Changes in phosphoproteins of chicken bone matrix in vitamin D-deficient rickets

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Vitamin D-deficiency and rickets was produced in growing chicks. The resulting decrease in mineralization of whole bone and of fractions separated by density centrifugation was accompanied by a very significant decrease in the contents of *O*-phosphoserine and *O*-phosphothreonine. Likewise, the total amount of *O*-phosphoserine and *O*-phosphothreonine and the concentrations of these phosphoamino acids in EDTA extracts and in fractions obtained by molecular sieving was also reduced. These data provide the first in vivo evidence that phosphoproteins may be critically involved in the calcification of bone.

Bone matrix *O-phosphoserine* *Phosphoprotein* *Vitamin-D* *Rickets*

1. INTRODUCTION

Phosphoproteins present in all of the normally and pathologically calcified vertebrate tissues have been postulated to play a significant role in the formation of a solid phase of calcium-phosphate in mineralized tissues [1–3]. To date, the only in vivo evidence in support of this thesis has been the finding that the concentration of organic phosphorous in the form of *O*-phosphoserine is reduced in the enamel proteins of rachitic, vitamin D-deficient rats [4], and the reported reduction in the amount of phosphoprotein found in human dentin in cases of dentinogenesis imperfecta, a condition in which dentin is less mineralized than normal [5].

To test this hypothesis in the case of bone, we have used the concentrations and amounts of *O*-phosphoserine and *O*-phosphothreonine in bone as an index of the content and extent of protein phosphorylation and have examined the changes in these parameters in normal and vitamin D-deficient, rachitic chickens. Vitamin D-deficient rickets is accompanied by a significant decrease in the *O*-phosphoserine and *O*-phosphothreonine contents of bone matrix, and by a decrease in the total amount and concentrations of these protein-bound phosphoamino acids extracted in EDTA.

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2. EXPERIMENTAL

For each experiment ~100 1-day-old white Leghorn male chicks obtained from Spafas Inc. (Norwalk CT) were kept in wire-bottom brooders with a constant heat source at 20°C. The experimental chicks were maintained on a special diet for 6 weeks [6]. Control chicks were fed a standard chick diet (no. 904603, ICN Pharmaceut.). Assessment of vitamin D-deficiency was done as in [6]. Results are reported from 7 separate expt.

2.1. *Preparation of bone*

The mid-diaphyses of the long bones and calvaria from 25 normal and rachitic chicks were separately pooled. The bones were frozen in liquid nitrogen, lyophilized and powdered in a liquid N_2 mill (Spex Industries, Metuchen NY) to a particle size of 1–10 μm. The bone powder was separated

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into fractions of different mineral content and density by a modification [6] of the procedure in [7].

2.2. *Weight and composition of whole bone*

Dry weight, wet weight, organic weight, total protein, collagen and non-collagenous protein, as well as the calcium magnesium and phosphorus concentrations of whole bone samples were determined as in [8].

2.3. *Amino acid analysis*

O-Phosphoserine and *O*-phosphothreonine were determined chromatographically [9] on a Beckman 121-M automatic amino acid analyzer (Beckman Instruments Inc.), after partial acid hydrolysis in triple-distilled constant boiling 6 N HCl at 106°C in vacuo [10]. Complete amino acid analyses were obtained on samples of protein hydrolyzed in triple-distilled constant boiling 6 N HCl, 106°C, in vacuo for 22 h, which were then chromatographed on the Beckman 121-M automatic amino acid analyzer.

2.4. *Extraction of EDTA-soluble phosphoproteins of chicken bone*

Bone powder was extracted at 4°C for 2 weeks by large volumes of 0.5 M EDTA (pH 7.4) containing the following protease inhibitors: phenylmethylsulfonyl fluoride (1 mM); ϵ-aminocaproic acid (50 mM); benzamidine hydrochloride (5 mM); and *p*-hydroxymercuribenzoic acid (1 mM) [6]. The pooled EDTA extracts were desalted by dialysis in Spectra-por I membrane tubing using water with the same inhibitors in the dialyzer reservoir, and were then lyophilized.

3. RESULTS AND DISCUSSION

Analyses of whole bone consistently demonstrated the following: rachitic bone contained less mineral and consequently more organic matrix and protein than normal bone; an elevated water content; a significant decrease in the *O*-phosphoserine and *O*-phosphothreonine contents (table 1). The decrease in total mineral deposited in bone was more clearly brought out by density centrifugation [6]. This also held true for the decrease in the *O*-phosphoserine contents (fig. 1). Table 1 is a typical experiment.

The decrease in the *O*-phosphoserine and *O*-phosphothreonine contents of vitamin D-deficient, rachitic bone was mirrored by a decrease (~40%) in the total amount of non-dialyzable, protein-bound *O*-phosphoserine and *O*-phosphothreonine which could be extracted from the vitamin D-deficient, rachitic bone by EDTA. This

Table 1

Composition of 6-week-old postnatal normal and vitamin D-deficient, rachitic chicken bone

Sample	Water (%)	Ash (% dry wt)	Organic (% dry wt)	Ca (mg % dry wt)	P (mg % dry wt)	Mg (mg % dry wt)	*O*-phosphoserine		*O*-phosphothreonine	
							residues/ 10^5 amino acids TP[c]	residues/ 10^5 amino acids NCP[d]	residues/ 10^5 amino acids TP[c]	residues/ 10^5 amino acids NCP[d]
Calvaria										
C[a]	40.3	60.7	39.3	23.7	11.0	0.44	83	670	21	167
R[b]	49.2	50.1	49.9	19.5	9.3	0.29	42	305	10	73
Tibiae and femora										
C	26.3	67.3	32.7	25.8	12.0	0.51	81	640	—	—
R	42.1	61.3	38.7	23.2	11.1	0.38	57	423	—	—
Metatarsals										
C	35.1	63.6	36.4	24.6	11.5	0.47	85	798	—	—
R	46.2	53.3	46.7	20.5	9.7	0.33	62	543	—	—

[a]C, control; [b]R, rickets; [c]TP, total proteins; [d]NCP, non-collagenous proteins

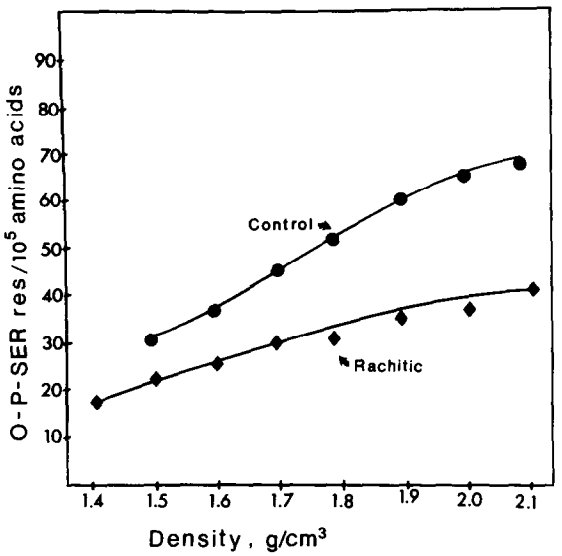

Fig. 1. The concentrations of *O*-phosphoserine in the density fractions of normal and rachitic chicken bone. Results of a typical experiment. Values represent average of two determinations which varied <5%.

was mirrored by the finding that the concentrations of *O*-phosphoserine and *O*-phosphothreonine in the crude EDTA extracts and in the two major G-100 fractions separated from the crude EDTA extracts [11] were also decreased by ~40% and ~60%, respectively, whether calculated as residues/10^5 total residues or as percent serine phosphorylated. However, none of the phosphoprotein fractions in the EDTA extracts were taken to purity as reported in [11]. Thus, it is not possible to accurately determine the extent of phosphoprotein phosphorylation in the various phosphoprotein species present in bone matrix.

The modification of the non-collagenous phosphoproteins by the action of vitamin D is not unique. Vitamin D has been shown to affect other bone non-collagenous proteins such as osteocalcin [12,13] and osteonectin [13], bone collagen [14–16] and cartilage proteoglycans [17]. The results of the present experiments demonstrate that the decreased rate in the amount of mineral deposited in vitamin D-deficient rickets in chickens is accompanied by an overall decrease in the protein-bound *O*-phosphoserine and *O*-phosphothreonine contents of bone and in the non-collagenous, EDTA-extractable phosphoproteins. These experiments provide the first in vivo evidence in bone to support the general hypothesis [1–3,18] that matrix-bound organic phosphorus plays a necessary role in the calcification of vertebrate tissues.

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