violet spectra were recorded with a double beam Hitachi Perkin-Elmer 124. Extinction coefficients were obtained from the absorbance at 250 nm of solutions whose concentrations were measured by amino acid analyses taking into account the respective contents of each protein in arginine.

**Immunoechemical methods.**

The antibodies were raised against the purified parvalbumins obtained as lyophilized powder.

Two rabbits per antigen were immunized according to an already described procedure [27]. The method consists to spill directly the lymphatic ganglions of the rear legs with 0.4 ml of 1 mg/ml antigen solution added to an equal volume of Freund's complete adju-
vant. The immunization is completed by three sube-

tantaneous injections in the back of 0.2 ml of the mi-

xiture. Three other injections of 1 ml each were made

at 15 days intervals. After this period ear bleedings

of 3 ml of blood allow semi-quantitative estimation

of the concentration of precipitating antibodies. Rab-

bits were then bled from the carotid artery. Antisera

were stored in 2 ml vials frozen at −32°C. Quantita-

tive precipitin reactions were performed according to

a previous method [28]. Precipitates dissolved in

0.1 M NaOH were analyzed by ultraviolet absorption

at 287 nm using $A = 0.0103 \mu g \text{mg}^{-1} \text{ml}^{-1} \text{cm}^{-1}$ [29]. The absor-

bance of the parvalbumin at this wavelength was

neglected.

Sometimes it was necessary to adjust to the same

test range the concentration of precipitating antiser-

um present in the sera. This was made by isolation of the

γ-γ-Immunoglobulins by Na$_3$SO$_4$ precipitation [28].

The precipitates were dissolved by dialysis against

0.05 M NH$_4$HCO$_3$ and the solutions were lyophylized.

Immunodiffusions and immunoelectrophoreses were
carried out on microscopic glass slides in 1.5 per cent
agarose gel in phosphate-citrate buffer pH 7.1.

Results and Discussion.

The elution profile of a fractionated extract of
lungfish white muscle, on Sephadex G75 is shown
in figure 1 A whereas figure 1 B gives the diagram
of the chromatography of the parvalbumin mixture
on DEAE-cellulose. Depending on the specimen
investigated the relative proportions of the five main
parvalbumins which could be isolated vary somewhat considerably especially at the level
of peaks corresponding to components IIIa and

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**Fig. 1.** — A. Elution profile of the 55-80 per cent acetone frac-
tion obtained from lungfish white muscle on a
Sephadex G75 column (45 × 6 cm) equilibrated
in 0.05 M NH$_4$HCO$_3$, Fractions of 11 ml.

B. Chromatography of the parvalbumins fraction
obtained on Sephadex G75 (fig. 1 A) on a DEAE-
cellulose column (30 × 2.5 cm) equilibrated in
0.015 M HCl-piperazine, pH 5.7. Eluting gradient
was : 400 ml buffer — 400 ml 0.15 M NaCl buffer.
Fractions of 1 ml.
II which can be much larger. Chromatographies of various unfractionated muscle extracts on A2A54 column (LKB — Bromma — Sweden) reveal that parvalbumins occur in the lungfish white muscle at a mean concentration of 250 ± 20 mg per 100 g of fresh tissue; this corresponds roughly to a 0.2 mM concentration in muscle.

The homogeneity of the isolated components was checked by starch gel electrophoresis (fig. 2). To obtain the component V and II in a relative pure state, it was necessary to rechromatograph the corresponding fractions of figure 1B in other conditions: component V was purified by DEAE-cellulose chromatography in 0.015 M HCl-piperazine pH 7 using a salt gradient of 400 ml buffer-400 ml buffer, 0.15 M NaCl; component II was rechromatographed on DEAE-cellulose in 0.05 M Tris-HCl pH 8 using a NaCl gradient of 0-0.15 M. Despite this, component V is often contaminated by denaturation products characterized by a faster rate of migration. It appears however chemically homogeneous by amino acid analysis (table I) and especially not contaminated by component IV.

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**Fig. 2.** — Starch-gel electrophoresis pattern at pH 8.4 illustrating the process of purification of the parvalbumins from lungfish white muscle. From left to right: slit 1 — parvalbumins from carp white muscle; slit 3 — lungfish parvalbumins fraction from Sephadex G75; slit 4 to 8 — components V, IV, IIIa, III and II.
Chemical and physical properties.

The amino acid compositions of the five components are shown in Table I. One can easily distinguish two families of parvalbumins on the basis of histidine and threonine contents; the first one contains components V, IV and IIIa and the second one components III and II.

The tryptic peptide maps also vary depending on the components investigated. Maps of component V, IV and IIIa are very similar; they differ drastically from the maps of component II and III (Fig. 3) which are somewhat different from each other despite an unquestionable relatedness. These two proteins are also extremely resistant to enzymic hydrolysis since apart from the usual denaturation performed in 6M guanidinium chloride and 0.03 M EDTA, it is necessary to heat the solution two minutes at 100°C just prior to the addition of 3 per cent of enzyme. A digestion time of at least 24 h is required.

### Table I.

**Amino acid composition of the performic acid oxidized parvalbumins from lungfish (Protopterus dolloi) white skeletal muscle.**

The values listed are averages of six analyses obtained from 24, 48 and 72 h of hydrolysis.

<table>
<thead>
<tr>
<th>Residues per mole of protein</th>
<th>Parvalbumin V</th>
<th>IV</th>
<th>IIIa</th>
<th>III</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amide acid</td>
<td>Calculated</td>
<td>Assumed</td>
<td>Calculated</td>
<td>Assumed</td>
<td>Calculated</td>
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<tr>
<td>Lys</td>
<td>15.8</td>
<td>16</td>
<td>14</td>
<td>14</td>
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<tr>
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<td>2</td>
<td>1.8</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>Arg</td>
<td>2.1</td>
<td>2</td>
<td>1.9</td>
<td>2</td>
<td>2.1</td>
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<td>15</td>
<td>15.9</td>
<td>16</td>
<td>15.2</td>
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<tr>
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<td>2</td>
<td>1.7</td>
<td>2</td>
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</tr>
<tr>
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<td>11</td>
<td>9.7</td>
<td>10</td>
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<tr>
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<tr>
<td>Pro</td>
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<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
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<tr>
<td>Gly</td>
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<td>8.1</td>
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<td>6.4</td>
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<tr>
<td>Ala</td>
<td>8.8</td>
<td>9</td>
<td>10.8</td>
<td>11</td>
<td>10.8</td>
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<td>0</td>
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<tr>
<td>Val</td>
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<td>7</td>
<td>7.3</td>
<td>7</td>
<td>6.2</td>
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<tr>
<td>Met (b)</td>
<td>2.7</td>
<td>3</td>
<td>0.0</td>
<td>0</td>
<td>1.9</td>
</tr>
<tr>
<td>Ile</td>
<td>5.6</td>
<td>6</td>
<td>7.0</td>
<td>8</td>
<td>7.9</td>
</tr>
<tr>
<td>Leu</td>
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<td>7.8</td>
<td>8</td>
<td>7.4</td>
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<tr>
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<tr>
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<td>9</td>
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<tr>
<td>Trp (c)</td>
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<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

| Total                       | 108          | 110 | 104  | 108 | 108 |

(a) Determined as cysteic acid.
(b) Determined as methionine sulfoxide.
(c) Determined from U-V spectra.
The five components contain 2 ± 0.15 Ca²⁺ per mole of protein.

The isoelectric points are distributed on 2.6 pH units, some of them reach particularly high values which have never been mentioned so far for parvalbumins.

Parvalbumin V IV IIIA III II

pI 6.0 6.5 4.3 4

The UV spectra are typical of this protein family. Molecular extinction coefficients at 250 nm determined from solutions, the concentrations of which were calculated by amino acid analyses are 2295, 2370, 2510 and 1988 mol⁻¹ cm⁻¹ for components V, IV, IIIA and III respectively. As an example the spectra of components V and III are illustrated in figure 4. The spectrum of compo-
Fig. 5. — Gel immunoelectrophoresis of the purified parvalbumins from the lungfish. The upper troughs were filled with 100 μl of a solution (15 mg/ml) of γ-G-immunoglobulin isolated from serum D51 by Na₂SO₄ precipitation; the lower troughs were filled with the same volume of a solution (4 mg/ml) of γ-G-immunoglobulins isolated from serum D31. The wells contain 2 μl of a parvalbumin solution (0.5 mg/ml) in phosphate buffer.
ponent V shows a high residual absorption above 275 nm despite the fact that this component is devoid of tyrosine. Tentatives to remove it using denaturing agents like urea, guanidinium chloride or detergents were unsuccessful. This contaminating chromogen has already been noticed in the case of some parvalbumins by ourself and other [3].

Antigenic properties.

Antisera against the parvalbumin III were obtained from two rabbits and were named D31 and D32. The amount of precipitating antibodies was estimated from the precipitating curves assuming a composition of the precipitating complex corresponding to a maximum molecular ratio $\frac{A_b}{A_g} = 2$. This figure was obtained from the zone of excess antibodies determined by the analysis of supernatants by the ring tests [31]. The experimental values of this ratio obtained by extrapolation at zero antigen concentration were 2.34 and 2.30 for sera D31 and D32 respectively. D31 contained 6 mg/ml of precipitating antibodies (MW = 158000) and D32 8.4 mg/ml.

Only one rabbit reacted significantly against the parvalbumin V. Trial bleedings revealed a concentration of precipitating antibodies comparable to those obtained with the parvalbumin III. Unfortunately the carotid bleeding made ten days after the last trial gave a serum (D51) much poorer in precipitating antibodies which did not exceed 0.7 mg/ml.

The results of the immunoelectrophoresis of the five components against appropriate solutions of $\gamma$-G-immunoglobulins isolated by Na$_2$SO$_4$ precipitation from sera D31 and D51 are shown in figure 5. The heterogeneity of component V is demonstrated by the slight alteration in the regularity of the precipitating arc. All the other components show rather regular patterns. Components V, IV and IIIa do not give any precipitation with

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Fig. 5. — Gel immunoelectrophoreses of synthetic parvalbumins mixture containing 1.5 mg/ml of each parvalbumin. The troughs were filled with $\gamma$-G-immunoglobulins as in figure 5.
serum D31; on the other hand, components III and II react exclusively with serum D31. Examples of cross reactivities are shown in figure 6. As expected (fig. 6A) components V, IV and IIIa manifest immunoreaction of nearly complete identity towards serum D31 except for a very weak spur corresponding to component IV and projecting beyond the point of partial coalescence with component IIIa.

Component III and II seem to have strictly equivalent determinants towards serum D31 (fig. 6B).

No cross reactions are observed between on one hand component IV and III and on the other hand between component IIIa and II (fig. 6C).

All these results confirm the existence of two well defined parvalbumins families in the lungfish white muscle.

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**Figure 7.** PAGE patterns of various muscle extracts obtained at pH 8.6. Total extracts.

1. Lungfish white muscle.
2. Sternohyoidan (head part).
3. Sternohyoidan (pink).
4. Sternohyoidan (red part).
5. Sternohyoidan (pale part).
7. Adductor hipericus.
8. Adductor superficialis.
11. Larval line.
12. Larval line.
13. Larval line.

**Abbreviations:**
- mb: myoglobin
- CK: creatine kinase
- gb: metmyoglobin
- mb: myoglobin
Parvalbumins in various organs and muscles.

Various organs and muscles have been examined in order to study the distribution of parvalbumins. Ten muscles, brain, kidney and liver were analysed by polyacrylamide gel electrophoresis, by double immunodiffusion and by immunoelectrophoreses.

In the order of increasing red colour the muscles can be classified as follows:

- trunk white muscle < sterno-hyo- (inferior jaw) < geno-hyo- (inferior jaw) < adductor inferior superficialis (jaw) < red part of sterno-hyo- (inferior jaw) < intermandibularis (inferior jaw) < protractor of cephalic rib < lateral line < adductor and adductor of the paired posterior fins < ventricle. The PAGE patterns are shown in Figure 7. All the muscles even heart contain parvalbumins but the patterns differ according to the muscle. White muscle along with geno-hyo- and sterno-hyo-, which are pink in colour, have the same assortments of parvalbumins, namely the five components, in comparable concentration. In the specimen investigated components III and IIIa occur in roughly similar amount. Component IV, not very abundant, is partly masked by three protein bands corresponding to creatine kinase, Met-myoglobin and myoglobin migrate on both side of this component.

The intermandibularis muscle (number 4) has a very weak amount of component III and its component II migrates faster than in the other muscles. The adductor inferior superficialis is very rich in components V and IIIa whereas ventricular muscle has a faint band migrating at the level of component IIIa. The double immunodiffusion tests confirm that all the extracts do contain parvalbumins giving precipitin reactions with sera D51 and D51. Even the extract from cardiac muscle gives a weak but quite discernible reaction especially with serum D51. The extracts from brain, kidney and liver do not show on PAGE visible traces of parvalbumins but double immunodiffusions carried out on these materials reveal in the brain extract the presence of parvalbumins reacting mainly with serum D51. Parvalbumins in the kidney were found more questionable.

Immunoelectrophoreses carried out on ventricle, brain, kidney and liver extracts show that parvalbumins V, IIa and III are present in roughly similar amount in ventricle and brain extracts. A component migrating at the level of component III but giving only immunoprecipitation with serum D51 can also be localized in the kidney extract. No parvalbumin could be detected in liver extract.

Conclusions.

Five parvalbumins isotypes have been isolated from the white muscle of the lungfish. Their global concentration amounts to about 0.2 mM; this is lower than the usual 0.4–0.5 mM concentration mentioned for other fish species [32, 35], much lower than the figure of about 1 mM reported for coelacanth white muscle [2] and roughly equivalent to parvalbumin concentration of frog white muscle [10, 22]. These five parvalbumins belong to two families differing from the points of view of amino acid composition, peptide map, C-terminal amino acid residue and immune cross-reactivity. They probably originate from an early gene duplication. As the lungfish possesses diploid cells of a very high DNA content [36], such a multiplicity of parvalbumin genes cannot be ascribed as in the Cyprinidae family to a polypliodization process [33, 37] but rather to a linear replication of some chromosomal regions followed by divergent evolution.

Comparison of various muscles shows on the other hand that, like in other fish species, the quantity of parvalbumins seems to be inversely related to the myoglobin content, the highest amount being found in white and the lowest in cardiac muscle [38].

The presence of parvalbumins in weak amount in fish cardiac muscle has already been pointed out earlier [39] and appears to be significant, their concentration in the heart is about ten times less than in the white muscle, in an Antarctic myoglobin-hemoglobin free fish [40]. Mammalian heart on the contrary seems to be completely devoid of parvalbumins [35].

The significance of the inverse relation parvalbumin-myoglobin is uncertain. The only clue to the physiological role of these proteins appears so far to reside in their ability to play the role of a soluble relaxing factor [16–18]. On the basis of this interpretation the lower parvalbumin content in the red muscles could be explained by their slower contractile activity and by their higher content in mitochondria. This Ca²⁺-trapping agent could be particularly efficient in cardiac muscle where mitochondria are particularly abundant [41] to lower the cytoplasmic Ca²⁺ concentration during the relaxation phase [42, 43].

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Lungfish parvalbumins.

REFERENCES.