A REAPPRAISAL OF THE RELATIONSHIP OF PHOSPHATE-ACCEPTOR PROTEIN TO PARVALBUMINS

J. Demaille, E. Dutruge, G. Baron, J.-F. Pechère* and E.H. Fischer**

Laboratoire de Biochimie Médicale, Université de Dakar (Sénégal), *Centre de Recherches de Biochimie Macromoléculaire, CNRS, Montpellier (France) and **Department of Biochemistry, University of Washington, Seattle, Wash. 98195 (USA).

Received October 7,1975

SUMMARY - A phosphate-acceptor protein thought to be related to parvalbumins was described from dogfish muscle (Blum, H.E. et al., 1974 Proc. Nat. Acad. Sci. USA 71, 2198-2202). Further examination of this material indicated that the fraction obtained contained mainly classical parvalbumin, contaminated by less than 5% of a true phosphate-acceptor protein of MW ca 18 000 that accompanies parvalbumin throughout its purification. No such acceptor could be found in hake subjected to identical purification procedures. It is concluded that a phosphate-acceptor protein such as found in dogfish muscle bears no relation to parvalbumins.

A protein readily phosphorylated by a c-AMP-independent dogfish protein kinase and thought to be related to parvalbumin was recently described from dogfish (Squalus acanthias) white skeletal muscle (1,2). Although this P-acceptor protein occurred in aggregated forms (MW ca 350 000, 75 000, 25 000), it appeared to dissociate in sodium dodecyl sulfate (SDS) gels into subunits of MW 11 000 and 13 000, and to display the same ultraviolet spectrum, Ca^{2+}-binding properties and immunological cross-reactivity with dogfish parvalbumin. It was thus suggested (2) that this protein might represent a physiologically active form of parvalbumins, and that the Ca^{2+}-binding properties of these proteins (3,4)

---

* Contribution n° 136 from the Centre de Recherches (CNRS)
**To whom correspondence and reprint requests should be addressed.

could perhaps be modulated by the covalent modification.

However, no phosphorylation of parvalbumins could be demonstrated under any experimental conditions (1,2,3,5,6).

This discrepancy prompted a reinvestigation of the relationship of the P-acceptor protein to parvalbumins in dogfish muscle, from which it was initially isolated, and in hake (Merluccius merluccius), because the properties and structure of the latter protein were known in great detail (3,7,8).

MATERIALS AND METHODS

Materials: Parvalbumins were prepared as previously described (9), and the P-acceptor protein was isolated by the heat-trichloroacetic acid (TCA) procedure II of Blum et al (2). Anti-hake 4.36 parvalbumin serum was obtained after repeated injections to rabbits of protein emulsions in Freund's complete adjuvant. [γ - ^{32}P] ATP (specific radioactivity about 270 mCi/mmole) was prepared according to Glynn and Chappell (10). Guinea pig complement was obtained from the Institut Pasteur, Paris, and alkaline phosphatase (from calf intestinal mucosa, type VII, 355 U/mg) from Sigma. Dogfish protein kinase was purified up to either the protamine or the DE 52 step, according to Blum et al (1).

Methods: Molecular weights were measured by SDS-10% polyacrylamide gel electrophoresis (11). Disc electrophoresis in 12% polyacrylamide gels at pH 8.9 was carried out as previously described (9). N-terminal analysis by the dansyl chloride procedure (12), amino acid analysis and selective tryptic cleavage of the succinylated protein at the single arginine, were performed as already described (3,13). Sequenator analysis and identification of the reaction products were carried out according to Hermodson et al (14). Immunological cross-reactions were studied either in 1% agarose gel double diffusion plates (15) or by the micro-complement fixation technique (16). Protein phosphorylation by dogfish protein kinase was carried out according to Blum et al (2).

RESULTS AND DISCUSSION

The heat-TCA procedure II, applied to hake muscle, gave a protein mixture, which SDS gel electrophoresis showed to be made essentially of two polypeptide chains of MW 13 000 and 11 000, as with the material obtained from dogfish. These low-MW components were further purified by DE 52 cellulose chromatography (2),

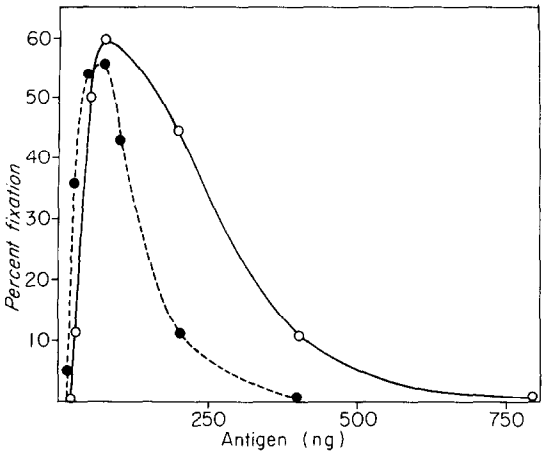

Fig.1: Immunological cross reaction of hake 4.36 parvalbumin (● ——— ●) and the hake heat-TCA protein purified up to the DE 52 cellulose step (o ——— o). The micro complement fixation experiment (16) was carried out in a final volume of 6 ml, with an anti-hake 4.36 parvalbumin serum (final dilution : 1/4 800).

and were eluted together as a sharp peak, with a yield of 1.04 g per kg fresh muscle, similar to the yield of the preparation of hake 4.36 parvalbumin (9). The spectrum (A_{259}/A_{278} = 9.55), the blocked NH_2-terminus and the amino acid composition† were closely similar to those of this parvalbumin (3). This last identity ruled out the possibility of two polypeptide chains, of about 113 and 96 residues, in roughly equimolar amounts. Furthermore the degradation in the sequenator, up to 19 steps, of the material after selective tryptic cleavage at the single arginine residue yielded one single amino acid sequence being identical to that of the hake 4.36 parvalbumin (8) from Arg^{75} to Asp^{94}†. The preparation finally exhibited a reaction of complete identity with hake 4.36 parvalbumin in immunodiffusion. Moreover, the pure parvalbumin and the purified heat-TCA preparation gave, in micro complement fixation, the same maximal fixation at the same antiserum dilution

†Data not reported here, but submitted for examination to the reviewers.

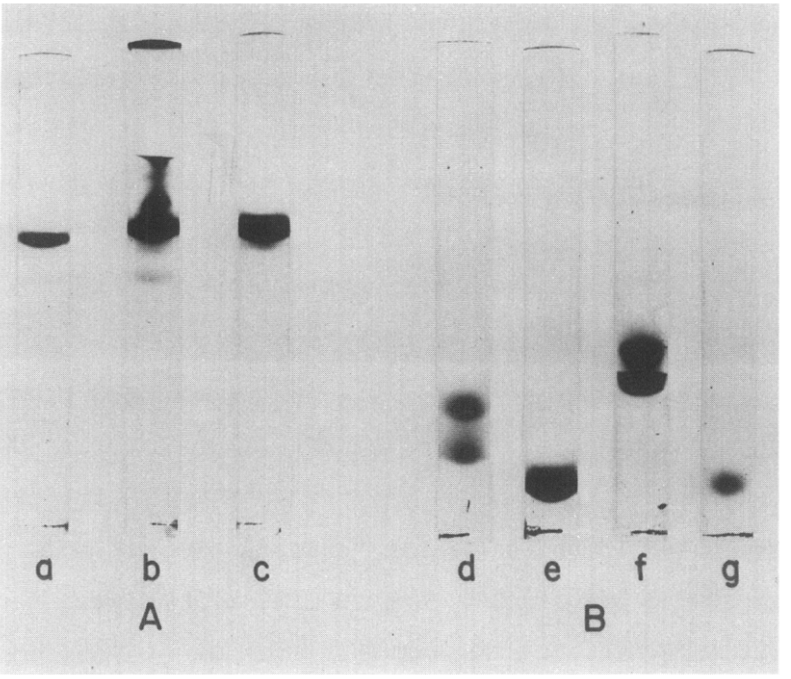

Fig.2: Polyacrylamide gel electrophoresis of hake 4.36 parvalbumin and heat-TCA proteins. -A : 12% polyacrylamide gel in Tris-glycine buffer pH 8.9; a) parvalbumin (25 µg), b) crude heat-TCA preparation (150 µg), c) major heat-TCA protein from the DE 52 step (67 µg). -B : SDS-10% polyacrylamide gel in phosphate buffer pH 7.0; d) and e) parvalbumin (33 and 25 µg respectively), f) and g) crude heat-TCA preparation (72 and 25 µg respectively). Samples d and f were dialyzed overnight vs SDS sample buffer, whereas samples e and g were boiled for 5 min in this buffer.

(Fig. 1), which clearly indicated that both proteins have exactly the same antigenic determinants. The small lateral shift, observed with the heat-TCA protein, could be easily explained by a general conformational change due to the drastic preparation protocol.

On the other hand, the electrophoretic mobilities of hake 4.36 parvalbumin and heat-TCA protein, in 12% polyacrylamide gel at pH 8.9, were identical (Fig 2). The hake heat-TCA protein, at variance with the dogfish one, thus showed no aggregation.

Another striking difference with dogfish is that no phosphoryla tion by protein kinase was observed in the crude heat-TCA prepara-

tion from hake, and in any of the fractions prepared therefrom. The possibility that the protein was already phosphorylated was ruled out by amino acid analyses and by prolonged incubation with alkaline phosphatase which did not change the electrophoretic mobility.

These facts suggested that the heat-TCA products were mostly parvalbumin, contaminated by low amounts of other material(s), among which a P-acceptor protein found in preparations from dogfish and not from hake. This implied that a) the doublet MW 13 000 - 11 000 is an artifact of SDS gel electrophoresis, b) the extent of phosphorylation of the dogfish material is not one mole phosphate bound per 12 000 g protein (2), but significantly lower.

In fact, pure hake 4.36 parvalbumin sometimes exhibited a similar double band pattern in SDS gels while a single band was occasionally observed with the heat-TCA material. This gel pattern was found to vary according to the conditions of formation of the SDS-protein complexes : boiling the sample in the presence of SDS appeared to give a single band, while an overnight dialysis *vs* the SDS buffer often led to a double band (Fig. 2). It was also impossible to separate two entities of different MW by chromatography on DE 52 columns under a variety of conditions.

On the other hand, in contrast with previous results (2), it was found that phosphate incorporation in dogfish heat-TCA material was actually consistently low (below 0.05 mole P_i/12 000 g protein). Moreover, the bulk of radioactivity could be separated from the main parvalbumin band in 12% SDS gels. Superimposition of Coomassie blue and radioactivity patterns showed a highly labelled, faint protein band, migrating slower than the unlabelled major parvalbumin band, with an approximate MW of *ca* 18 000 (Fig. 3). Inclusion of 0.1 mM Ca^{2+} in buffers used during the preparation resulted

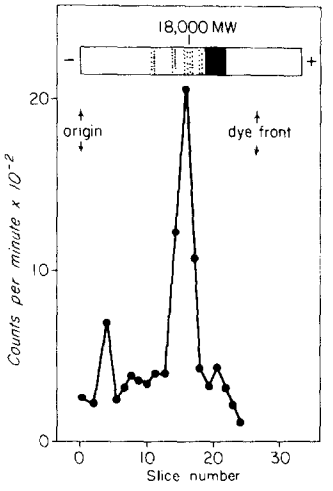

Fig.3: SDS-12% polyacrylamide gel electrophoresis of a ^{32}P-labelled P-acceptor preparation from dogfish white muscle. Labelling with protein kinase and [γ-^{32}P] ATP was performed on a heat-TCA preparation, and the protein fraction with the highest specific activity was purified by DE 52 cellulose chromatography (2). 45 µg protein were loaded on the gel, which was sliced after staining with Coomassie Blue. Slices were dissolved in 30% hydrogen peroxide at 100°C, and counted in 10 ml of scintillant (8 g of Omnifluor and 125 g of naphtalene per liter of dioxane). The main protein band is parvalbumin. The arrows indicate the top of the gel and the migration of the marker dye, respectively.

in a protein mixture which accepted much less phosphate than that obtained under the usual Ca^{2+}-free conditions, suggesting that the P-acceptor protein might be able to bind Ca^{2+}.

In summary, the original conclusion of a close relationship between P-acceptor protein and parvalbumins (2) resulted from the fact that the former material was present in small amounts and carried along by parvalbumin throughout its purification. This specific conveying effect remains rather puzzling however, since it has been observed also in the rabbit, where, again, the material exhibits a higher MW than parvalbumin (unpublished). This and the data above suggest that the P-acceptor might be a Ca^{2+}-binding, acidic protein whose presence warrants further investigation.

ACKNOWLEDGEMENTS -

This work was supported in part by the Fondation pour la Recherche Médicale Francaise, the Institut National de la Santé et de la Recherche Médicale (C.R.L. n° 72.1.018.2 to J.D.), the Délégation Générale à la Recherche Scientifique et Technique (contract n° 72.7.0398 to J.F.P.), the National Institutes of Arthritis and Metabolic Diseases (AM 07902) NIH, USPHS, the National Science Foundation (GB 20482) and the Muscular Dystrophy Association of America. The authors thank Dr. L.H. Ericsson and Dr. S. Pocinwong, Department of Biochemistry, U. of W., for sequenator analysis and for stimulating discussions, respectively.

REFERENCES

1. Blum, H.E., Pocinwong, S. and Fischer, E.H. (1974) in Metabolic Interconversion of Enzymes 1973 (Fischer, E.H., Krebs, E.G., Neurath, H. & Stadtman, E.R. eds) pp. 197-208. Springer-Verlag, Berlin, Heidelberg and New York.
2. Blum, H.E., Pocinwong, S. and Fischer, E.H. (1974) Proc. Nat. Acad. Sci. USA 71, 2198-2202.
3. Pechère, J.F., Capony, J.P. and Ryden, L. (1971) Eur. J. Biochem. 23, 421-428.
4. Benzonana, G., Capony, J.P. and Pechère, J.F. (1972) Biochim. Biophys. Acta 278, 110-116.
5. Baron, G., Demaille, J. and Dutruge, E. (1975) FEBS-Lett., 56, 156-160.
6. Pechère, J.F., Demaille, J., Dutruge, E., Capony, J.P., Baron, G. and Pina, C. (1975) in 1st Symposium on Calcium Transport in Contraction and Secretion, Bressanone, 11-16 May 1975, North-Holland, Amsterdam, in press.
7. Capony, J.P. and Pechère, J.F. (1973) Eur. J. Biochem. 32, 88-96.
8. Capony, J.P., Rydèn, L., Demaille, J. and Pechère, J.F. (1973) Eur. J. Biochem. 32, 97-108.
9. Pechère, J.F., Demaille, J. and Capony, J.P. (1971) Biochim. Biophys. Acta 236, 391-408.
10. Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147-149.
11. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
12. Gray, W.R. (1972) in Methods in Enzymology (Hirs, C.H.W. & Timasheff, S.N. eds), vol. 25, pp 121-138, Academic Press, New York and London.
13. Capony, J.P., Demaille, J., Pina, C. and Pechère, J.F. (1975) Eur. J. Biochem., 56, 215-227.
14. Hermodson, M.A., Ericsson, L.H., Titani, K., Neurath, H. and Walsh, K.A. (1972) Biochemistry, 11, 4493-4502.
15. Crowle, A.J. (1973) Immunodiffusion, 2nd ed., p. 247, Academic Press, New York and London.
16. Levine, L. and Van Vunakis, H. (1967) in Methods in Enzymology (Hirs, C.H.W., ed.) vol. 11, pp. 928-936, Academic Press, New York and London.