THE CALCIUM BINDING PROPERTIES OF A GLYCOPROTEIN ISOLATED FROM PRE-OSSEOUS CARTILAGE

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Summary: The Ca^{++} binding properties of a glycoprotein purified from pre-osseous cartilage have been investigated. This protein of M.W. 200,000 has two classes of Ca^{++} binding sites, with dissociation constants of 10^{-7}M and 10^{-4}M respectively. About 10 nmoles of Ca^{++} may be bound per mg of protein at the high and 3,000 at the low affinity sites. The Ca^{++} binding ability is pH dependent and it is maximum at pH 8.3. Lanthanum and butacaine have no inhibitory effect on Ca^{++} binding, whereas ruthenium red strongly inhibits both the high and low affinity sites. Possible functions of this glycoprotein in cartilage matrix are discussed.

INTRODUCTION

In order to explain the early mineral depots in calcifying tissues,the existence of a component with high calcium affinity has been postulated by many investigators in the past (for review see 1,2,3). In spite of the intensive investigations along this line, the identification of such a substance is still lacking. During the early phase of calcification in cartilage, " roundish" globules containing small mineral crystallites have been observed by Bonucci (4). Upon demineralization of the specimen, an organic network remains where glycoprotein material has been demonstrated histochemically (4). Glycoprotein with calcium binding properties has been recently isolated from different sources such as liver mitochondria (5-8), serum of Xenopus laevis (9) and byssus of Anomia ephippium L. (10). In a recent paper (11) we have reported the isolation of a calcium containing glycoprotein from pre-osseous cartilage. Since the presence of calcium in this preparation was observed in spite of the extraction procedure from the tissues (4M guanidinium chloride,dialysis, 8M urea treatment) and persisted even after gel electrophoresis using an anionic system (12), a strong affinity for the ion has been suspected.

In the present paper we report the results of a study upon the calcium binding properties of this glycoprotein isolated both from resting and ossifying regions of calf scapula cartilage.

MATERIALS AND METHODS

Glycoproteins were purified from Protein Polysaccharide Complex (PPC) extracts of the resting and ossifying regions of calf scapula cartilage as described in detail elsewhere (11). The material extracted according to Sajdera and Hascall (13) was subjected, in 0.4M guanidinium chloride and 8M urea, to 3.5% polyacrylamide gel electrophoresis and a single peak was collected after 5-7 hours of continuous recording of absorbance at 280 nm (11). This fraction was used for the different analysis.

Analytical disc electrophoresis on polyacrylamide has been carried out using a standard apparatus with the anionic buffer system described by Davis (12). Staining of the gels was performed as previously reported (11). The molecular weight of the isolated glycoproteins has been determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) according to Weber and Osborn (14) using for comparison suitable standard proteins supplied by Boehringer.

Calcium binding ability has been investigated following the technique described by Colowick and Womack(15). The dialysis system consisted of an upper chamber with a final volume of 2ml, separated from the lower chamber of 0.6 ml by a dialysis membrane (Kalle, Wiesbaden-Biebrich). The flow rate through the lower chamber was adjusted to 1 ml per minute. The reaction mixture contained, in 2 ml, 200-500 µg of protein, 10 µmoles of tris-glycine buffer pH 8.3 and 1.5 nmoles of $^{45}Ca^{++}$. Isotope counting was performed with a Philips end-window counter.

In the experiments of Ca^{++} binding at different pH values, tris-glycine buffer was adjusted at the electrode by adding a few microliters of 0.2M HCl or NaOH; pH was checked also at the end of the dialysis experiments. Experiments with inhibitors of Ca^{++} binding were performed after pre-incubation, at room temperature for 3 hours, of the glycoprotein in 5mM tris-glycine buffer pH 8.3 in the presence of Lanthanum chloride, butacaine or ruthenium red. Protein was estimated by the biuret reaction according to Gornall et al. (16).

RESULTS

The degree of homogeneity of glycoproteins extracted and purified from

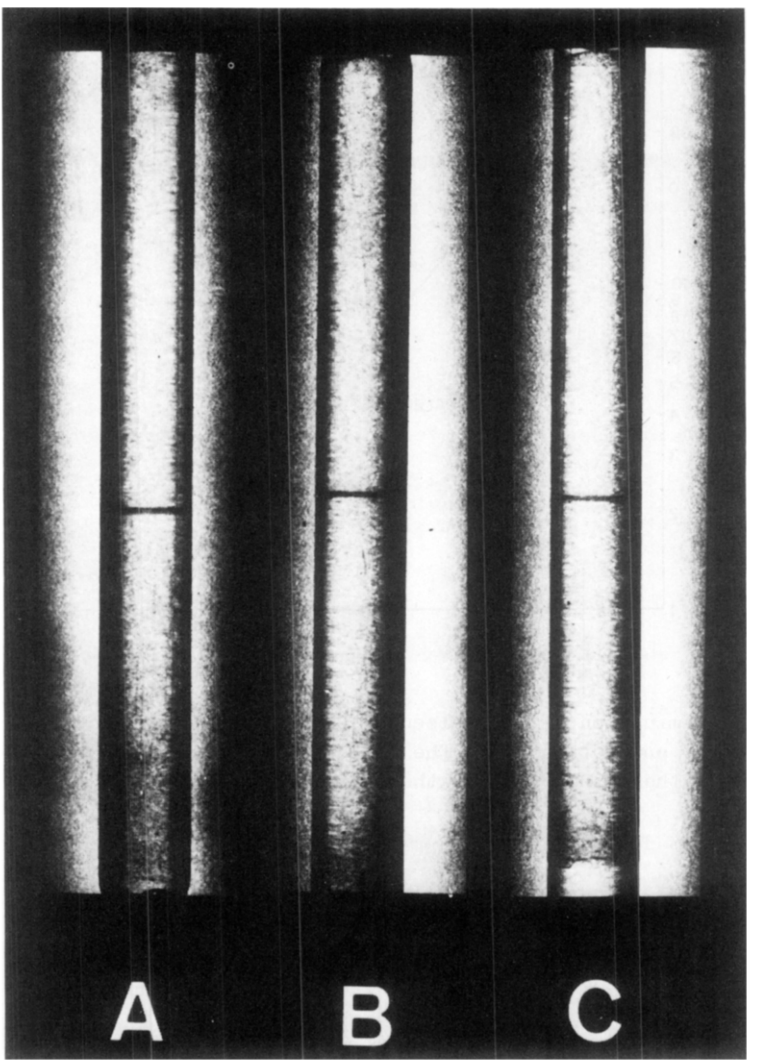

Fig. 1 - Disc electrophoresis of GPO (A), GPR (B) and combined GPO and GPR (C)
Twenty µg of protein were loaded on a 7% polyacrylamide gel and electrophoresis was performed for 45 min at 5 mA/gel.
Gels were stained in 0.01% toluidine blue, 0.1M acetate buffer pH 4.5 and destained by prolongued immersion in the same buffer.
The direction of migration was from top to the bottom of the figure.

resting and ossifying zones of calf scapula cartilage was assessed by disc electrophoresis in 7% polyacrylamide gel with and without 8M urea. A single band was obtained both with the material prepared from the resting zone of cartilage (GPR) and with that from the ossifying one (GPO). The bands, stained for protein with Coomassie blue, showed the same electrophoretic mo-

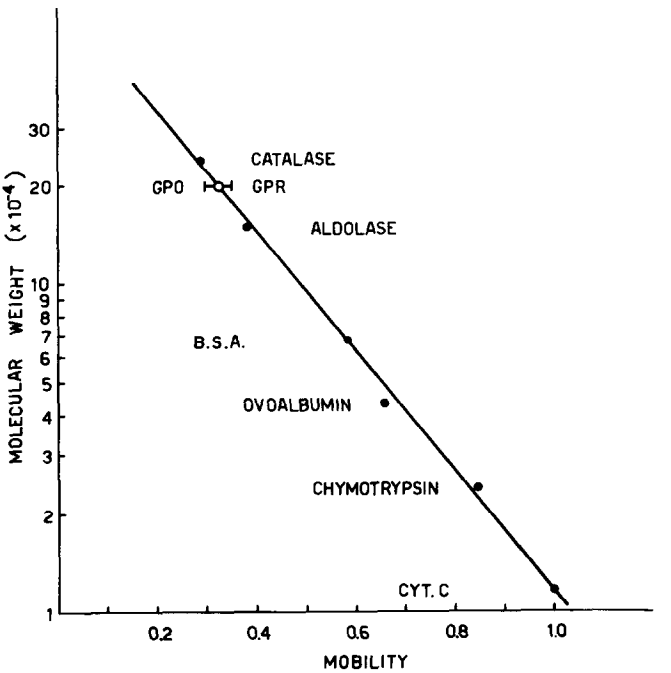

Fig. 2 - Determination of the molecular weight of GPO and GPR according to Weber and Osborn (14). The molecular weights of marker proteins were those indicated by the supplier (Boehringer).

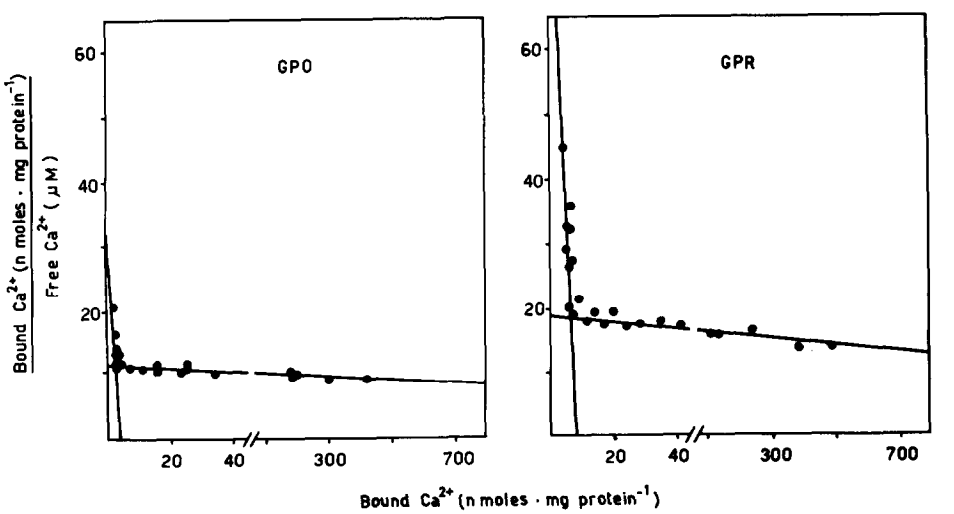

Fig. 3 - Scatchard plots of Ca^{++} binding to GPO and GPR. Data were obtained from at least five experiments.

bility. The same bands gave a positive metachromatic reaction with toluidine blue at pH 4.5 (Figure 1, a and b) and reacted also with alcian blue and with the periodic acid-Schiff reagent. The fact that the glycoproteins derived from the two zones of cartilage have the same electrophoretic mobility and staining properties points to a strict similarity, if not identity of them. A further piece of evidence in favour of identity was obtained by loading the analytical gel with a mixture of the two glycoproteins: again a single band was obtained (Figure 1, c). It was then predictable that also the molecular weight of the two glycoproteins was similar. This was determined according to Weber and Osborn (14) by polyacrylamide gel electrophoresis, in the presence of 1% SDS and, within the limits of this technique (17), a molecular weight of approximately 200,000 Daltons for either species was derived. (Figure 2).

Molecular weight, electrophoretic mobility and staining properties seem then to indicate that the same glycoprotein is embedded in the matrix of the two zones of cartilage.

It was therefore interesting to compare the Ca^{++} binding properties of the two glycoproteins; this is illustrated in Scatchard plots, (18,19) reported in Figure 3. Both preparations display two classes of Ca^{++} binding sites, one with low and one with high affinity. In both cases the values of the two dissociation constants are of the order of 10^{-4} and 10^{-7} M respectively. The high affinity portion of the curve may be extrapolated to approximately 4 nmoles of Ca^{++} bound per mg of protein for GPO and to approximately 8 nmoles of Ca^{++} per mg of protein for GPR. Since the molecular weight of the glycoproteins has been determined to be 200,000, it follows that 1 mole binds approximately 2 moles of Ca^{++} with high affinity, assuming an extrapolated value of 10 nmoles of high affinity Ca^{++} bound per mg protein. The difference in the number of high affinity sites between the two glycoproteins may not be the expression of an intrinsic difference in the molecular structure. The fact that the glycoprotein from the ossifying region has approximately half the number of sites that the other glycoprotein may only indicate that a portion of the sites are already loaded with endogenous Ca^{++} or with other ions of similar or higher affinity. As a matter of fact a higher amount of

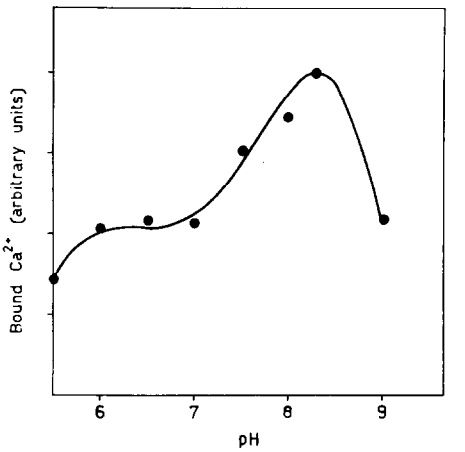

Fig. 4 - Effect of pH on binding of Ca^{++} by GPO. Each point represents the mean of two experiments. Each experiment was done with about 400 µg of GPO in a 2 ml system containing 10 µmoles of tris-glycine at the various pH values.
Ordinate: Total Ca^{++} bound to the high and low affinity sites.

Ca^{++} has been found in the calcifying zone of cartilage than in the resting region (20). The total number of low affinity binding sites can be extrapolated, in both cases, to about 3,000 nmoles of Ca^{++} bound per mg of protein. The studies of Ca^{++} binding ability were carried out at pH 8.3, the pH of the buffer of the preparative electrophoresis. The Ca^{++} affinity was found to be optimal at this pH, whereas it decreases at higher or lower pH values. The pH dependence was particularly evident for the low affinity sites. The pattern of Ca^{++} affinity as a function of the pH is illustrated by Figure 4. The pH dependence seems to be a common feature of Ca^{++} binding proteins since it has been reported also by Sottocasa et al. (21) for mitochondrial glycoprotein, by Molinari-Tosatti et al. (22) for elastin, by Fuchs et al. (23) for troponin and by MacLennan and Wong (24) for calcium-sequestering protein from sarcoplasmic reticulum. The presence of ionized groups in the protein or in the glucidic moiety of the molecule may be responsible of this pH sensitivity of Ca^{++} binding. Aminoacid and sugar analyses of the purified glycoprotein is now in progress.

Table I shows the effect of some chemicals which are known inhibitors of

EFFECT OF INHIBITORS ON HIGH AND LOW AFFINITY Ca^{2+} BINDING

Inhibitor	% Inhibition	
	High affinity	Low affinity
La^{3+} (10 μM)	0	7.3
Butacaine (100 μM)	0	12.3
Ruthenium red (25 μM)	63.8	63.8

Ca^{++} binding for other glycoproteins (7,25). Lanthanum is an inhibitor of neither the high nor of the low affinity sites,at least at the concentration used in these experiments. Ten μM Lanthanum has been found to be a good inhibitor of the mitochondrial Ca^{++} binding glycoprotein recently isolated by Sottocasa et al. (25). This glycoprotein was also sensitive to butacaine which is not the case with the cartilage protein. As already reported for the mitochondrial glycoprotein (25) ruthenium red, on the contrary, significantly inhibits both classes of sites. Ruthenium red is considered a specific dye for glycosaminoglycans (26). Its inhibitory effect, therefore, suggests the involvement of carbohydrate moiety of the molecule in the binding of Ca^{++}.

DISCUSSION

In a previous paper (11) we have reported the isolation of a glycoprotein from the protein polysaccharide complexes (PPC) extracted from the resting and transforming-ossifying regions of calf scapula cartilage. A function was soon identified for this protein, i.e. the ability to facilitate the aggregation of proteoglycan subunits, which are also part of PPC (11). This interesting relationship between glycoprotein and proteoglycan subunits was first described by Hascall and Sajdera (27) in the non ossifying nasal cartilage and thus may be a general feature of cartilage matrix. Furthermore this inte-

raction seems to be relevant to the kinetics of collagen fibril formation (28). But besides this important role in the formation and control of the architecture of cartilage matrix, a high Ca^{++} binding ability is now to be ascribed to this glycoprotein on the basis of our data.

Interactions of proteoglycans with Ca^{++} were recently studied also in other Laboratories (29,30) in the attempt to identify a component of the matrix with high and specific Ca^{++} binding ability. The extraction procedure used by these authors is not the dissociative technique reported by Sajdera and Hascall and used by us. A comparison is therefore difficult between their and our data. The dissociative procedure has allowed us to obtain a solubilized fraction which was purified to the stage of a single molecular species as assessed by disc gel electrophoresis. Thus for the first time a well defined component of cartilage matrix is available for calcium binding measurements. From the data reported by Smith and Lindenbaum (30) it appears that the PPL 6 fraction may perhaps be compared to our glycoprotein. Their preparation in fact shows the highest Ca^{++} affinity among the subfractions obtained from nasal and scapula cartilage. The dissociation constant for Ca^{++} binding to our glycoprotein is of the order of 10^{-7} M, which is not an unusual value for ligand binding to binding proteins. As compared to other Ca^{++} binding proteins (5-10,22-25,31), however, this glycoprotein has an unusually high molecular weight. Indeed this glycoprotein has the ability to promote aggregation and to form larger polymers by itself. On the other hand the failure of SDS or 8M urea in lowering the molecular weight seems to exclude simple aggregation phenomena.

At present we do not yet know whether this Calcium-binding protein is an unique feature of ossifying cartilage or it is a common component of all cartilagenous tissue. Smith and Lindenbaum have found that protein-polysaccharide fractions from resting and ossifying scapula cartilage have a greater affinity for Ca^{++}, than those from nasal septum (30). On the other hand the function of this glycoprotein in the two types of cartilage is possibly not only determined by the Ca^{++} affinity but also by its degree of polymerization and by its relationship with the other components of cartilage matrix. These are the aspects to be considered in the studies now currently in progress in our Laboratory.

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