Isolation and Characterization of Parvalbumins from the Skeletal Muscle of Higher Vertebrates*

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SUMMARY

Parvalbumins, i.e. the low molecular weight, calcium-binding muscle proteins isolated heretofore exclusively from fish and amphibians, have been obtained in sizable amounts from the skeletal muscle of turtle, chicken, rabbit, and man. The finding that these proteins are not confined to lower vertebrates but have been conserved throughout evolution strongly suggests that they must possess a definite physiological function possibly related to the contractile process.

Parvalbumins are low molecular weight, soluble, calcium-binding proteins found hitherto exclusively in the muscle of lower species (fish and amphibians). They display an unusual ultraviolet spectrum due to a high phenylalanine to tyrosine and tryptophan ratio. Their amino acid sequence has been determined (1-3), as well as their tertiary structure by x-ray crystallography (4-7). However, no physiological function could be ascribed to them as yet.

Recently, a protein related to parvalbumin was isolated from dogfish skeletal muscle with the main difference that it could be readily phosphorylated by an endogenous protein kinase (8). When preliminary data indicated that such phosphate acceptor proteins existed in mammalian systems, there was a strong suspicion that classical parvalbumins might also be present. This manuscript describes the isolation of parvalbumins from turtle, chicken, rabbit, and human skeletal muscle. The finding that these low molecular weight, calcium-binding proteins are not confined to aquatic animals strongly suggests that they must possess a definite physiological function possibly related to the contractile process.

MATERIALS AND METHODS

White female rabbits (New Zealand) and chicken (crossbred between white rock and game hen) were obtained from Totem Farms, Inc., Seattle, Wash., and killed by intravenous injection of 25 mg per kg of sodium pentobarbital. Frozen turtle muscle (Wisconsin turtle) was purchased from a local market. Human skeletal muscle (musculus pectoralis and iliopsoas) was obtained (l-3), as well as their tertiary structure by x-ray crystallography (4-7). However, no physiological function could be ascribed to them as yet.

Purification of Rabbit Muscle Parvalbumin—All steps were carried out at 4°C. Fresh rabbit skeletal muscle (1 kg) was ground in a meat grinder, homogenized for 1 min in a Waring Blender at full speed in 2.5 volumes of distilled water, pH 7.0, and centrifuged for 30 min at 14,000 × g; the supernatant was lyophilized. The residue was dissolved in 100 ml of 10 mM sodium acetate, pH 5.7, and dialyzed against the same solution. The dialyzed material (after clarification by centrifugation) was applied to a column (65 × 5 cm) of DE52-cellulose equilibrated in the same solution; elution was carried out with a linear gradient of 10 to 500 mM sodium acetate, pH 5.7. The fractions containing the low molecular weight protein (see "Materials and Methods") were pooled and the solution was lyophilized. The residue was dissolved in 10 mM imidazole HCl, pH 6.8, and passed through a column (2.5 × 100 cm) of Sephadex G-75 equilibrated in the same solution. The pure fractions as judged by gel electrophoresis in the presence of sodium dodecyl sulfate and their characteristic ultraviolet spectra were pooled; following dialysis against 5 mM ammonium acetate, pH 8.0, and lyophilization, a salt-less powder was obtained. From 1 kg of fresh rabbit muscle, 100 to 300 mg of pure parvalbumin can be isolated.

Test of Purity and Molecular Weight—Parvalbumine from the turtle, chicken, rabbit, and man were pure as judged by the criteria of polyacrylamide gel electrophoresis (Fig. 1), sedimentation velocity, and equilibrium experiments in the ultracentrifuge (not illustrated). The faster migration observed on normal gels (Fig. 1B) for turtle and human (versus chicken and Clinical Pathology, University of Washington. Sephadex was obtained from Pharmacia, Piscataway, N.J.; DE52-cellulose from Whatman; and Chelex-100 (200 to 400 mesh, sodium form) from Bio-Rad Laboratories. *4CaCl₂ was purchased from New England Nuclear Corp. (specific radioactivity about 15 mCi per mg). Polyacrylamide gel electrophoreses were carried out either in Tris-glycine buffer, pH 8.3, according to Ornstein (9) and Davis (10) or in the presence of 0.1% sodium dodecyl sulfate according to Weber and Osborn (11); 10% gels were used throughout.

Protein was determined using the biuret method (12). Amino acid analyses were carried out according to Moore and Stein (13) using norleucine as an internal standard (14); hydrolyses were performed in sealed, evacuated tubes after repeated flushings with nitrogen in 5.7 n HCl at 108°C for periods of 24, 48, and 72 hours; the hydrolyzed samples were analyzed on a Beckman model 120 C automatic amino acid analyzer. Ultracentrifuge analyses were carried out at 6° in 0.1 M potassium phosphate buffer, pH 6.8, in a Spinco model E analytical ultracentrifuge. Calcium and other metals were measured by atomic absorption using a Perkin-Elmer model 303 spectrometer; calcium-binding experiments were performed according to Briggs and Plesiluman (15). Parvalbumins were identified during purification by gel electrophoresis in the presence of sodium dodecyl sulfate where they migrate as a major band close to the dye front. Upon further purification, they could also be characterized by their unusually high (greater than 1) 260:280 nm absorbance ratio, and by their characteristic ultraviolet spectra.

RESULTS

Since the purification and properties of the parvalbumins from turtle, chicken, rabbit, and human skeletal muscle are very similar, only the characterization of the rabbit protein will be described in some detail.

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FIG. 1. Polyacrylamide gel electrophoresis patterns in the presence (A) and absence (B) of 0.1% sodium dodecyl sulfate of parvalbumins (~10 µg of protein each) from the skeletal muscle of turtle (I), chicken (6), rabbit (9), and man (4). Origin (top) and front (bottom) are indicated by arrows.

Fig. 2. Ultraviolet absorption spectrum of rabbit skeletal muscle parvalbumin (6.6 mg per ml) in 5 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, adjusted to pH 7.0 with NaOH. Identical spectra were obtained in either 0.1 N NaOH or HCl.

Amino acid composition of rabbit parvalbumin

Results are the average of 24, 48, and 72 hours of hydrolysis on material obtained from two different preparations.

| Amino acid    | Residues per molecule | Assumed No. of residues per molecule |
|---------------|-----------------------|-------------------------------------|
| Lysine        | 16.1 ± 0.2            | 16                                  |
| Histidine     | 2.0                   | 2                                   |
| Arginine      | 1.15 ± 0.05           | 1                                   |
| Aspartic acid | 12.15 ± 0.15          | 12                                  |
| Threonine     | 5.15 ± 0.05           | 5                                   |
| Serine        | 8.0 ± 0.2             | 8                                   |
| Glutamic acid | 12.65 ± 0.05          | 13                                  |
| Proline       | 0.75 ± 0.05           | 1                                   |
| Glycine       | 9.4 ± 0.1             | 9                                   |
| Alanine       | 11.3 ± 0.2            | 11                                  |
| Valine        | 5.45 ± 0.3            | 5                                   |
| Methionine    | 2.75 ± 0.15           | 3                                   |
| Isoleucine    | 6.0 ± 0.0             | 6                                   |
| Leucine       | 8.95 ± 0.3            | 9                                   |
| Tyrosine      | 0                     | 0                                   |
| Phenylalanine | 9.1 ± 0.2             | 9                                   |
| Half-cystine  | 0                     | 0                                   |
| Tryptophan    | 0                     | 0                                   |

Total residues: 110

* Based on 2.0 histidines per molecule.
* Extrapolated to zero time of hydrolysis.
* Values from a single preparation only; in the other preparation obtained by a different purification procedure, involving trichloroacetic acid precipitation and a step, values obtained for proline and methionine were 2 and 2 residues per mole, respectively.
* Determined as cysteic acid according to Hirs (16).
* Measured spectrophotometrically according to Bencze and Schmid (17).

Metal Analysis—The four parvalbumins described here were isolated as calcium metalloproteins even though the purification was carried out in the absence of added Ca2+ ions or even in the presence of 0.1 mM EDTA (for the turtle and chicken proteins). The purified materials were free of magnesium, manganese, iron, cobalt, copper, and zinc as determined by atomic absorption. Calcium-binding measurements by equilibrium dialysis and the Chelex partition procedure (15) indicated that all four parvalbumins display two strong binding sites for calcium with a $K_{dss}$ of 0.2 µM or below, an affinity similar to that previously reported for hake parvalbumin (20).

H. E. Blum, S. Pochinwong, and E. H. Fischer, unpublished data.
The finding that parvalbumins are present in sizable amounts in the muscle of higher vertebrates including man raises more than ever the question of their physiological function. While 100 to 300 mg of the low molecular weight calcium-binding proteins could be isolated per kg of fresh rabbit muscle, more than 1 g per kg was obtained from the turtle. Assuming a conservative yield of 50\% during purification, the concentration of parvalbumins would be approximately \( \frac{1}{10} \) to \( \frac{1}{3} \) that of TN-C\(^*\) (21). Evidence for a relationship between these two proteins was recently presented (18, 22), based on a degree of homology between their amino acid sequences. On the other hand, parvalbumins cannot originate as mere breakdown products of the larger TN-C. First, while the greater similarity was observed between the COOH-terminal portion of TN-C and parvalbumins (22), the latter proteins could only come from the NH\(_2\) end of TN-C since all but one (18) have a blocked NH\(_2\) terminus. Second, the rabbit parvalbumin described here contains 16 lysyl residues per molecule while only 9 are found in rabbit TN-C (22).

The role of parvalbumins is still unclear. There was no phosphorylation of any of the parvalbumins described here with either dogfish or rabbit muscle protein kinase or phosphorylase kinase. The fact, however, that parvalbumins are not restricted to aquatic, lower vertebrates but have been conserved through close to 500 million years of evolution strongly suggests that they must serve some basic physiological function possibly related to the contractile process. This conclusion seems further supported by the finding that in man they are present in heart and smooth (uterus) muscle, as well as in skeletal muscle.

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