ALKALINE PHOSPHATASE ACTIVITY ASSOCIATED TO A CALCIUM BINDING GLYCOPROTEIN FROM CALF SCAPULA CARTILAGE

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1. Introduction

The macromolecular organisation of cartilage matrix represents a challenging problem and its solution will provide useful information to understand both the physiology of the tissue and the mechanism of its calcification. One of the macromolecular components of this matrix, a glycoprotein, was recently isolated in our laboratory [1,2] from the calcifying cartilage of calf scapula. Electrophoretically homogeneous, the preparation is endowed with some interesting features. It can easily undergo self aggregation and promotes aggregation of the proteoglycan subunits, acting as the glycoprotein-link described by Hascall and Sajdera [3]. The most important feature of our glycoprotein, however, is the high calcium binding affinity ($K_D = 10^{-7}$M), which in a tissue to be calcified is of particular relevance. An interesting fact in regard to this is that glycoprotein material has been histochemically identified in the Bonucci globules of calcifying cartilage [4], where the very early depots of apatite cristallites have been recognized by electron microscopy. These structures have been found also by other investigators [5,6]. They have been recently isolated and analyzed for their catalytic functions by Ali et al. [7]. One of the enzymic activities bound to the globules, is alkaline phosphatase [7]. This enzyme was considered by Robison [8], to play an essential role in calcification. Since Robison's time many alkaline phosphatases have been extracted and purified (for a review see [9,10]) and for many of them a glycoprotein nature has been established [10]. It appears logical, therefore, our interest in studying the catalytic function of our glycoprotein preparation, alkaline phosphatase activity 'in primis'. In this communication we report preliminary results concerning some properties of the alkaline phosphatase activity (EC 3.1.3.1) exhibited by the calcium-binding glycoprotein isolated from scapula cartilage.

2. Experimental

For this study we have used the glycoprotein isolated from the resting region of calf scapula cartilage purified as previously reported to the stage of electrophoretic homogeneity [2]. Enzymic activity was measured according to Bessey et al. [11] using *p*-nitrophenylphosphate as substrate (Sigma, USA). Release of *p*-nitrophenol was followed by measurement of absorbance at 410 nm in a temperature regulated recording-spectrophotometer at 37°C. Specific activity is expressed as μmoles of *p*-nitrophenol released $min^{-1}mg^{-1}$ of protein. Protein concentration was determined according to Schacterle and Pollack [12].

3. Results and discussion

The influence of pH upon the enzymatic activity is illustrated by fig. 1. As for other alkaline phosphatases [9,10], the optimum pH is in the high alkaline regions. The maximal activity was found around pH 10, both in glycine–NaOH and carbonate–bicarbonate buffer (not shown). The enhancement of the activity at this pH, however, is not particularly pronounced as compared to lower more physiological values, both in the absence or in the presence of ions. Howell et al. [13] have shown that the extracellular fluid of epiphyseal cartilage has pH 7.8. Thus from

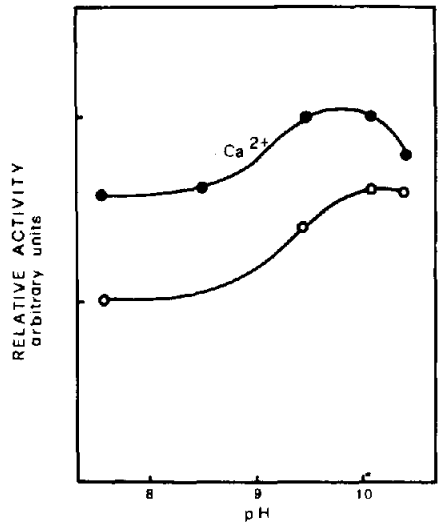

Fig. 1. Effect of pH on phosphatase activity of cartilage glycoprotein. The reaction mixture was formed by 45 mM glycine–NaOH buffer, 4.55 mM *p*-nitrophenylphosphate, 9.1 mM $CaCl_2$. Reaction was started by the addition of the enzymatic protein (62 μg/ml of the medium). (o-o-o) enzymatic activities without Ca^{2+} addition. The activity values at different pH are expressed as percentage of the activity measured at pH 10 in the presence of Ca^{2+} (= 100%)

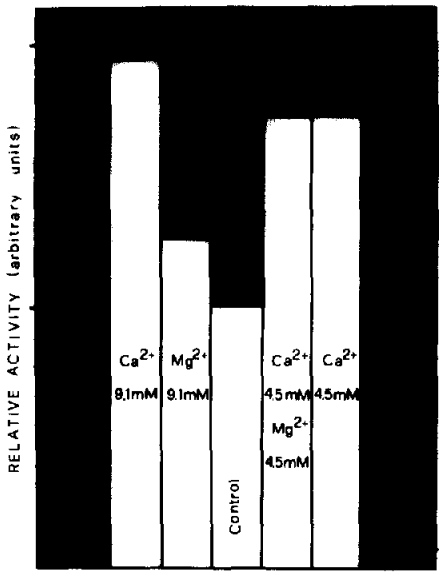

Fig. 2. Influence of Ca^{2+} and Mg^{2+} on alkaline phosphatase activity of the glycoprotein. Assay conditions: as indicated in fig. 1.

our graph a good activity of alkaline phosphatase appears possible, not considering of course the influence of the type of substrate and of its concentration. From a Lineweaver plot we have calculated a K_M for *p*-nitrophenylphosphate of 5.9×10^{-4}M, a value consistant with that of other phosphatases [14].

Different specific activity values are reported in literature for various purified alkaline phosphatases. Our best glycoprotein preparations show a specific activity of 1920 U mg^{-1} of protein, using the enzymatic unit reported by Dabich and Neuhaus [14]. These investigators have purified a phosphatase from bovine synovial fluid 2286-fold, obtaining a specific activity of 16 000 U mg^{-1} of protein.

One of the cardinal features of alkaline phosphatases is their activation by divalent metal cations [10]. As illustrated in fig. 2 the activity of our preparation is slightly enhanced by Mg^{2+}. Also in calcifying hamster molars [15] alkaline phosphatase activity is not stimulated by this cation. On the contrary, a marked activation is produced by equimolar additions of Ca^{2+}. This effect is not influenced by Mg^{2+} added at the same concentration of calcium. Interestingly Mg^{2+} is a poor competitor also for Ca^{2+} binding to this glycoprotein [16,17]. The reciprocal of the reaction velocity is a linear function of the reciprocal of Ca^{2+} concentration in the range of concentrations plotted in fig. 3. Another characteristic of alkaline phosphatases is the sensitivity to some inhibitors [10].

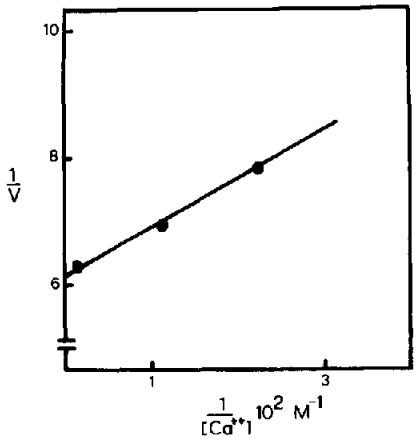

Fig. 3. Effect of Ca^{2+} concentration on the alkaline phosphatase activity. Assay conditions as in fig. 1.

Table 1
Inhibitory effect of some chemicals on the phosphatase activity of the glycoprotein preparation.

Compounds	Percent of inhibition
EDTA (9.1 mM)[a]	100
KCN (10 mM)	50
PO_4^{3-} (4.5 mM)[b]	50
L-Phenylalanine (5 mM)[c]	8.6

Assay conditions as in fig. 1, in the presence of 9.1 mM Ca^{2+}, with the following modification: [a]Glycoprotein was preincubated with EDTA for 30 min before addition to the assay mixture. [b] Ca^{2+} was omitted in the reaction mixture. [c] The buffer was 50 mM carbonate–bicarbonate.

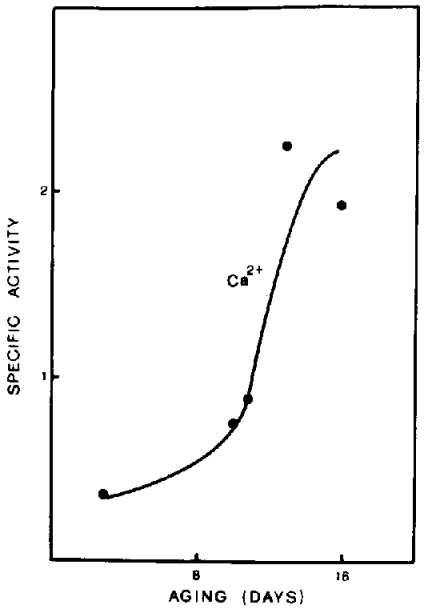

Fig. 4. Effect of aging upon the phosphatase activity of the glycoprotein. A preparation containing 0.22 mg protein/ml was aged at 4°C. Enzymatic activity was determined as indicated in fig. 1: Aliquots of the solution were used, containing 88 μg of protein.

As with other phosphatases, chelating agents, such as EDTA or cyanide, strongly depress the activity as indicated in table 1. A good inhibition is also demonstrated by inorganic phosphate, one of the reaction products, as reported in literature for this enzyme [18]. L-Phenylalanine is considered an organo-specific and stereo-specific inhibitor of the intestine alkaline phosphatase [19]. On the contrary, phosphatases of tissue different from intestine, are insensitive to this L-amino acid [20]. The enzymatic activity of our preparation also is not influenced by 5 mM L-phenylalanine. The informations thus far collected seem then to indicate that the catalytic activity of our glycoprotein preparation may be referred to as a typical alkaline phosphatase (EC 3.1.3.1) activity, with the special feature of being activated by Ca^{2+} and insensitive to Mg^{2+}. Further work is designed in order to better define the natural substrate, considering the fact that the enzyme plays its role in the matrix of a calcifying cartilage. ATP and pyrophosphate are particularly interesting in this regard. There is, in fact, some evidence that Ca^{2+}-activated ATPase and alkaline phosphatase might be the same enzyme [21]. The function of splitting pyrophosphate has been also ascribed to alkaline phosphatases [22]. Biochemical analysis of isolated Bonucci vescicles has demostrated the presence of alkaline phosphatase, ATPase and pyrophosphatase activities [7]. These catalytic functions might be ascribed to the same enzyme. There is an appealing coincidence of biochemical finding in the Bonucci globules and our glycoprotein preparation, as if the latter material is presumably an essential part of the former. This hypothesis is sustained also by the very recent observation by Ali and Evans [23] that isolated vescicles can form hydroxyapatite under alkaline conditions in the presence of Ca^{2+} and ATP. Both preparations therefore, cartilage vesicles and our glycoprotein preparation, show alkaline phosphatase activity and Ca^{2+} binding capability.

The fundamental question as to whether we are dealing with an enzyme associated with the glycoprotein preparation or with a catalytic function of the glycoprotein itself, remains still open. Nevertheless there is a fact which, besides the homogeneity of the preparation, seems highly interesting in this regard. We have previously reported that the calcium affinity of glycoprotein increases upon aging of its solutions in the cold [16]. This increment has now been found to be paralelled by an enhancement of phosphatase activity, as illustrated in fig. 4. As reported [16,17] aging is accompanied by the association of the material into aggregates: apparently this event, which may occur also in vivo, increases both Ca^{2+} binding and phosphatase activity. Where the two functions, Ca^{2+} binding and phosphatase activity, exerted by two

different proteins, these should possess a strong tendency to associate. In cartilage matrix this association would produce a useful cooperation in the calcification process: calcium trapping, from one side, and phosphate ions availability, from the other; both events occurring in a microenvironment.

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