Alkaline phosphatase binds to collagen; a hypothesis on the mechanism of extravesicular mineralization in epiphyseal cartilage[1]

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Summary. Affinity chromatography on Sepharose 4B-collagen gels was used to test the affinity of alkaline phosphatase for collagen. Results indicate that 1) alkaline phosphatase of preosseous cartilage binds to collagen probably by electrostatic interactions, 2) this interaction is inhibited by proteoglycan subunits. These results suggest that, in vivo, the formation of a collagen-alkaline phosphatase complex may be a step of the process leading to cartilage calcification.

It is widely accepted that alkaline phosphatase is involved in the process of calcification of various mineralizing tissues[3]. Its activity has been shown to increase during the process of transformation of connective tissues to cartilage induced by a matrix of demineralized bone[4]. Unfortunately the role played by the enzyme is still not clear, although its presence in the matrix vesicles (MV), where early mineral formation is described, suggests that it may function for calcium and/or phosphate fixation. However, since minerals are formed also outside matrix vesicles, if the enzyme is connected with the process it also has to be present outside matrix vesicles in order to diffuse to collagen, where the highest amount of hydroxyapatite is formed in the latest stages of calcification.

Two lines of investigation have therefore been set up to study whether the properties of the enzyme are compatible with the presumed double location, and evidence has been obtained that a portion of the enzyme may diffuse from MV in the surrounding area since it is removable from isolated MV simply by washing them with isotonic KCl[5].

The aim of this work is to study the interaction of alkaline phosphatase, purified from epiphyseal cartilage, with type II collagen, extracted from the same tissue, and the influence of proteoglycans on this interaction. Results indicate that collagen binds alkaline phosphatase by electrostatic forces and that proteoglycans interfere with the binding.

Materials and methods. Alkaline phosphatase (E.C.3.1.3.1.), collagen and proteoglycans were purified from preosseous cartilage of calf scapulas excised from the animals at the slaughterhouse and immediately transferred in ice to the laboratory. Alkaline phosphatase was extracted from matrix vesicles with deoxycholate-butanol according to Hsu et al.[6]. MV were isolated from the ossifying region of the cartilage by following the procedure described by Ali et al.[7]. Matrix vesicles were first washed with 0.15 M KCl at 4 °C for 30 min, spun down in a Spinco SW 50.1 rotor (50,000 rpm for 20 min) and then exposed to the deoxycholate-butanol extraction.

Collagen and proteoglycans were extracted from the zone of resting cartilage. By using a freezing microtome, 200-nm-thick slices were prepared which were then lyophilized and stored at − 20 °C until used.

Collagen was prepared by papain digestion of this material according to the procedure of Lee-Own and Anderson[8].

Proteoglycans were obtained by treating cartilage slices with 4 M guanidinium chloride in 50 mM Tris-HCl buffer pH 7.4, in the presence of the usual mixture of protease inhibitors (100 mM ε-aminohexanoic acid, 5 mM benzamidine chloride, 10 mM sodium EDTA, mM sodium iodoacetate and mM phenylmethyl-sulphonyl fluoride). Proteoglycan subunits were purified by CsCl density gradient centrifugation in dissociative conditions, according to the one step procedure of Sajdera and Hascall (PGS – D_1)[9].

Collagen was coupled to CNBr-Sepharose 4B (Pharmacia) according to Bauer et al.[10]. In these conditions, 1.6 mg of collagen was bound per 1 ml of wet gel, as indicated by the amount of hydroxyproline on the hydrolyzed resin, determined with the method of Jamall et al.[11]. 10–15 ml columns, containing Sepharose 4B or Sepharose 4B-collagen equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $CaCl_2$, were loaded with 0.2–0.4 ml of PGS (3.75 mg·ml^{-1}) and 1 ml of alkaline phosphatase dissolved in the same buffer (100 μg prot·ml^{-1}). Columns were washed with the Tris buffer and eluted with the same buffer containing 1M NaCl, at the constant rate of 6 ml/h, at room temperature. Fractions of 0.5–0.7 ml were collected and analyzed for their content of uronate and phosphatase activity, according to the method of Bitter and Muir[12] and of Stagni et al.[13], respectively.

Results. Alkaline phosphatase and proteoglycan subunits (PGS) extracted from epiphyseal cartilage were chromatographed on Sepharose 4B and on Sepharose 4B-collagen. The elution profiles are clearly different, as illustrated by figure 1. With the Sepharose 4B-collagen procedure a true affinity chromatography is performed, both the enzyme and PGS being removed from the column after the addition of 1 M NaCl to the eluting buffer. Preliminary experiments, however, showed that 0.3 M NaCl was sufficient to eluate both the phosphatase and proteoglycans from the column. With the simple Sepharose 4B chromatography (upper portion of fig. 1) the 2 cartilage components are eluted by the Tris buffer around the void volume.

With the aim of assessing whether proteoglycans may influence

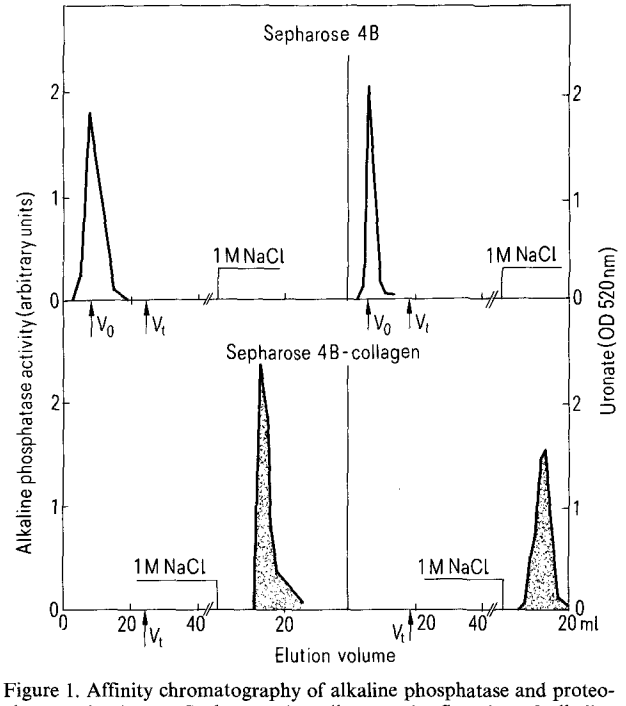

Figure 1. Affinity chromatography of alkaline phosphatase and proteoglycan subunits on Sepharose 4B-collagen gels. Samples of alkaline phosphatase and proteoglycan subunits were chromatographed on columns of identical size containing Sepharose 4B (upper panels) or Sepharose 4B-collagen (lower panels). Left panels: alkaline phosphatase elution profiles. Right panels: proteoglycan elution profiles. Elution was performed as described in 'Materials and methods'.

the binding of the phosphatase to collagen, a different set of experiments was performed. Two identical columns of Sepharose 4B-collagen were prepared (V_t = 10 ml), but only 1 was preloaded 3 times, each time with 3.75 mg of PGS, in 1 ml. The column was then washed with Tris-HCl buffer till no uronate was detectable in the eluate. Both columns were then loaded with the same amount of alkaline phosphatase (100 µg protein), washed with the buffer and then eluted with 1 M NaCl. The elution profiles are reported in figure 2. From the Sepharose 4B-collagen column (upper graph) only a small percentage of the total enzyme activity is recovered before the addition of 1 M NaCl (27 vs 73%). This amount of the enzyme activity represents the excess of loading in respect of the capacity of the column. On the contrary, from the column previously loaded with PGS, the elution profiles shows that 64% of the total enzyme activity is eluted with the buffer and only 34% is desorbed by 1 M NaCl. No trace of uronate was found in the eluate after addition of the enzyme.

Experiments were also made to assess whether calcium may influence collagen-alkaline phosphatase interaction. The elution profiles were not modified by the absence of 5 mM $CaCl_2$ in the elution buffer (data not shown).

In the experiments described above (n = 15) recovery of alkaline phosphatase was 85 ± 8% and the recovery of the uronate for PGS was 85 ± 6%.

Discussion. The ability of collagen to interact with macromolecules such as proteoglycans, glycosaminoglycans, fibronectin, chondronectin, osteonectin, laminin is well documented[14–16]. Various enzymes are also reported to bind in vitro to collagen, e.g. collagenase[10], collagen-glycosyl-transferase[17]. As far as we know this is the first evidence that alkaline phosphatase also has the property of binding to collagen. The fact that by addition of NaCl the enzyme is desorbed suggests that the binding may be due to ionic forces, which appear to sustain a strong bond. It is known that proteoglycans bind to collagen by ionic interactions with the core protein of PGS[18]. The strength of binding is similar to that observed for the phosphatase, an acidic glycoprotein[19]. The fact that PGS and alkaline phosphatase may compete for the same site(s) of collagen, would indicate that in vivo the enzyme would bind to the fibrous protein more easily if proteoglycans were not present. It is relevant in this regard to mention that during the process of calcification of cartilage a significant decrement of proteoglycans is reported[20,21]. Dissolution of matrix vesicles is also observed; remnants of vesicles such as proteolipids and enzymes may spread in the surrounding area[22].

In conclusion the finding that, in cartilage, collagen binds alkaline phosphatase provides a new insight into the mechanism of calcification outside matrix vesicles. By forming a complex with collagen, alkaline phosphatase may promote the formation of ion clusters by reaction of the phosphorylated enzyme with calcium ions of the interstitial fluid.

Indeed, cartilage alkaline phosphatase does show calcium binding properties[3]; by suspending small mineral particles, the enzyme acts as a nucleator in matrix vesicles and permits the circulation of a preformed mineral phase from sites of initial deposition into hardly accessible 'holes' of matrix collagen[3]. Collagen, regularly arranged, would provide the insoluble organic support for crystal growth and orientation.

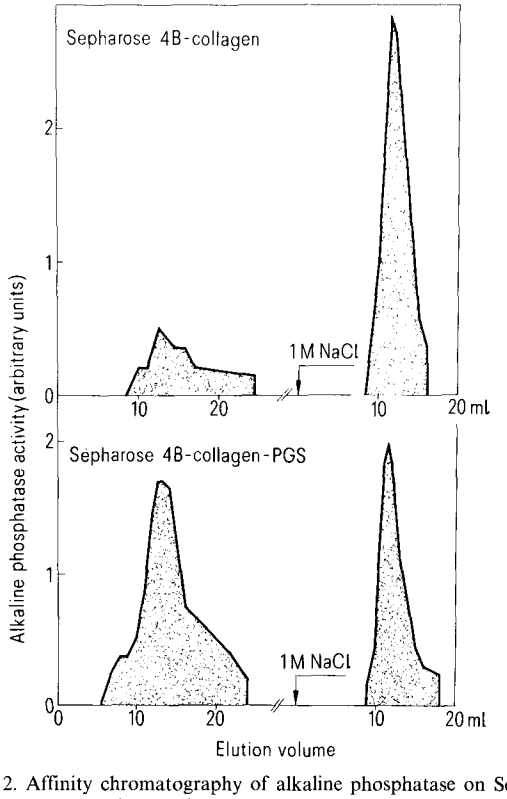

Figure 2. Affinity chromatography of alkaline phosphatase on Sepharose 4B-collagen (top) or Sepharose 4B-collagen partially saturated with proteoglycan subunits (PGS) (bottom). Conditions are as described in 'Materials and methods'.

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