Activation and specificity of alkaline phosphatase of a mineralizing collagen-rich system

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Dedicated to Prof. Dr. G. Pfefferkorn on the occasion of his 65th birthday.

Summary. Alkaline phosphatase from tibia tendon of *Meleagris gallopavo* L. was highly purified. The enzyme activation by different ions was measured. Mg^{2+} showed a high activation with a broader spectrum of phosphomonoester hydrolization. The in vivo Mg^{2+} concentration was an optimum for in vitro activation.

Alkaline phosphatase (APase) EC 3.1.3.1. is a characteristic enzyme in the process of biomineralization, but its real function is not known. Topochemical analyses of naturally mineralizing turkey tibia tendons have shown that this collagen-rich mineralizing system is an excellent model to study hard tissue formation[2]. Mineralization of turkey tibia tendon starts at about 11 weeks, and is always connected with an increase in APase activity. In the proximal front region of the mineralizing tendon, a peak of APase activity occurs. In just the same area, the concentration of magnesium increases. Though it is generally assumed that Mg^{2+} plays an important role in hard tissue formation, its real function with regard to APase activity is not exactly known[3]. Thus the main topics of our experiments were to find out whether there is a characteristic relationship between the APase activity and divalent ions.

Materials and methods. APase had been purified with improved methods. 13-17 weeks old tendons were deep-frozen immediately after dissection. The mineralizing areas of the tendons were sliced and put into buffered 0.2 M NaCl-solution. After ultrasonic treatment and centrifugation the supernatant was concentrated by ultrafiltration. By using ion exchange chromatography, DEAE-cellulose (Whatman), and molecular sieve chromatography, Sephadex G-100 (Pharmacia), we obtained a purified enzyme fraction. SDS-gel electrophoresis[4] indicated a mol. wt of about 38,000 daltons.

During the purification procedure, APase activity was measured against p-nitrophenylphosphate (pNPP) as substrate. Adenosine-3'-monophosphate (3'-AMP), adenosine-5'-monophosphate (5'-AMP), adenosine-5'-diphosphate (ADP), adenosine-5'-triphosphate (ATP) and pNPP were products of Boehringer, Mannheim. Protein was determined by the method of Lowry[5]. The hydrolytic activity of APase against the nucleotides was measured by high-voltage paper electrophoresis.

Results and discussion. The activation of APase by divalent ions was studied. Magnesium, calcium, sodium and potassium were chosen for these experiments, since we found that they are enriched in the proximal mineralizing region[2]. Assuming a water content of about 50-60%, the highest elemental content per dry weight for Na, K, Mg and Ca in the mineralizing region would correspond to the following concentrations in mmoles per 1 kg wet wt: 250 Na, 60 K, 80 Mg and, mainly as mineral, 1,200 Ca.

The results of the activation experiments are summarized in figure 1. The presence of Mg^{2+} results in a characteristic activation with a pH optimum of about 10.2. Ca^{2+}, Na^{+} and K^{+} ions activated the enzyme to a less degree. The activation curves for Mn^{2+} and Zn^{2+} ions corresponded to that of Mg^{2+}, but their total effect was less and the pH optimum somewhat lower (pH 7-8).

It is generally assumed that in the epiphyseal plate the initial mineralization is connected with socalled matrix vesicles. In activation experiments on isolated matrix vesicles (a system which most probably contains Ca and Mg itself) Hsu and Anderson[6] observed increasing APase and ATPase activities by the addition of Mg^{2+} and Ca^{2+} respectively.

Figure 2 shows the enzyme activation depending on the Mg^{2+} concentration. Increasing Mg^{2+} content results in an increasing APase activity. The activation curve reaches a maximum when the molar ratio of Mg^{2+}: $ROPO_3^{2-}$ ($ROPO_3^{2-}$ = phosphoric acid monoester) is 1:1.

Furthermore, the hydrolytic activity of APase was measured against 5'-AMP, 3'-AMP, ADP and ATP in the absence as well as in the presence of Mg^{2+} ions. Without Mg^{2+} only 5'-mononucleotides were split instantly; but in the presence of Mg^{2+} even 3'-AMP, ADP and ATP were hydrolized. These results mean that the specificity of APase activity depends on the presence of divalent ions.

While the pH-optimum in the in vitro experiments was observed at pH 10.2 in the presence of Mg^{2+}, we have found pH values between 7.5 and 8.5 in the mineralizing region of the tendon.

It can therefore be deduced from figure 1 that only Mg^{2+} is a potent activator of APase in this pH range.

Wöltgens et al.[7] have extracted 2 enzymatic activities from the molars of 3-day-old hamsters. This extract showed optimal APase activity at pH 10.3 and optimal pyrophosphatase (PP_iase) activity at 8.7. They assumed that these 2 activities were due to the same enzyme. We have found that the APase from turkey tibia tendon, activated by Zn^{2+} and Mn^{2+}, can also split pyrophosphate bonds in the pH range 7-8. Linde et al.[3] have checked crude extracts from odontoblasts, enamel organ and mineralizing cartilage with regard

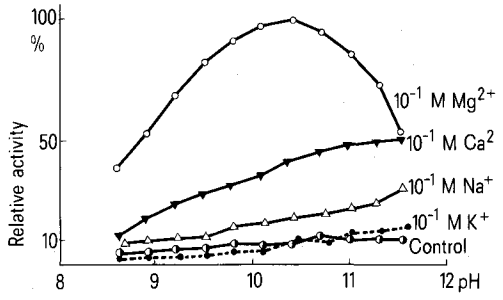

Fig. 1. APase activity depending on pH in the presence of different ions.

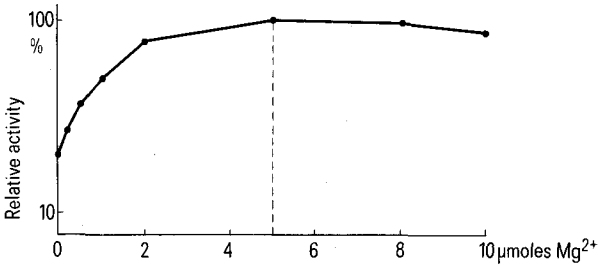

Fig. 2. Mg^{2+} activated APase; substrate concentration 5 µmoles.

to their APase activities. They have found a pH optimum at pH 10.3 for all extracts. A strong Mg^{2+}-activation was also observed.

On the other hand, Felix and Fleisch[8,9] have separated an inorganic pyrophosphatase from an APase by Sephadex gel filtration of a pig scapula cartilage extract. They assume that the PP_iase and the APase are isoenzymes, rather than different enzymes.

Concerning the function of the APase, there is good reason to assume - on the basis of Robison's theory[10] - that this enzyme forms orthophosphate groups resulting in mineral formation. Such an assumption might be supported by our results showing that at least 66% of the total phosphate amount, in the mineralizing region of the turkey tibia tendon as well as in the predentine[11], exists in the form of orthophosphate groups. Considering our results and those of the other authors, one might assume that divalent ions which activate the APase appear, in certain microcompartments in the mineralizing area, to induce the next 'push' of calcification, and so control the mineralization process.

1 We thank Deutsche Forschungsgemeinschaft for support.
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Glycosaminoglycans in separated tubules of the guinea-pig and rat kidney medulla*

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Summary. Isolated tubules of the renal medulla of guinea-pig and rat contained glycosaminoglycans. 20-25% of the uronic acids corresponded to hyaluronic acid. In the guinea-pig, chondroitin and dermatan-sulfates accounted for at least 50% of the uronic acids, whereas, in the rat, heparan sulfates comprised 65-70% of them.

Although glycosaminoglycans have been isolated from kidneys of various mammals[1-4], little is known about their distribution in renal tissues. It has been assumed that they are components of the medullar stroma, even though histochemical data indicated that certain renal tubules also contained glycosaminoglycans[5-7]. The present report deals with the isolation and characterization of these glycoconjugates from the tubules of renal medulla of guinea-pig and rat.

Materials and methods. Separation of tubules. 370 guinea-pigs and 220 rats were bled and perfused through the left ventricle with cold 10 mM sodium phosphate buffer, pH 7.4 and through the abdominal aorta, with 0.05% collagenase in the same buffer[8]. The kidney medulla was excised and incubated for 3 h at room temperature with collagenase[8]. Renal tubule preparations thus obtained were controlled by phase and light microscopes[9] (figure).

Isolation of glycosaminoglycans. The tubule preparations were digested with papain (EC 3.4.4.10) for 6 h at 65 °C in 0.1 M phosphate buffer, pH 6.5[10], treated with cold trichloroacetic acid (10% final concentration) and centrifuged. The supernatants were neutralized with saturated NaOH, concentrated in a rotatory evaporator and run through a Sephadex G-50 column (2.5 × 30 cm) eluting with distilled water. The excluded material was treated with deoxyribonuclease (EC 3.1.4.5) and ribonuclease (EC 2.7.7.17)[11]. 2 volumes of chloroform-methanol (2:1, by volume) were added. Crude preparations obtained by rechromatography on Sephadex G-50, were dried, and fractionated on DEAE-Sephadex A-25 (0.25 × 20 cm) in a cold room[12], eluting with distilled water (Fraction I), 0.1 and 0.5 M NaCl (II-III), 1.0, 1.5 and 2.0 M NaCl containing 0.01 M HCl (IV-VI) and 6.0 M HCl (VII). These fractions were concentrated and salted out by chromatography on Sephadex G-25.

Analytical assays. Uronic acids[13], hexosamines[14,15], sulfates[16,17], sialic acids[18], neutral sugars[19], methylpentoses[20] and proteins[21] were assayed by duplicate.

Monosaccharides were identified by paper chromatography using and ethyl acetate pyridine-water (12:5:4, by volume) system[22], after 4 h hydrolysis[14]. Chromatograms were developed with benzidine or nynhydrin.

DEAE-Sephadex A-25 fractions were electrophoresed on cellulose acetate paper[23,24]. Strips were stained with 1% alcian blue pH 2.5 or pH 1.2 for 30 min, with the PAS procedure[25] or with Coomassie brilliant blue R[26]. After 3 washings with ethanol for 10 min they were cleared with methanol-acetic acid glycerol (85:14:1, by volume).

Results and discussion. The separated renal tubules of both species contained several carbohydrates (figure, table 1). In the rat, there were less uronic acids and hexoses than in the

Table 1. Carbohydrate and sulfate content of separated tubules of renal medulla of guinea-pig and rat

Component	Guinea-pig (5)*			Rat (2)*		
	Homogenate	Crude preparation	%	Homogenate	Crude preparation	%
Uronic acids	-	33± 3	-	-	17± 2	-
Hexosamines	377± 11**	107± 12**	28***	307± 73**	98± 23**	32***
Sulfates	-	21± 7	-	-	10± 1	-
Sialic acids	90± 26	21± 2	23	73± 3	26± 1	35
Hexoses	1810± 320	281± 32	15	1951± 440	50± 8	3
Methylpentoses	330± 110	12± 4	3	-	-	-

* Number of experiments. Each tubule preparation consisted of a pool of kidneys, from not less than 50 animals. All determinations were made in duplicate. ** µg/100 mg dry weight. Values are means ± SE. *** Percent carbohydrate of the crude preparations in respect to the homogenate.