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Identification of γ-glutamyl phosphate in the α2 chains of chicken bone collagen

(organic phosphorus/protein/sodium borohydride/acyl phosphate)

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ABSTRACT **Purified components of chicken bone collagen contain approximately 4 atoms of organic phosphorus per mol of collagen, located principally in the α2 chains. Previous analyses have demonstrated the absence of *O*-phosphoserine, *O*-phosphothreonine, and other phosphorylated hydroxy amino acids, phosphoamidated amino acids, and phosphorylated sugars. In the present report we establish that chicken bone collagen contains γ-glutamyl phosphate. This was accomplished by the isolation of tritiated α-amino-δ-hydroxyvaleric acid after reductive cleavage with NaB[^{3}H]H$_4$ of the γ components, the α2 chains, and peptides enriched in organic phosphorus that were derived from the α2 chains. Tritiated α-amino-δ-hydroxyvaleric acid was not detected in any of the following unphosphorylated proteins after cleavage with NaB[^{3}H]H$_4$: albumin and lysozyme, the α2 chains of several unmineralized tissues, and, most importantly, dephosphorylated α2 chains of chicken bone collagen. The α2 chain of chicken bone collagen is the first structural protein found to contain an acyl phosphate.**

The extracellular organic matrix of chicken bone contains organic phosphorus (1, 2), the majority of which is present in noncollagenous phosphopeptides that are not covalently bound to the collagen (3, 4). The noncollagenous phosphopeptides contain both *O*-phosphoserine (3, 4) and *O*-phosphothreonine (2, 5). Recent studies (5) have confirmed earlier work that organic phosphorus is also present in the purified components of chicken bone collagen, principally in the α2 chains (2). However, in contrast to the noncollagenous phosphopeptides, the α2 chains contain neither phosphoserine nor phosphothreonine (2). Indeed, no phosphorylated hydroxyamino acid, phosphoamidated amino acid, or phosphorylated sugar could be identified in highly purified components of the chicken bone collagen including the α2 chains which contained approximately 4 to 5 atoms of organic phosphorus per mol of collagen (2). Peptides containing organic phosphorus were isolated from partial acidic hydrolysates and enzymatic digests of purified collagen components that contained an unidentified cationic amino acid. On the basis of the pH stability of the organic phosphorus moiety in the α2 chain and the composition of the organic phosphorus-containing peptides derived from enzymatic degradation and partial acidic hydrolysis, it was suggested that the organic phosphorus moiety in the α2 chains was γ-glutamyl phosphate (2, 6). In this paper, we report the identification of γ-glutamyl phosphate in the purified α2 chains of chicken bone collagen based on the isolation of ^{3}H-labeled α-amino-δ-hydroxyvaleric acid (AHOVl) after reductive cleavage with NaB[^{3}H]H$_4$ (7).

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MATERIALS AND METHODS

Protein and Peptide Samples. For the reductive cleavage experiments with NaB[^{3}H]H$_4$ (7) the following proteins and peptides from chicken bone collagen were used: α2 chains and phosphorus-enriched peptides derived from them (2, 8–10), purified γ components, and α2 chains that had been completely dephosphorylated by heating in 10% acetic acid for 4 hr at 60°C and for 10 min at 100°C. α2 chains of several unmineralized collagens (namely, from rat tail tendon, rat skin, and chicken skin) were prepared from 9 M LiCl extracts by agarose gel filtration and CM-cellulose and DEAE-cellulose ion exchange chromatography (2). These α2 chains contained less than 0.2 atom of organic phosphorus per mol of collagen. Bovine albumin (fraction V, Sigma Chemical Co., St. Louis, MO) and lysozyme (Worthington Biochemical Corp., Freehold, NJ), containing no organic phosphorus were also used for the reductive cleavage.

The acid stability of the phosphate bond in protein-bound acyl phosphates was determined on adenosine 5′-triphosphatase (EC 3.6.1.3) from pork cerebral cortex microsomes (Sigma) and acetate kinase (EC 2.7.2.1) from *Escherichia coli* (Boehringer Mannheim). The enzymes were phosphorylated with [γ-^{32}P]ATP (20 μCi/μmol of ATP; 1 Ci = 3.7 × 10^{10} becquerels) by methods described for these enzymes by Nagano *et al.* (11) and Anthony and Spector (12, 13), respectively.

Sodium Borohydride Reduction. To a solution of about 10 mg of protein in 1 ml of dimethyl sulfoxide, 0.35 ml of NaB[^{3}H]H$_4$ (0.15 M; ≈100 mCi/mmol) was added and the reaction was allowed to proceed for 1 hr at room temperature with stirring (7). The reaction was stopped by acidification with formic acid to destroy the excess NaBH$_4$, and the dimethyl sulfoxide was removed by repeated lyophilization and finally by ion exchange chromatography on a 1 × 6 cm column of Dowex 50, H$^+$ resin. Lyophilized protein samples were hydrolyzed in 6 M HCl at 108°C for 24 hr. The HCl was removed by repeated evaporation under reduced pressure, and the dried samples were washed with water several times, dissolved in 0.25 M NaOH and kept at room temperature for 72 hr or at 60°C for 1 hr; then they were brought to pH 6.0 and dried. Samples of the acidic hydrolysates of the protein and of the fractions obtained by preparative amino acid chromatography in the region of glutamic acid and proline were subsequently subjected to high-voltage electrophoresis.

Abbreviation: AHOVl, α-amino-δ-hydroxyvaleric acid.

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Preparative Amino Acid Chromatography. The acidic hydrolysates of the NaB[^{3}H]H$_4$-reduced samples of collagen and collagen peptides were chromatographed on a 0.9 × 60 cm column of MR-201 resin (Mark Instrument Co., Villanova, PA) by using a gradient similar to that described by Piez and Morris (14) or else on a 0.9 × 90 cm column of Technicon Chromabeads type A (Technicon) by using the gradient described by Hamilton (15). The elution characteristics were established with a mixture of standard amino acids. Authentic AHOVl (Vega-Fox, Tucson, AZ) was found to coelute with glutamic acid. Aliquots were removed for ninhydrin analyses, and their radioactivity was measured using a Beckman LS-8000 scintillation counter. Peaks eluting in the regions corresponding to glutamic acid and proline were collected; in several instances these were rechromatographed to ensure homogeneity of the fractions. Chromatography of aliquots of these fractions on a Beckman 121-M amino acid analyzer confirmed that they contained only single peaks that eluted with proline and glutamic acid, respectively.

High-Voltage Paper Electrophoresis. High-voltage paper electrophoresis was carried out at 2500 V on Whatman no. 1 paper at pH 1.7 in 7% (vol/vol) formic acid (2). Approximately 100 nmol of unlabeled AHOVl was added to the sample eluted by preparative acid chromatography in the region corresponding to glutamic acid before the high-voltage electrophoresis was carried out. In order to reduce the amount of glutamic acid present in these samples, in some instances they were dissolved in 60% ethanol (which precipitated a significant amount of the glutamic acid). After the electrophoresis, guide strips 2.3 mm wide were cut from the edges of the electrophoretogram bands of each sample, and the standards of AHOVl, glutamic acid, and proline were stained with ninhydrin. The remaining strips of each sample were cut into consecutive 1-cm segments. The segments were eluted with 1 ml of 6 M HCl. A 0.2-ml aliquot was evaporated, 10 ml of Instagel was added, and the radioactivity was measured in the scintillation counter. To confirm further the identity of the [^{3}H]-AHOVl and [^{3}H]proline, the segments of the experimental samples corresponding to AHOVl and proline were eluted and subjected to a second high-voltage electrophoresis. In several instances, acidic hydrolysates of the peptides that were enriched in organic phosphorus and reduced with NaB[^{3}H]H$_4$ were subjected to high-voltage electrophoresis without prior preparative amino acid chromatography.

Stability of Phosphate Bond in Protein-Bound Acyl Phosphates. Precipitates of phosphorylated adenosine 5′-triphosphatase and lyophilized samples of acetate kinase, respectively, were dissolved in 0.5–1.0 ml of 11 M HCl and hydrolyzed under reduced pressure for 40 hr at 37°C. An aliquot was removed for the determination of total radioactivity, and the HCl was then removed under reduced pressure at 37°C. In order to determine how much of the acyl phosphate had been cleaved to inorganic phosphate, aliquots of the enzymes and of the enzymes after acidic hydrolysis were subjected to high-voltage paper electrophoresis at a pH corresponding to the most stable condition for the phosphate bond in the specific enzyme: pH 3.5 for the adenosine 5′-triphosphatase, and pH 6.4 for the acetate kinase. Standard [γ-^{32}P]ATP and ^{32}P$_i$ were identified by autoradiography. Consecutive segments of the paper strips were eluted with 50% acetic acid and the total radioactivity was determined in each of the following: the enzymes prior to hydrolysis, peptides produced by the acid hydrolysis, [γ-^{32}P]ATP, and P_i.

Degradation Products after Acidic and Alkaline Hydrolysis of AHOVl. Samples of AHOVl were hydrolyzed in 6 M HCl at 105°C for 24 or 72 hr. When samples were chromatographed on the analytical or preparative columns of an amino acid analyzer, most of the material chromatographed as two peaks coincident with glutamic acid and isoleucine, the latter absorbing strongly at 440 nm after reaction with ninhydrin. A small amount of material coeluted with proline. The material coeluting with proline gave the high 440/570 nm ratio of absorbances typical of proline. Samples hydrolyzed for 72 hr contained more material eluting with isoleucine than did samples hydrolyzed for 24 hr. In a typical experiment using authentic AHOVl hydrolyzed in 6 M HCl at 105°C for 24 hr and chromatographed on the amino acid analyzer, approximately 36% of the original AHOVl was recovered as AHOVl, 4% was recovered as proline, and 61% eluted with isoleucine. When aliquots of the acidic hydrolysates were treated with alkali and then chromatographed, approximately 59% was recovered as AHOVl and 41% as proline. There was no detectable material eluting with isoleucine. There was no conversion of AHOVl to proline or to material eluting with isoleucine when AHOVl was treated with alkali alone.

High-voltage electrophoresis of acid hydrolysates not subjected to subsequent alkali treatment resolved components coincident with authentic AHOVl and proline, plus four spots that migrated more rapidly (more basic) than proline and AHOVl. For example, in one high-voltage electrophoresis experiment carried out for 45 min with authentic AHOVl hydrolyzed in acid without further treatment with alkali, there was a major component that ran 22.0 cm (similar to authentic AHOVl), a minor component that ran 22.6 cm (similar to authentic proline), and four spots that ran 30.0 (pink when stained with ninhydrin), 32.0 (yellow with ninhydrin), 33.0 (faint pink with ninhydrin), and 35.0 cm (faint pink with ninhydrin). The spot that ran with proline also stained yellow with ninhydrin, similar to authentic proline. When the acidic hydrolysates were treated with 0.25 M NaOH for 24 hr at room temperature or for 1 hr at 60°C and then subjected to high-voltage electrophoresis, the four basic, more positively charged components seen on electrophoresis all disappeared. More material now eluted with proline and AHOVl. High-voltage electrophoresis of the peak eluting with isoleucine demonstrated that this material did indeed correspond to the four basic components observed when the whole hydrolysate was subjected to electrophoresis. Similarly, when the four more positively charged components obtained by preparative high-voltage electrophoresis were eluted, treated with alkali, and reelectrophoresed, material now appeared that migrated with proline and with authentic AHOVl, and the spot eluting with proline stained yellow with ninhydrin, similar to authentic proline.

The data are consistent with the findings of others (7, 16, 17) that the principal degradation products of AHOVl after standard acidic hydrolysis are cyclic lactones such as α-aminovalerolactone. However, in addition to the lactones, a small amount of AHOVl is converted to proline (16). The present study also demonstrates that the major degradation products of AHOVl after acidic hydrolysis are converted to proline (≈60%) and back to AHOVl (≈40%) when the acid hydrolysate is subsequently treated with alkali.

RESULTS

As reported (2, 6), highly purified α2 chains of chicken bone collagen were found to contain approximately 4 atoms of organic phosphorus per mol of collagen, but no phosphorylated hydroxy amino acid, phosphoamidated amino acid, or phosphorylated sugar could be identified that would account for this organic phosphorus content. Moreover, peptides were recovered from both partial acidic and enzymatic digests of the α2 chains that were enriched in organic phosphorus (Table 1). The two major amino acids present in the peptides obtained from the α2 chains by both enzymatic and acidic hydrolysis were glu-

Table 1. Content and distribution of organic and inorganic phosphorus in α2 chains of chicken bone collagen and in peptides derived from them by partial acidic hydrolysis and enzymatic digestion

	Organic phosphorus, μg/100 mg α2	Inorganic orthophosphate, μg/100 mg α2
Enzymatic digestion		
α2 chains	142	0
α2 chains after digestion	89	54
Chromatographic fractions from Dowex 50, H^+ resin:		
E-1*	72	70
E-2	≈3	0
E-3	0	0
E-4	0	0
E-5	0	0
11 M HCl digestion		
α2 chains	142	0
Partial acidic hydrolysate	75	65
Chromatographic fractions from Dowex 50, H^+ resin:		
A-1*	45	80
A-2*	10	Trace
A-3	Trace	0
A-4	5	0

* Peptides in these fractions were used for reductive cleavage with NaB[^{3}H]H$_4$.

tamic acid and glycine. Hydroxyproline was also present in significant amounts in the peptides containing the highest concentrations of organic phosphorus, consistent with their being derived from collagen. The major amino acids (namely, those comprising approximately 50–80% of the total amino acids present in the peptides) in the three peptides utilized for reductive cleavage with NaB[^{3}H]H$_4$ are presented in Table 2. All of the peptides in the fractions containing organic phosphorus had high concentrations of an unknown peak eluting before cysteic acid when chromatographed, *without further hydrolysis*, in pH 1.5 0.2 M citrate buffer on the Beckman 121-M automatic amino acid analyzer (2, 5). No γ-carboxyglutamic acid was identified in the α2 chains or in the phosphorus-enriched peptides.

Reaction of the γ-components, the α2 chains, and the peptides enriched in organic phosphorus obtained from the enzymatic digests of the α2 chains (2) with NaB[^{3}H]H$_4$ released 60–80% or more of the organic phosphorus as inorganic orthophosphate.

Preparative amino acid chromatography of alkali-treated acidic hydrolysates of the NaB[^{3}H]H$_4$-reduced γ components, α2 chains, and peptides isolated after acidic and enzymatic digestion of the α2 chains yielded significant amounts of radioactivity in all peaks eluting with proline and glutamic acid. For example, in a typical experiment, 53% of the radioactive counts were recovered in the peak eluting with glutamic acid and 47% with proline when the α2 chains were reduced with NaB[^{3}H]H$_4$, acid hydrolyzed, and then treated with alkali before preparative amino acid chromatography. High-voltage electrophoresis of the peaks eluting with proline and with glutamic acid showed the presence of ^{3}H-labeled material corresponding to proline and to AHOVl, respectively (Fig. 1).

Table 2. Major amino acids present in peptides of several fractions isolated from α2 chains enriched in organic phosphorus

	Residues per 1000 total amino acid residues		
	From enzymatic digestion	From partial acidic hydrolysates	
	Fraction E-1	Fraction A-1	Fraction A-2
Hydroxyproline	50	21	64
Serine	62	151	33
Glutamic acid	396	116	604
Glycine	167	213	88

In contrast to the α2 chains of chicken bone collagen, no radioactivity was recovered in fractions corresponding to authentic proline or AHOVl when albumin, lysozyme, α2 chains of chicken skin, rat tail tendon, or rat skin, or dephosphorylated α2 chains of chicken bone collagen were treated with NaB[^{3}H]H$_4$.

After reaction with 11 M HCl for 40 hr at 37°C, approximately 25–50% of the organic phosphorus content of the α2 or γ components of chicken bone collagen was recovered as organic phosphorus (Table 1). Similarly, however, 12–15% of the acyl phosphate bonds (β-aspartyl phosphate) (7, 11, 16, 18) in adenosine 5′-triphosphatase and ≈60% or more of the acyl phosphate bonds (γ-glutamyl phosphate) (12, 13, 17) in acetate kinase resisted cleavage to inorganic phosphate under identical conditions.

DISCUSSION

The present study—which has demonstrated the recovery of tritiated α-amino-δ-hydroxyvaleric acid and [^{3}H]proline after reductive cleavage with NaB[^{3}H]H$_4$ of highly purified α2 chains, of peptides derived from them, and of γ-polymers and has identified proline as a major degradation product of AHOVl after acidic hydrolysis and subsequent alkali treatment of the hydrolysate—clearly establishes that chicken bone collagen does indeed contain γ-glutamyl phosphate. The conversion of 60–80% or more of the organic phosphorus to inorganic orthophosphate suggests that all of the organic phosphorus in the α2 chains is present in this form.

The identification of glutamyl phosphate in the peptides isolated from the purified α2 chains cleaved enzymatically at neutral pH as well as from the peptides obtained after partial hydrolysis in 11 M HCl makes it unlikely that the glutamyl phosphate bond was formed by an internal rearrangement such as an acyl shift (19–21). It is also unlikely that the formation of [^{3}H]AHOVl and [^{3}H]proline is an artifact resulting from the interaction of the protein with NaB[^{3}H]H$_4$ because neither [^{3}H]AHOVl nor [^{3}H]proline was recovered after reaction of NaB[^{3}H]H$_4$ with several other nonphosphorylated proteins such as albumin, lysozyme, the α2 chains of soft tissue collagens, and, most importantly, the dephosphorylated α2 chains of chicken bone collagen.

The relative stability of the glutamyl phosphate bond in the α2 chains of chicken bone collagen to hydrolysis in 11 M HCl is at first glance somewhat surprising because most acyl phosphate bonds are highly reactive and rapidly cleaved in acid (11–13). However, the acyl phosphates of adenosine 5′-triphosphatase and especially of acetate kinase were also not completely converted to inorganic phosphate under identical acidic conditions. Indeed, the glutamyl phosphate bond of acetate kinase was, if anything, slightly more resistant to cleavage in 11 M HCl at 37°C than was the glutamyl phosphate bond of the α2 chains of chicken bone collagen, and both were considerably more stable than the aspartyl phosphate bond of adenosine 5′-triphosphatase. Although it is not possible to conclude from these limited data that aspartyl phosphate bonds in general are less stable in 11 M HCl at 37°C than glutamyl phosphate bonds, such structural factors undoubtedly play a role in determining the stability of the acyl phosphates (11–13) as evidenced, for example, by the increased acid stability of the

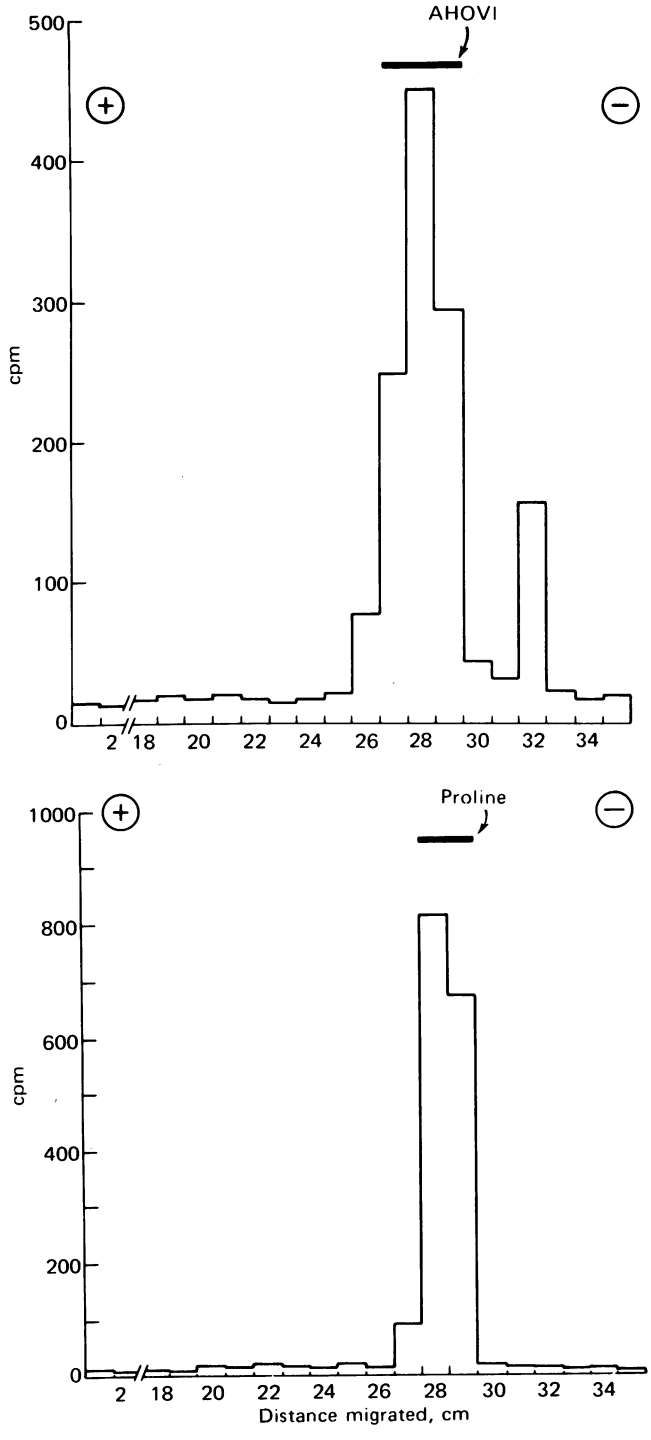

FIG. 1. High-voltage paper electrophoresis of acid hydrolysate of NaB[^{3}H]H$_4$-treated α2 chains of chicken bone collagen. After reaction with alkali, the hydrolysate was chromatographed on a preparative amino acid analyzer. (*Upper*) Only the fraction eluting, on preparative amino acid chromatography, with glutamic acid was electrophoresed. Authentic AHOVl was located by reaction with ninhydrin. (*Lower*) Fraction eluting on preparative amino acid chromatography with proline was electrophoresed. Authentic proline was located by reaction with ninhydrin.

mixed phosphate anhydrides of succinic (3-carboxypropionyl) and maleic (3-carboxyacrylic) acids (22). In addition, the local chemical environment around the acyl phosphate bond as reflected by the chemical nature, interactions, and configuration of the specific amino acids in the immediate vicinity of the glutamyl phosphate also undoubtedly exerts a profound effect on the hydrolytic behavior of the acyl phosphate bond. This would also account in part for differences in the acid stability of acyl phosphate bonds in different proteins. It is also interesting that, when inorganic orthophosphate is incorporated as organic phosphorus by collagen fibrils *in vitro*, the phosphate moiety formed is not a phosphorylated hydroxy amino acid, phosphoamidated amino acid, or phosphorylated sugar (23–25). The possibility that an acyl phosphate bond is formed has not been pursued.

The possible functions of the γ-glutamyl phosphate can only be speculative at this time: direct involvement in the process whereby a solid phase of calcium phosphate is deposited in bone (heterogeneous nucleation, oriented overgrowth, crystal size and shape, and so forth), or indirectly in mineralization or other tissue functions by conferring an enzymatic activity to the collagen molecules or fibrils, such as the ATPase activity noted previously in decalcified bone and reconstituted collagens (25).

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