Calcif. Tiss. Intl. 27, 281-284 (1979)

Calcified Tissue International

Rapid Communications

Phosphopeptides and γ-Carboxyglutamic Acid-Containing Peptides in Calcified Turkey Tendon: Their Absence in Uncalcified Tendon

Melvin J. Glimcher, Diane Brickley-Parsons and Dora Kossiva

Department of Orthopaedic Surgery, Harvard Medical School, Children's Hospital Medical Center, Boston, Massachusetts 02115

SUMMARY

Uncalcified samples of turkey tendon obtained prior to calcification, and other samples from areas of tendon that never calcify, contain little or no O-phosphoserine [Ser(P)], O-phosphothreonine [Thr(P)] and γ-carboxyglutamic acid (Gla). Significant amounts of all three of these Ca^{2+}-binding amino acids, which are found in EDTA-extractable, non-collagenous proteins, are detected coincident with the onset of mineralization of a tendon and increase in concentration as mineralization proceeds.

INTRODUCTION

The calcification and subsequent ossification of certain avian tendons has been well described (3,10-12,17,18). Because the ossification of this tissue proceeds relatively slowly, it is possible to obtain samples of tendon at various stages of calcification before ossification begins. There are a number of other advantages which make this biological system an excellent model with which to study various aspects of tissue calcification. For example, not all of the tendons calcify and not all locations in those tendons which eventually calcify become calcified at early stages. Thus it is possible to obtain samples of tendon none of which ever calcifies, specific portions of tendons which are uncalcified but which eventually do calcify, as well as calcified portions of tendons containing varying amounts of the solid mineral phase of Ca-P.

As early as 1930 (17), investigators noted that calcification of avian tendon is preceded by changes in its histochemical staining properties as well as changes in the size and shape of the cells (10). Biochemical analyses have demonstrated that calcification begins in a tissue matrix that contains the same collagen fibers prior to calcification (11). Therefore, it seems reasonable to conclude that if the components of the matrix of the tendon play a role in the nucleation of the mineral phase or other aspects of the tissue calcification, they do so either (a) by changing certain of their interaction properties (matrix constituents already present), (b) by adding or removing certain components, or (c) both. Because of the recent interest focussed on the possible role in mineralization of the Ca^{2+}-binding, non-collagenous phosphopeptides (4,6,16) and the peptides containing γ-carboxyglutamic acid (Gla) (8,9,13,14) we have analyzed the following turkey tendon tissues for the presence of phosphopeptides and Gla-containing peptides: (a) tissue from tendons that normally never calcify; (b) from calcified tendon, tissue from those parts that normally never calcify; (c) tissue taken prior to the onset of calcification from the parts that normally calcify; and (d) calcified tendons in various stages of calcification. We have also analyzed ossified turkey tendons and turkey bone for comparison purposes.

MATERIAL AND METHODS

A. Animals

Female Nicholas Big White turkeys, raised on the same standard diet, were obtained from Wampler Food, Inc., Hinton, VA. The age of the animals was precisely known since they were raised from the time of hatching. Animals aged 7 weeks to 1 year were used for the histological studies. Only tissues from animals aged 7 weeks, 10 weeks & 2 days, 15 weeks & 5 days, 17 weeks, and 1 year were used for the biochemical studies. In these particular animals most of the calcified tendons examined had small but significant amounts of bone replacing the calcified tendon tissue shortly after the animals reached 17 weeks of age. Ossification thus begins earlier in the calcifying tendons of these animals than in those employed in other studies (10-12). Consequently it is important to note that chemical or biophysical studies without morphological control may be quite misleading, since it is not possible to tell from gross inspection whether the calcified tissue in the tendon consists of calcifying tendon, bone or a mixture of the two tissues.

0008-0594/79/0027-0281 $01.00

B. Tissue Preparation

The tibialis caudalis tendons in the tibiotarsus region of the lower extremities were used as the source of potentially calcifying tendons and of calcified tendons. Portions of the tendon in the vicinity of the joints were found not to mineralize at least up to the age of 1 year and were used as sources of unmineralizing tendon from a tendon that did mineralize in other locations. The neck tendons were used as sources of uncalcified tendons that do not calcify anywhere along their length up to the age of 1 year. The tibialis caudalis tendon of 7 week old animals was found to be unmineralized at this stage of development, and portions of this tendon from regions which eventually became mineralized in older animals were used as sources of calcifying tendon prior to the onset of calcification.

Representative tendons were x-rayed in order to determine the age of onset of calcification and to mark the locations where calcification begins and where it extends. This also proved to be very useful in isolating those portions of the calcifying tendons where calcification did not occur at least up to the age of 1 year.

Cortical bone was obtained from the same animals from the midshaft of the tibiae. The endosteal and periosteal surfaces were very carefully cleaned and scraped free of soft tissue and cancellous bone.

Samples of undecalcified calcified tendon and bone were frozen in liquid nitrogen, freeze-dried and ground in a Spex nitrogen mill in 20 sec bursts to a fine powder. Pieces of frozen uncalcified tendon were cut by hand to approx. 1 mm^3 and then similarly ground in the Spex nitrogen mill.

C. Analytical Procedures

The dry weight, ash weight, and the calcium and phosphorus contents of the ash were determined on aliquot samples of the undecalcified samples as previously described (2). The soluble phosphoproteins and Gla-containing proteins were extracted as described (1,2,15). The methods of analysis for Ser(P) and Thr(P) (1,2,15) and for Gla (7) have recently been summarized in this journal (5). Similarly, traces of Gla and phosphorylated amino acids were detected in uncalcified tissues as described in the same article (5).

RESULTS AND DISCUSSION

The non-collagenous proteins in the EDTA extracts represented from ∿2-5% of the total protein of the tendon. Only trace amounts of Ser(P), Thr(P) and Gla were present in any of the uncalcified samples of whole turkey tendon or in the proteins extractable in EDTA, regardless of their source (Tables 1 & 2). Once mineralization begins, however, significant concentrations of these Ca^{2+}-binding amino acids are detected in the whole native tissue and in the proteins extracted from them in EDTA (Tables 1 & 2). In addition to their chromatographic behavior, Ser(P) and Thr(P) were positively identified by the analytical chromatographic behavior of the fractions isolated by preparative amino acid chromatography and by the release of serine, threonine and P_i from these fractions on complete acid hydrolysis (1,2). Calcified tendon which is heavily but somewhat less mineralized than bone from the same animals contains a significantly lower concentration of the Ca^{2+}-binding amino acids than bone. On the other hand, the bone in the ossified tendon, and bone from the mid-portion of the diaphyses of the tibiae of the same animal both contain lower concentrations of Ser(P) and Thr(P) (but not of Gla) than younger bone of very similar mineral content (Table 1).

Table 1. THE CONCENTRATIONS OF O-PHOSPHOSERINE, O-PHOSPHOTHREONINE AND γ-CARBOXYGLUTAMIC ACID IN UNCALCIFIED, CALCIFIED AND OSSIFIED TURKEY TENDON AND IN BONE (Residues/10^5 amino acid residues)

Sample Age, Location	Ser(P)	Thr(P)	Gla	Ca (wt %)	P (wt %)
TENDONS:					
7 wk (a)*	1	trace	trace	0.06	0.10
" (b)*	1	--**	--	0.04	0.10
" (c)*	3	trace	--	0.06	0.15
10 wk 2 d (a)	11	2	11	9.0	4.4
" " (b)	1	trace	--	0.08	0.10
" " (c)	2	trace	--	0.01	0.07
15 wk 5 d (a)	32	3	31	21.9	10.1
" " (b)	1	--	∿1	0.4	0.25
" " (c)	2	--	∿0.5	0.03	0.1
17 wk (a)	33	4	49	21.0	9.7
" (b)	1	--	∿0.5	0.7	0.3
" (c)	3	2	trace	0.03	0.1
1 year (a)***	30	4	92	23.0	10.4
" (b)	trace	--	--	0.04	0.05
" (c)	2	--	trace	0.05	0.09
BONE:					
12 wk	78	12	79	25.5	12.4
17 wk	79	11	132	26.4	12.1
1 year	48	5	126	25.8	11.6

*(a) Portion of tibialis caudalis tendon which calcifies with increasing age of animal
(b) Portion of tibialis caudalis tendon which does not calcify
(c) Neck tendons none of which calcify
**Double hyphens (--) signify undetected.
***Ossified tendon

Histological examination of a large number of calcified and decalcified longitudinal and cross-sections of all the tendons reveals that, except for the tissue removed from 1 year old animals, all of the samples of the tendon tissue used for biochemical analyses were composed solely of tendon and contained no bone. On the other hand, all of the mineralized portions of the tendons from 1 year old animals consisted of bone tissue, al-

though there was a small amount of uncalcified tendon tissue in the periphery and in small "pockets" within the body of the tendon. Histological examination of various tendons at different ages makes it very clear that there are marked variations as far as the onset and extent of ossification of the tendon is concerned. These variations depend upon the particular strain of turkey used, the diet and conditions under which the animals are raised, the specific tendons examined, the precise locations within the tendon examined, and so forth. This makes it absolutely mandatory that careful histological examination of multiple sections be carried out in order to establish with certainty that the tissue analyzed is calcified

Table 2. THE CONCENTRATIONS OF O-PHOSPHOSERINE, O-PHOSPHOTHREONINE AND γ-CARBOXYGLUTAMIC ACID IN THE EDTA-EXTRACTABLE, NON-COLLAGENOUS PROTEINS OF UNDECALCIFIED, CALCIFIED AND OSSIFIED TURKEY TENDON AND BONE (Residues/10^5 amino acid residues)

Sample Age, Location	Ser(P)	Thr(P)	Gla
TENDONS:			
7 wk (a)*	46	trace	trace
" (b)*	47	trace	--**
" (c)*	40	trace	--
10 wk 2 d (a)	176	10	340
" " (b)	32	trace	trace
" " (c)	62	trace	--
15 wk 5 d (a)	244	80	396
" " (b)	39	--	22
" " (c)	44	trace	14
17 wk (a)	392	120	400
" (b)	21	--	24
" (c)	20	--	14
1 year (a)***	380	40	1880
" (b)	4	--	--
" (c)	2	--	trace
BONE:			
12 wk	570	120	680
17 wk	540	120	1170
1 year	380	44	1950

*(a) Portion of tibialis caudalis tendon which calcifies with increasing age of animal
(b) Portion of tibialis caudalis tendon which does not calcify
(c) Neck tendons none of which calcify
**Double hyphens (--) signify undetected
***Ossified tendon

tendon and not bone. In our own studies reported here, the histological observations that the tissue consisted wholly of tendon in the younger animals and wholly of bone in the case of calcified tissue from the tendon of the 1 year old animals, were consistent with their hydroxylysine contents (6).

Although the presence of any component in a mineralized tissue does not a priori establish that it is involved in the mineralization of that tissue, the virtual absence of Ser(P), Thr(P) and Gla from tendon tissue which is not mineralized, and their presence in increasing amounts coincident with the onset of mineralization, and as a direct function of the mineral content of the tissue, suggest that the non-collagenous peptides which contain these Ca^{2+}-binding amino acids are either associated with tissue mineralization, with the mineral phase per se, or with the synthesis and degradation of the tissue matrix.

ACKNOWLEDGMENTS: We wish to thank Ms. Beatrice Lefteriou for her expert technical assistance, Dr. Peter V. Hauschka for helpful discussions and counsel and for the analyses and identification of γ-carboxyglutamic acid, and Ms. Mariana Sybicki for her histolgical advice and skilled preparation of the samples. We gratefully acknowledge the help of Dr. Walker Thompson of Wampler Food, Inc., Hinton, VA in obtaining the samples of turkey tendon.

Supported in part by grants from the National Institutes of Health (AM 15671) and the New England Peabody Home for Crippled Children, Inc.

REFERENCES

1. Cohen-Solal, L., Lian, J.B., Kossiva, D., Glimcher, M.J.: The identification of O-phosphothreonine in the soluble non-collagenous phosphoproteins of bone matrix, FEBS Lett. 89: 107-110, 1978
2. Cohen-Solal, L., Lian, J.B., Kossiva, D., Glimcher, M.J.: Identification of organic phosphorus covalently bound to collagen and non-collagenous proteins of chicken bone matrix: the presence of O-phosphoserine and O-phosphothreonine in non-collagenous proteins and their absence from phosphorylated collagen. Biochem. J. 177: 81-98, 1979
3. Engstrom, A.: Apatite-collagen organization in calcified tendon, Exp. Cell Res. 43:241-245, 1967
4. Glimcher, M.J.: Composition, structure and organization of bone and other mineralized tissues and the mechanism of calcification. In R.O. Greep, E.B. Astwood (eds.): Handbook of Physiology, Endocrinology, vol. 7, pp. 25-116. Am. Physiol. Soc., Washington, D.C. 1976
5. Glimcher, M.J., Kossiva, D., Roufosse, A.: Identification of phosphopeptides and γ-carboxyglutamic acid-containing peptides in epiphyseal growth plate cartilage. Calcif. Tissue Int., in press, 1979
6. Glimcher, M.J., Krane, S.M.: The organization and structure of bone, and the mechanisms of calcification. In G.N. Ramachandran, B.S. Gould (eds.): Treatise on Collagen, Vol. 2B, pp. 68-251. Academic Press, London and New York, 1968
7. Hauschka, P.V.: Quantitative determination of γ-carboxyglutamic acid in proteins, Anal. Biochem. 80:212-223, 1977
8. Hauschka, P.V., Lian, J.B, Gallop, P.M.: Direct identification of the calcium-binding amino acid, γ-carboxyglutamate, in mineralized tissue, Proc. Nat. Acad. Sci. USA 72: 3925-3929, 1975

9. Hauschka, P.V., Lian, J.B., Gallop, P.M.: Vitamin K and mineralization, Trends Biochem. Sci. 3:75-85, 1978
10. Johnson, L.C.: Mineralization of turkey leg tendon. I. Histology and histochemistry of mineralization. In R.F. Sognnaes (ed.): Calcification in Biological Systems, pp. 117-128. Am. Assoc. Adv. Science, Washington, D.C. 1960
11. Likins, R.C., Piez, K.A., Kunde, M.L.: Mineralization of turkey leg tendon. III. Chemical nature of the protein and mineral phases. In R.F. Sognnaes (ed.): Calcification in Biological Systems, pp. 143-149. Am. Assoc. Adv. Science, Washington, D.C., 1960
12. Nylen, M.U., Scott, D.B., Mosley, V.M.: Mineralization of turkey leg tendon. II. Collagen-mineral relations revealed by electron and x-ray microscopy. In: R.F. Sognnaes (ed.): Calcification in Biological Systems, pp. 129-142. Am. Assoc. Adv. Science, Washington, D.C. 1960
13. Price, P.A.: Comparison of γ-carboxyglutamic acid-containing proteins from bovine and swordfish bone: Primary structure and Ca^{++} binding. In R.H. Wasserman et al. (eds.): Calcium Binding Proteins and Calcium Function, pp. 333-337. Elsevier/North Holland, Amsterdam and New York, 1977
14. Price, P.A., Otsuka, A.S., Poser, J.W., Kristaponis, J., Raman, N.: Characterization of a γ-carboxyglutamic acid-containing protein from bone, Proc. Nat. Acad. Sci. USA 73:1447-1451, 1976
15. Spector, A.R., Glimcher, M.J.: The extraction and characterization of soluble anionic phosphoproteins from bone, Biochim. Biophys. Acta 263:593-603, 1972
16. Veis, A., Perry, A.: The phosphoprotein of the dentin matrix, Biochemistry 6:2409-2416, 1967
17. Weidenreich, F.: Das Knochengewebe (Grossgebündelte Grundsubstanz-Faserknochen). In von Möllendorf (ed.), Handbuch der mikroskopischen Anatomie des Menschen, 2nd ed'n., Pt. 2 pp. 409-416. Springer, Berlin 1930
18. White, S.W., Hulmes, D.J.S., Miller, A., Timmins, P.A.: Collagen-mineral axial relationship in calcified turkey leg tendon by X-ray and neutron diffraction, Nature 266:421-425, 1977

Received October 20, 1978 / Revised January 2, 1979 / accepted January 16, 1979