GENERATION OF PARVALBUMIN-LIKE PROTEINS FROM TROPONIN*

Claus W. Heizmann†, Dean A. Malencik‡, and
Edmond H. Fischer

Department of Biochemistry, University of
Washington, Seattle, Washington, 98195.

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Summary: Parvalbumin-like proteins could be generated from the calcium binding subunit of the troponin complex isolated from dogfish skeletal muscle. These proteins displayed several similar properties with dogfish parvalbumins, including atypical ultraviolet spectrum and molecular weights; furthermore, whereas native troponin or its calcium-binding subunit do not cross-react immunologically with antibodies prepared from dogfish parvalbumins, the breakdown products show strong cross-reactivity.

Parvalbumins are water soluble, acidic, low molecular weight calcium-binding proteins present in the skeletal muscle from lower vertebrates; their physical and chemical properties have been extensively studied (1) including amino acid sequence (2) and determination of tertiary structure (3). While purifying the troponin complex from the skeletal muscle of the Pacific dogfish (*Squalus sucklii*) we have attempted to establish a relationship, if any, between parvalbumins and the Ca^{2+}-binding component of troponin (TN-C), since they have similar affinities for divalent metal ions and spectral properties. Troponin together with tropomyosin controls the interaction of actin and myosin necessary for muscle contraction (4). This publication demonstrates that a protein having many characteristics of dogfish parvalbumin can be generated from pure dogfish TN-C by limited proteolysis.

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Materials and Methods

Dogfish were netted in the waters of Puget Sound and kept in a 2000 gallon, donut shaped tank with circulating, aerated sea water. Troponin was isolated from fresh dogfish skeletal muscle and separated into its component parts; the Ca^{2+} binding subunit (TN-C) was pure as judged by polyacrylamide gel electrophoresis in the presence of Na dodecylsulfate. Calcium-binding properties and amino acid composition are described elsewhere (5). Dogfish parvalbumin was purified from skeletal muscle according to Péchère (6), and myosin light chains according to the procedure of Lowey and Holt (7). Electrophoreses were carried out on 10% polyacrylamide gels in the presence of 0.1% Na dodecylsulfate according to Weber and Osborn (8). Ca^{2+} was determined on a Perkin Elmer atomic absorption spectrophotometer. Antibodies against homogeneous dogfish parvalbumin were elicited in the rabbit.

Results and Discussion

Purified dogfish troponin showed only three bands on gel electrophoresis corresponding to TN-T (60,000 mol. wt.), TN-I (31,000 mol. wt.), and TN-C (20,500 mol. wt.) (5); it did not cross-react with antiparvalbumin antibodies. Storage of this material for 2-3 weeks either in solution or in lyophilized form resulted in a slow degradation, as shown by the appearance of a number of low molecular weight peptides. Unlike the original material, this degraded preparation cross-reacted strongly with parvalbumin antibodies; the presence of a single precipitin line on Ouchterlony plates (Fig. 1) with total absence of spurs suggested that a single antigen was involved.

Purification of the peptide mixture on a column of Sephadex G-75 according to the procedure developed for the isolation of parvalbumins (6) gave 2 major peaks. Material from the first peak did not cross-react and proved to be undegraded troponin while material from the

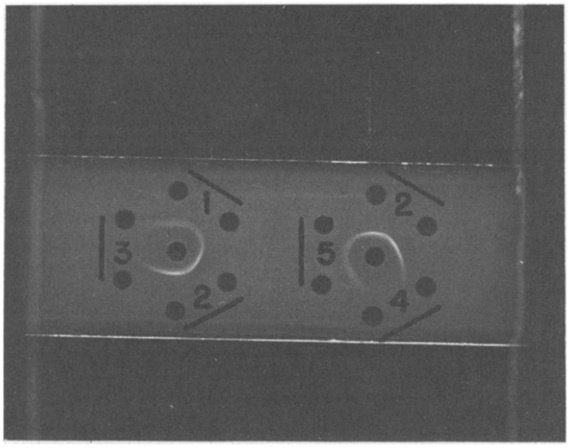

Fig. 1. Immunodiffusion patterns. Center wells contain antibody against homogenous dogfish parvalbumin. Peripheral wells contain (1) Degraded Troponin (2) Parvalbumins (3) Undegraded Troponin (4) Undegraded TN-C (5) Degraded TN-C.

second peak gave a strong precipitin reaction with antiparvalbumin (Fig. 1); gel electrophoresis showed the presence of two proteins with molecular weights of 11,000 and 13,000, respectively, similar to dogfish parvalbumins. Contamination of purified troponin by parvalbumin can be discounted since (a) parvalbumins are water soluble and troponin is washed extensively before extraction with 1 M KCl; and (b) it shows no immunological cross-reactivity with antiparvalbumin.

Purified dogfish TN-C binds 2 Ca^{2+} ions per molecule with a K_{diss} of 9.1×10^{-7} M (5). Like intact troponin, it displays no cross-reactivity with antiparvalbumin. It is stable when kept frozen in the presence of Ca^{2+} ions (Fig. 2A), but when dialyzed against 1 mM EGTA to remove more than 90% of the bound Ca^{2+}, it undergoes slow autodigestion (Fig. 2, B, C, and D). Within 3 days at room temperature, the band corresponding to TN-C vanishes almost completely while two new proteins with molecular weights of 11,000 and 13,000 can be seen on gel electrophoresis (Fig. 2D).

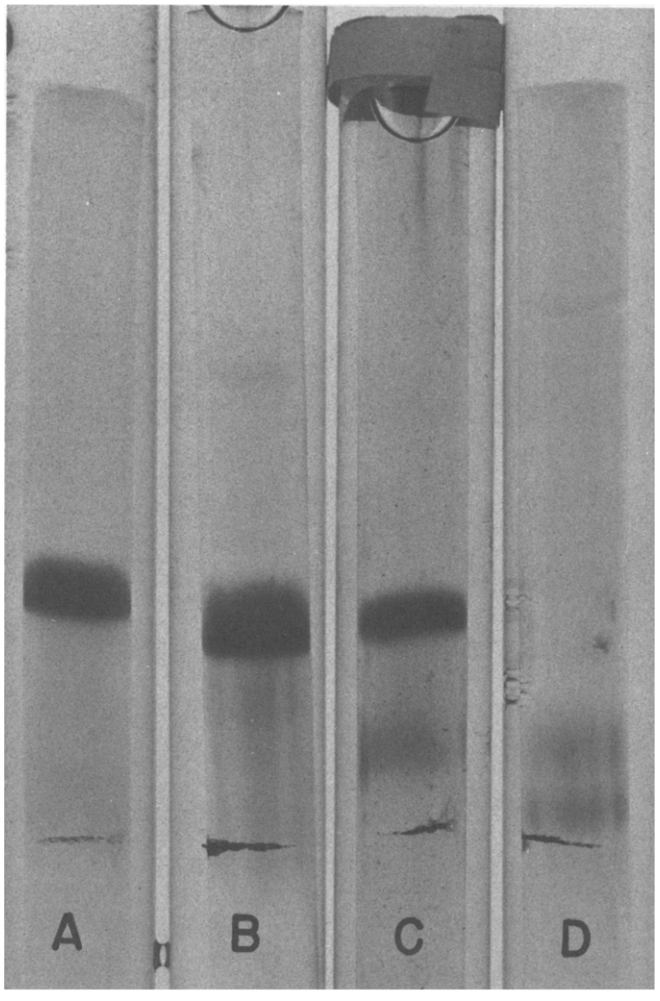

Fig. 2. Polyacrylamide gel electrophoresis (10%) in the presence of 0.1% sodium dodecylsulfate of calcium free TN-C (*ca.* 20 µg protein) after (A) 0 hrs. (B) 12 hrs. (C) 48 hrs. (D) 96 hrs.

The 11,000 and 13,000 molecular weight species gave a strong immunological cross-reactivity with antiparvalbumin but only in the presence of Ca^{2+} (Fig. 1); removal of the divalent metal ion resulted in a loss of immunological reaction suggesting either that the antibodies are directed towards the Ca^{2+} binding sites or that the tertiary structure is disrupted in the absence of Ca^{2+} ions. Several reports of partial disruption of conformation of Ca^{2+} -free TN-C have been

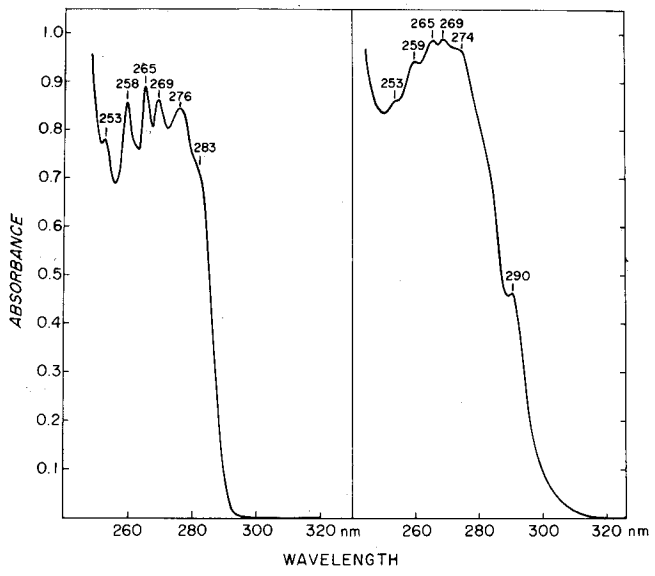

Fig. 3. Ultraviolet spectrum of dogfish parvalbumins (left) and dogfish TN-C (right) in 20 mM Tris, pH 7.5.

published using circular dichroism (9), or in the case of parvalbumins by NMR spectroscopy (10).

As was reported for other parvalbumins, the ultraviolet spectrum of the dogfish protein is atypical. Maximum absorbance occurs at 265 nm and shows considerable vibrational substructure due to a high phenylalanine/tyrosine ratio and complete absence of tryptophan (Fig. 3). Pure dogfish muscle TN-C contains 2 tryptophanyl residues but still displays the vibronic structure in the phenylalanine region of the spectrum (Fig. 3); it has the same phenylalanine/tyrosine ratio as dogfish parvalbumin.

Dogfish myosin light chains were also isolated and found to have molecular weights of *ca.* 23,000 and 16,000, respectively, as determined by sodium dodecylsulfate gel electrophoresis. Neither protein bound Ca^{2+} nor cross-reacted with antiparvalbumin antibodies. Furthermore, no stable 11,000 and 13,000 molecular weight intermediates could be generated upon limited proteolysis.

These results indicate that material having identical molecular weight, absorption spectrum, calcium-binding properties and immunological reactivities as the parvalbumins can be generated from pure TN-C by limited proteolysis. The appearance of protein fragments throughout the troponin preparation suggests that proteolytic enzymes (11), such as cathepsins must be present as trace contaminants (12). Nevertheless, in spite of this close relationship, it is unlikely that parvalbumins result principally from proteolytic degradation of TN-C.

Several questions remain, however. Apparently, why does red muscle from fish contain troponin but no parvalbumin (1)? Various parvalbumin isotypes have been isolated from other fish species (1), but not characterized in detail; what relationship exists among them and TN-C?

Other Ca^{2+}-binding proteins with somewhat analogous properties but uncertain function have been reported from brain (13), intestine (14), and muscle (15); their possible structural relationship is presently being investigated.

References

1. Demaille, J., Dutruge, E., Capony, J.-P. and Péchère, J.-F. (1973) Jablonna Symposium, Elsivier, in press.

2. Capony, J.-P., Ryden, L., Demaille, J. and Péchère, J.-F. (1973) Eur. J. Biochem. 32, 97.

3. Kretsinger, R. H. and Nockolds, C. E. (1973) J. Biol. Chem. 248, 3313.

4. Ebashi, S., Endo, M. and Ohtsuki, I. (1969) Quar. Rev. Biophys. 2, 351.

5. Malencik, D. A., Heizmann, C. W., and Fischer, E. H. (1973), to be published.

6. Péchère, J.-F., Demaille, J. and Capony, J.-P. (1971) Biochim. Biophys. Acta 236, 391.

7. Lowey, S. and Holt, J. C. (1972) Cold Spring Harb. Symp. 37, 19.

8. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.

9. Murray, A. C. and Kay, C. M. (1972) Biochemistry 11, 2622.

10. Parello, J., Cave, A., Puigdomenech, P., Maury, C., Capony, J.-P. and Péchère, J.-F. Biochimie, in press.

11. Dabrowska, R., Barylko, B., Nowak, E., and Drabikowski, W. (1973) FEBS Letter 29, 239.

12. Hartshorne, D. J. and Dreizen, P. (1972) Cold Spring Harb. Symp. 37, 225.

13. Stewart, J. A. (1972) Biochim. Biophys. Acta 263, 178.

14. Fullmer, C. S. and Wasserman, R. H. (1972) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 31, 693 Abs.

15. Blum, H. E., Pocinwong, S. and Fischer, E. H. (1973) Third Intl. Symp. on Metabolic Interconversion of Enzymes, Springer-Verlag, in press.