THE IDENTIFICATION OF *O*-PHOSPHOTHREONINE IN THE SOLUBLE NON-COLLAGENOUS PHOSPHOPROTEINS OF BONE MATRIX

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1. Introduction

To date, the only phosphorylated amino acid identified in the non-collagenous phosphoproteins of the organic matrices of enamel [1], dentin [2–4] and bone [5,6] has been *O*-phosphoserine [2,6–11]. Although the organic phosphate of rat dentinal phosphoprotein [11] and bovine enamel [13,14] can be accounted for by *O*-phosphoserine [ser(P)], analyses of the phosphoproteins of chicken and bovine bone suggest that the total phosphorus content is greater than can be accounted for by ser(P) alone [5]. In the present study, the EDTA-soluble, non-diffusible phosphoproteins of eight species are found to contain *O*-phosphothreonine [thr(P)] as well as ser(P). Essentially all of the protein-bound, organic phosphate of the EDTA-soluble proteins are accounted for by ser(P) and thr(P). This represents the first instance in which thr(P) has been identified in the structural proteins of mineralized tissues. Moreover, the absence of thr(P) in the phosphoproteins of enamel and dentin is a characteristic feature which distinguishes the phosphoproteins of bone from those of dentin and enamel.

Note added in proof to page 110, line 7: While no thr(P) was detected on ordinary runs, if the column is overloaded with massive amounts of a partial acid hydrolysis of dentinal phosphoprotein, a tiny peak is seen eluting after ser(P), slightly earlier than standard thr(P). If this trace peak proves to be thr(P), it would still only amount to at most 1 residue of thr(P) for every 50–100 ser(P) residues

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2. Materials and methods

2.1. *Tissues*

Cortical, diaphyseal bone from the metatarsals of 10 week chickens, 12 week and 17 week turkeys, tibiae and femora of 2 year steers, 7–8 week rabbits, 3–4 month rats, 1 year pigs and lambs, and 50–65 year humans, were washed with cold 1 M NaCl and quickly split into small pieces by hand. Except in the case of human bone, all bones used were dissected immediately after death. Human bone was obtained from fresh amputations. *p*-Chloromercurobenzoate (0.1 M) and *p*-toluenesulfonyl-fluoride (10 μM) were added to all solutions. The bone pieces were frozen in liquid N_2, freeze dried and ground in a Spex nitrogen mill in 20 s bursts to a fine powder passing a no. 140 mesh screen (100 μm particle size). Bovine enamel proteins [1] and the purified phosphorylated polypeptides, E_3 and E_4 [13,14] (the latter gifts from Elsa Strawich and Jerome Seyer), were prepared as in [1]. Dentinal phosphoprotein [2,3] was a gift of Arthur Veis and Sandra Lee.

2.2. *Extraction of soluble bone phosphoproteins*

Samples of bone powder were determined in 0.5 M EDTA, pH 7.5, at 2°C for 3–5 weeks. The EDTA extracts, the salt and H_2O washes were desalted at 2°C using an Amicon Dialyzer/Concentrator DC2 (Amicon Corp., Lexington, MA) with cartridge type H1P5, against 0.01 M NH_4HCO_3. The solution was then freeze dried.

2.3. *Fractionation of EDTA-soluble proteins*

Samples, 150–300 mg, of the EDTA extracts

were dissolved in 20 ml 0.1 M NH_4HCO_3, pH 8.0 and applied to a 25 × 100 cm column of Sephadex G-100 equilibrated in the same buffer [5] and eluted with 0.1 M NH_4HCO_3, pH 8.0, at a flow rate of 12 ml/h. Fractions, 6 ml, were collected and read manually at A_{230}. The fractions enriched in organic phosphate [5] were used for further analysis.

2.4. *Analysis for* O-*phosphoserine and* O-*phosphothreonine*

2.4.1. EDTA-soluble components of bone

Aliquot samples of the EDTA-soluble components were hydrolyzed in 4 N HCl, 106°C, 6 h and chromatographed on a Beckman 121M automatic amino acid analyzer using 0.2 M Na-citrate, pH 1.5, as the eluting buffer. Authentic ser(P) and thr(P) (Sigma Chemical Co., St Louis, MO) were used as standards. To confirm the identity of components eluting in the position of ser(P) and thr(P), 25–50 mg aliquot samples were hydrolyzed in 4 N HCl, 106°C, 6 h and chromatographed on a preparative amino acid analyzer consisting of MR-201 resin (Mark Instrument Co., Villanova, PA) in a 0.9 × 60 cm column equilibrated and eluted with 0.2 M Na-citrate at pH 1.5, 54°C, and fractions eluting in the region of ser(P) and thr(P) collected. Aliquot samples were rechromatographed on the Beckman 121M amino acid analyzer to con-

Table 1
The concentrations of serine, threonine, *O*-phosphoserine and *O*-phosphothreonine in whole bone and in the EDTA-extractable, non-collagenous phosphoproteins (EDTA-NCP) of bone matrix

(residues/1000 amino acid residues)

		Serine[a]	*O*-Phospho-serine[b]	Threonine[c]	*O*-Phospho-threonine[b]
Chicken	Whole bone	31.0	0.7	20.5	0.1
	EDTA-NCP	64.0	6.5	36.4	2.0
Turkey					
17 week	Whole bone	30.5	0.8	20.2	0.1
	EDTA-NCP	64.0	5.4	34.6	1.2
12–13 week	Whole bone	30.3	0.7	20.1	0.1
	EDTA-NCP	62.9	5.7	34.2	1.2
Rat					
50 day	Whole bone	42.1	0.9	21.3	0.2
	EDTA-NCP	65.7	3.9	30.0	0.8
5 month	Whole bone	44.3	1.2	23.1	0.2
	EDTA-NCP	66.9	4.5	44.8	1.2
Rabbit	Whole bone	48.2	0.6	24.3	0.1
	EDTA-NCP	58.9	4.6	40.0	0.8
Lamb	Whole bone	33.4	0.3	20.2	0.08
	EDTA-NCP	42.9	2.8	29.6	0.6
Pig	Whole bone	35.2	0.4	18.4	0.08
	EDTA-NCP	48.0	4.8	29.1	0.9
Bovine	Whole bone	34.0	0.5	18.6	0.09
	EDTA-NCP	51.3	3.8	37.6	0.8
Human	Whole bone	36.7	0.6	19.5	0.07
	EDTA-NCP	56.4	7.4	44.2	1.1

[a] Includes *O*-phosphoserine
[b] Corrected for destruction of *O*-phosphoserine and *O*-phosphothreonine due to hydrolysis
[c] Includes *O*-phosphothreonine

firm that they chromatographed as ser(P) and thr(P). The rest of the fraction was further hydrolyzed in 6 N HCl for a total of 24 h, 106°C, and its amino acid composition and its phosphorus content determined. Identification of ser(P) and thr(P) was confirmed by the liberation of serine and threonine, respectively [10].

To correct for the degradation of serine, threonine, and the liberation of phosphorus from ser(P) and thr(P), 5 mg samples were hydrolyzed in 4 N HCl, 106°C for 4 h, 6 h, 8 h and 24 h, respectively and the contents of ser(P), thr(P), serine and threonine determined from amino acid analyses. From these data and from the total contents of serine and threonine obtained from complete amino acid analyses, graphs of the rates of hydrolysis of ser(P) and thr(P) were constructed and extrapolated to zero time. Thr(P) was found to be degraded much more rapidly than ser(P) (~80% versus ~40% in 4 N HCl at 106°C for 8 h).

2.4.2. Bone; before and after demineralization

Specimens of whole, undemineralized bone powder, and EDTA-demineralized bone powder were placed in 4 N HCl (1 ml/4 mg bone powder) at 2°C in vacuum sealed tubes under N_2 and shaken at intervals for 12–16 h. The tubes were then placed in a bath at 106°C for 6 h and aliquots used for the identification of ser(P) and thr(P). Other aliquots were quantitatively removed, brought to 6 N HCl and hydrolyzed for an additional 18 h at 106°C and used for complete amino acid analyses. The ser(P) and thr(P) contents/1000 total amino acids were thus calculated.

3. Results and discussion

Ser(P) and thr(P) were identified in whole bone and in the EDTA-soluble, non-diffusible, non-collagenous proteins of all 8 bone species by their elution positions on automatic amino acid analyses (table 1), and by the liberation of only serine and threonine, respectively, after acid hydrolysis of the fractions isolated by preparative ion exchange chromatography. Recovery of the total ser(P) and thr(P) contents of whole, undecalcified bone was excellent: approx. 90–95% of the ser(P) and thr(P) contents of whole bone could be accounted for in the insoluble residue and non-diffusible, EDTA-extractable proteins. Ser(P) and thr(P) were also detected in the EDTA-soluble, non-collagenous proteins obtained by molecular sieving the EDTA solubilized, non-collagenous proteins through G-100 Sephadex columns of 3 of the species. Examples are shown in tables 2 and 3. Analyses of several of the whole EDTA extracts and G-100 fractions revealed that 98–100% of their total, protein-bound organic phosphorus contents could be

Table 2
Concentrations of serine, *O*-phosphoserine, threonine and *O*-phosphothreonine in fractions enriched in organic phosphate obtained by molecular sieving through Sephadex G-100

(residues/1000 total amino acid residues)[a]

	Chicken bone	Fetal calf bone
Serine[b]	78	83
O-Phosphoserine	22	13
Threonine[c]	49	45
O-Phosphothreonine	9	1.3

[a] Corrected for destruction of *O*-phosphoserine and *O*-phosphothreonine due to hydrolysis
[b] Includes *O*-phosphoserine
[c] Includes *O*-phosphothreonine

Table 3
Concentrations of *O*-phosphoserine and *O*-phosphothreonine in the G-100 fractions of an EDTA extract of chicken bone

(residues/1000 total amino acid residues)

Amino acid	Whole EDTA extract	Sephadex G-100 fractions					
		A	B	C	D	E	F
Ser(P)	6.5	5.6	21.6	10.4	3.1	8.6	12.0
Thr(P)	2.0	0.9	8.6	2.5	0.9	2.2	1.7

accounted for by ser(P) and thr(P). G-100 fractionation of the EDTA extracts of calf bone and chicken showed ser(P) and thr(P) in several fractions. One fraction, shown in table 2, accounted for 20% of the phosphorylated protein in the extract.

The soluble phosphoproteins of bovine enamel and dentin contained ser(P) but no thr(P).

Preliminary results obtained from chicken bone, in which case most of the collagen can be dissolved as the gelatin in denaturant solvents [20,21], have shown that essentially all of the ser(P) and thr(P) is located in non-collagenous proteins. However, purified, collagen $\alpha 2$ chains were also found to contain organic phosphate, not as ser(P) or thr(P), but possibly in an acyl linkage [23].

The results of the present study establish for the first time the presence of thr(P) in bone and specifically in the EDTA-soluble proteins of the organic matrix of bone. Failure to detect thr(P) previously may have been due to the relatively small amount of thr(P) present, the marked destruction of thr(P) which occurs during acid hydrolysis, and the fact that ser(P) and thr(P) are difficult to separate on routine amino acid analysis. Further work is in progress to establish how much, if any, of these phosphorylated amino acids are covalently linked to collagen, either directly in the primary structure of the α chains, or indirectly via branched peptides, as occurs in bovine bone [6] and dentin [12].

The presence of thr(P) in the phosphoproteins of bone and their absence in the phosphoproteins of dentin and enamel serves to further distinguish these proteins. The presence of the ser(P) and thr(P) and the specific Ca^{2+}-binding amino acid, γ-carboxyglutamic acid [24,25], in the organic matrix of bone and their spatial relationships to one another may be critical for a number of the processes involved in the deposition and removal of the solid mineral phase.

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