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ALKALINE PHOSPHATASE FROM ADULT RAT FEMUR

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Four alkaline phosphatase forms from adult rat femur were distinguished on polyacrylamide gel electrophoresis: two soluble forms of $M_r$ 165 000 and 110 000 in the water extract, and three membrane-bound forms of $M_r$ 130 000, 110 000 and 100 000 extractable with deoxycholate.

Alkaline phosphatase after SDS-treatment disintegrated into three kinds of monomers: of $M_r$ 80 000, 65 000 and 50 000. The soluble fraction (extract I) contained subunits of $M_r$ 80 000 and 55 000 — whereas the pellet fraction (extract II), subunits of $M_r$ 65 000 and 50 000. Since for native forms only three types of subunits were found it seems that, apart from homodimers, there are also some heterodimers composed of the $M_r$ 65 000 and 50 000 subunits forming the native enzyme of $M_r$ 110 000 — 115 000.

Two denatured monomers: of $M_r$ 80 000 and 50 000 may form two native homodimeric forms of $M_r$ 165 000 and 100 000 while in the pellet two monomers: of $M_r$ 65 000 and 50 000 may correspond to three native alkaline phosphatase forms: of $M_r$ 130 000, 110 000 — 115 000 and 100 000. Probably the $M_r$ 110 000 — 115 000 form is a heterodimer composed of subunits of $M_r$ 65 000 and 50 000.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, AP, EC 3.1.3.1) belong to glycoproteins which catalyse in vitro the hydrolysis of various monophosphate esters.

The heterogeneity of alkaline phosphatase in different animal tissues has been demonstrated by biochemical and immunological methods [1]. Genetic evidence testifies to a three gene loci code for each protein moiety of the three isoenzymes of AP: placental, intestinal and tissue unspecific found in liver, bone and kidney, L-B-K [2]. In addition, different molecular forms of each AP isoenzyme were identified and are assumed to be posttranslational products [3]. The distribution and properties of the placental and intestinal isoenzymes are well established.
Data on the number of the enzyme forms, their molecular weights and subunit structure of the tissue-unspecific L-K-B isoenzymes are limited and contradictory. This especially concerns bone AP since its analysis has been limited only to the characteristics of enzyme activity in various types of bone [4, 5] and to developmental changes [6].

The active centre of AP contains serine, which is known to be essential for phosphate binding [8, 9]. The intermediate formed during catalysis can be demonstrated. Each form of AP consists of two monomers, each of them containing a separate active centre with OH-serine [8, 9]. Both OH groups are phosphorylated during enzymic catalysis, forming a transient intermediate.

The present experiments were undertaken to determine the distribution and subunit structure of AP isoenzymes in crude adult rat femur extracts.

MATERIALS AND METHODS

Reagents. [γ-32P]ATP was purchased from Amersham. All other chemicals were of the highest analytical grade available.

Material. For the experiments male Wistar rats were used. The animals, 5 weeks old, were killed. The part of femur containing the metaphysis, epiphyseal cartilage and epiphysis was removed, freed from bone marrow, washed with distilled water, ground and suspended in cold chloroform-saturated water. The suspension was homogenized for 60 s in an Ultraturrax homogeniser (crude homogenate). Alternatively, the same suspension was left for 24 h extraction, then centrifuged and the sediment reextracted with 0.9% NaCl for another 24 h. Both extracts were combined and centrifuged at 160 000 × g for 1 h (Extract I). This extract containing the soluble fraction of AP was stored at −20°C. The pellet was subsequently extracted with 0.5% deoxycholate (DOC) for 1 h at 4°C and again centrifuged at 160 000 × g for 1 h (Extract II).

Enzyme assay. Total AP activity was measured using p-nitrophenylphosphate (PNPP) as a substrate, according to Bessey et al. [10].

Protein determination. Protein was determined by the method of Bradford [11] using bovine serum albumin as a standard.

Molecular weight. Molecular weight (Mₜ) of proteins was determined by polyacrylamide gel electrophoresis using high and low molecular weight kits (Sigma).

Phosphorylation procedure. Native AP forms in the crude homogenate, extract I and extract II were incubated at 30°C in 100 μl of solution containing: 50 mM Hepes buffer, pH 7.1, 50 mM KCl, 0.125 mM EGTA, 6.25 mM EDTA, 0.5 μCi [γ-32P]ATP (specific activity 3000 Ci/mmol). The reaction was started by addition of the enzymic preparation and stopped after 60 s by addition of 5 μl of ice-cooled 10% trichloroacetic acid containing 50 mM inorganic phosphate and 0.5 mM ATP. The precipitate was washed several times with the above solution and then dissolved in a mixture containing 5% glycerol, 50
mM Tris/HCl buffer, pH 7.8, 20% SDS and 5 mM dithiothreitol. The mixture was heated for 5 min in a boiling water bath and then cooled.

Polyacrylamide gel electrophoresis. Aliquots of native and phosphorylated, SDS-denatured material were loaded on 8.5% polyacrylamide gel with or without SDS [12]. Electrophoresis was carried out at 60 V for 3-4 h, \( \gamma^{32}P \)-phosphorylated AP was detected on the gel by autoradiography using X-OMat Kodak films. Native AP were stained for the activity with \( \alpha \)-naphthylphosphate.

RESULTS AND DISCUSSION

The results of polyacrylamide gel electrophoresis of AP are shown in Fig. 1. The crude homogenate (lane 1) contains several bands of AP not well separated. The calibration of polyacrylamide gel electrophoregrams with

![Fig. 1. Alkaline phosphatase activity in adult rat femur. Polyacrylamide gel electrophoresis. 50 µg of protein. Activity detected on the gel with \( \alpha \)-naphthylphosphate as substrate. 1, Crude homogenate; 2, extract I; 3, extract II](image)
standard protein markers yielded four bands of $M_r$ 165000, 130000, 110000 — 115000 and 100000, respectively. The protein band of 110000 — 115000 showed the highest AP activity. In extract I (lane 2), only two bands of $M_r$ 110000 — 115000 and of 165000 were found, both present in crude homogenate. In extract II (lane 3) three bands of AP of $M_r$ 100000, 110000 — 115000 and 130000 were present. Phosphorylated monomers detected on gel by autoradiography indicate clearly that alkaline phosphatase of rat femur, forms with ATP as substrate enzyme-substrate (Pi) intermediates with each subunit (Fig. 2). As previously, none of the proteins of AP extracts, except AP apoprotein, was phosphorylated under the experimental conditions used [13]. No phosphorylated proteins were found on autoradiograms of native or SDS-treated samples on polyacrylamide gels when phosphorylation

Fig. 2. Phosphorylated monomers of alkaline phosphatase from adult rat femur. Autoradiograms of monomers of alkaline phosphatase phosphorylated by $[^{32}\text{P}]$ATP separated on polyacrylamide gel. 1, Crude homogenate; 2, extract I; 3, extract II
was carried out in the presence of AP competitive inhibitors: \( \beta \)-glycerophosphate or inorganic phosphate [14]. Therefore this procedure enables investigation of both AP forms and subunit composition in crude extracts.

As shown in Fig. 2, in crude extracts (lane 1) three bands, corresponding to the proteins of \( M_r \) 80,000, 65,000 and 50,000 were present. In extract I (lane 2) two radioactive bands of \( M_r \) 80,000 and 55,000 whereas in extract II two bands of \( M_r \) 65,000 and 50,000 were detected.

Although these results proved the presence of subunits of defined molecular weight they do not provide any information on the native molecular forms in the non-purified materials. However, at present some suggestions seem to be justified. In extract I the monomer of \( M_r \) 75,000 — 80,000 derived from AP of \( M_r \) 165,000, whereas the monomer of \( M_r \) 55,000 from AP form of \( M_r \) 110,000. In extract II three AP forms were present; they dissociated probably into two kinds of monomers: of \( M_r \) 65,000 and 55,000. Therefore it may be assumed that some forms of the enzyme are built up of two different kinds of subunits. In addition to three homodimeric forms (of \( M_r \) 165,000, 130,000 and 100,000) the form of \( M_r \) of 110,000 — 115,000 may consist of \( M_r \) 65,000 and \( M_r \) 50,000 subunits.

There are some suggestions that AP plays a key role in bone mineralization [15]. The enzyme is considered to be a useful marker for studying bone development, as the highest peak of AP activity is found to precede an increase in the calcification [16, 17]. It has not so far been elucidated which AP form is responsible for bone mineralization.

Several properties of AP from bone and cartilage reported by various authors are divergent. Also our results concerning the number of AP forms and the molecular weight of their native and monomeric subunits are not consistent with the literature data. Some workers postulated that in bone only one [18], while others that two AP forms are present [19]. The occurrence of either a dimeric or a tetrameric structure has been also postulated [19, 20]. The molecular weight reported for native and monomeric forms of AP ranged from \( M_r \) 65,000 to 205,000 and from 18,000 to 70,000, respectively [19, 20, 21]. It seems that these divergences of the molecular weight of AP from bone and cartilage are due to the difference in isolation procedures, to the use of protease digestion or to differences in the type of bone and its developmental stage. Precise characterizatons of AP from bone requires further studies.

REFERENCES


