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Histidinoalanine, a Naturally Occurring Cross-Link Derived from Phosphoserine and Histidine Residues in Mineral-Binding Phosphoproteins†

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ABSTRACT: Native mineral-containing phosphoprotein particles were isolated from the Heterodont bivalve *Macrocallista nimbosa*. The native particles are discrete structures about 40 nm in diameter which migrate as a single band during electrophoresis in agarose gels. Removal of the mineral component with ethylenediaminetetraacetic acid dissociates the native protein into nonidentical subunits. The lower molecular weight subunits, representing 8% of the total protein, were obtained by differential centrifugation. The native protein is characterized by a high content of aspartic acid, phosphoserine, phosphothreonine, histidine, and the bifunctional cross-linking residue histidinoalanine. The low molecular weight subunits have the same amino acid composition except for a reduction in histidinoalanine and a corresponding increase in phosphoserine and histidine residues, demonstrating that the alanine portion of the cross-link is derived from phosphoserine residues. Ion-exchange chromatography and molecular sieve chromatography show that the low molecular weight subunits have a similar charge density but differ in molecular weight, and the relative mobilities of the subunits on agarose gels indicate that they are polymers of a single phosphoprotein molecule. The minimum molecular weight of the monomer is about 140000 on the basis of the amino acid composition. The high molecular weight subunits are rich in histidinoalanine and too large to be resolved by either molecular sieve chromatography or gel electrophoresis. On the basis of the ultrastructural, electrophoretic, chromatographic, and compositional evidence, native phosphoprotein particles are composed of subunits ionically cross-linked via divalent cations. These subunits are variable molecular weight aggregates of a single phosphoprotein molecule covalently cross-linked via histidinoalanine residues. Evidence for a nonenzymatic cross-linking mechanism is discussed.

Histidinoalanine or N^{τ}-(2-amino-2-carboxyethyl)histidine is a bifunctional cross-linking amino acid. It was first identified in dentin and bone (Fujimoto et al., 1982) and then in other vertebrate connective tissues including tendon, cartilage, and aorta (Fujimoto, 1982a; Fujimoto & Yu, 1984). In dentin, histidinoalanine occurs exclusively in free phosphophoryn and an insoluble phosphophoryn–protein conjugate (Kuboki et al., 1984). Phosphophoryns are high-capacity calcium-binding proteins in which about 80% of the amino acid residues are phosphoserine and aspartic acid (Lee et al., 1977). The conjugated form of phosphophoryn is probably complexed to collagen (Dimuzio & Veis, 1978).

Histidinoalanine also occurs in another group of high-capacity calcium-binding proteins called phosphoprotein particles (Sass & Marsh, 1983; Marsh & Sass, 1985). The phosphoprotein particles sequester calcium, magnesium, and inorganic phosphate ions and exist in a stable colloidal suspension in the blood and extrapallial fluid of Heterodont bivalves (Marsh & Sass, 1983, 1984, 1985). The particles are rich in phosphoserine, aspartic acid, and histidine residues and have a histi-

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dinoalanine content ranging from about 10 to over 100 residues per 1000 depending on the species. Here histidinoalanine occurs as both the N^{τ} and N^{π} isomers. The phosphoprotein particles are compact high molecular weight structures about 40 nm in diameter. The purpose of this paper is to demonstrate that the particles are composed of lower molecular weight subunits which are ionically cross-linked via mineral ions and covalently cross-linked via histidinoalanine residues.

It has been postulated that histidinoalanine is formed during alkaline hydrolysis of proteins by β-elimination of H_2O from serine or H_2S from cysteine to give dehydroalanine which subsequently reacts with an imidazole nitrogen on histidine (Finley & Friedman, 1977). Fujmoto (1984) demonstrated that small amounts of histidinoalanine are formed when various proteins such as bovine serum albumin and casein are heated at neutral pH. This study demonstrates that the histidinoalanine residues in the bivalve phosphoprotein particles are not formed during hydrolysis but are naturally occurring cross-links derived from phosphoserine and histidine residues.

Materials and Methods

Preparation of Native Phosphoprotein Particles and Isolation of Low Molecular Weight Subunits. The Sun Ray Venus clams *Macrocallista nimbosa* were obtained live from the Gulf Speciman Co. (Panacea, FL). All fluids were collected which drained from the tissues when the adductor muscles were cut, and the clam was removed from its shells. The fluid was centrifuged for 10 min at 600*g* to remove cells and filtered successively through 5.0- and 0.45-μm filters to remove smaller particulate material. Then the native phosphoprotein particles were sedimented from the fluid by centrifugation at 150000*g* for 40 min. A thin coating of less dense material which covered the particles was dislodged by gentle shaking for a few seconds with water. The coat and water were then removed by aspiration. Finally, the particles were washed by resuspending in water and sedimenting as before.

The particles were demineralized by dialysis against 0.2 M ethylenediaminetetraacetic acid (EDTA),[1] pH 8.3, and then 50 mM NaCl. The demineralized protein was centrifuged for 3 h at 400000*g*. The low molecular weight subunits were recovered in the supernatant fluid, and the high molecular weight subunits were in the pellet.

Chromatography of Low Molecular Weight Subunits. The low molecular weight subunits were charged to a 1.6 × 22 cm column of DEAE-cellulose equilibrated with 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.3, containing 0.2 M NaCl. The column was eluted with 100 mL of the same solution and then with 300 mL of a linear gradient ranging from 0.2 to 0.4 M NaCl in 50 mM Tris, pH 8.3. Fractions of 2 mL were collected at a flow rate of 50 mL/h, and the absorbance was determined at 230 nm. Pooled fractions from the single phosphoprotein band were concentrated and equilibrated with 50 mM NaCl by diafiltration using an Amicon YM 10 membrane. The concentrate was charged to a 1.6 × 100 cm column of Sephacryl S-500 (Pharmacia) equilibrated with 50 mM NaCl. The column was eluted with the same solution, and 3-mL fractions were collected at a flow rate of 40 mL/h.

Agarose Gel Electrophoresis. The native phosphoprotein particles were run on 1% agarose gels 2 mm in thickness; the tank buffer and gel buffer were 0.25 M Tris–0.192 M glycine, pH 8.3, containing 10 mM $CaCl_2$. The demineralized proteins and subunit fractions were run under the same conditions, but $CaCl_2$ was omitted. The gels were stained with Stains All as described by Green et al. (1973).

Electron Microscopy. Aqueous solutions of the native and demineralized phosphoprotein (2 mM in organic phosphate) were applied directly to carbon-coated electron microscope grids. The bulk of the solution was withdrawn with a strip of filter papter, and the grids were allowed to dry. The protein was examined and photographed in a Hitachi 11E electron microscope operated at 75 kV.

Amino Acid Analysis. The demineralized protein (unfractionated) and the low molecular weight subunit fraction were hydrolyzed in 6 N HCl for 24 h at 106 °C and analyzed on a Glenco amino acid analyzer equipped with a 0.32 × 30 cm column of DC-4A resin and Pico-Buffer System II (Pierce Chemical Co.). Residues were detected by postcolumn reaction with ninhydrin. Phosphoserine and phosphothreonine were resolved in a separate analysis on the same instrument using 0.2 M sodium citrate buffer at pH 1.5. When authentic samples of phosphoserine and phosphothreonine were hydrolyzed under the same conditions, 13.5% of the serine and 12.6% of the threonine were destroyed. Serine and threonine losses in the protein hydrolysate were assumed to be similar, and the appropriate corrections were made. Phosphate residues were determined by summing the inorganic phosphate content of the hydrolysate, measured as described by Ames and Dubin (1960), with the residual phosphoserine and phosphothreonine content measured with the amino acid analyzer.

Determination of the Ninhydrin Color Yield for Histidinoalanine. N^{τ}- and N^{π}-histidinoalanine were isolated from *Macrocallista* phosphoprotein particles as previously described (Sass & Marsh, 1983). Nuclear magnetic resonance spectra were obtained on D_2O solutions of histidinoalanine containing a measured amount of sodium 3-(trimethylsilyl)tetradeuteriopropionate (TSP) with a JEOL FX90Q instrument using 20–80-ms pulse delays. The two β-methylene hydrogens of histidinoalanine generated two signals that were 3.30 and 3.38 ppm downfield from the single resonance of the nine methylene hydrogens in TSP. The molar concentration of histidinoalanine (M_H) was estimated by the equation:

$$M_H = 9(I_{3.30} + I_{3.38})M_T/2I_0$$

where M_T is the molar concentration of TSP, I is the intensity of the signals measured by peak areas, and the subscripts refer to the chemical shifts relative to TSP. Values of M_H were independent of the pulse delay in the interval of 20–80 ms and were reproducible within a 5–10% accuracy. The molar absorptivity of both N^{τ}- and N^{π}-histidinoalanine was about 1.7-fold greater than that of leucine after reaction with ninhydrin using the amino acid analyzer described previously.

Results

The native phosphoprotein, which is complexed with mineral ions, exists as discrete particles about 40 nm in diameter when examined in the electron microscope (Figure 1a). When the protein is demineralized, its viscosity in solution increases, and in the electron microscope it appears as an amorphous mass of low electron density containing poorly defined centers of higher electron density (Figure 1b). The native particles migrate as a single band with a relative mobility of about 0.25 with respect to bromphenol blue in 1% agarose gels when subjected to electrophoresis at pH 8.3 in the presence of calcium ions (Figure 2a). When the protein is demineralized with EDTA, it dissociates into heterogeneous subunits. The majority of the subunits do not penetrate the agarose gel during electrophoresis (see below). The small amount of protein

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TSP, sodium 3-(trimethylsilyl)tetradeuteriopropionate.

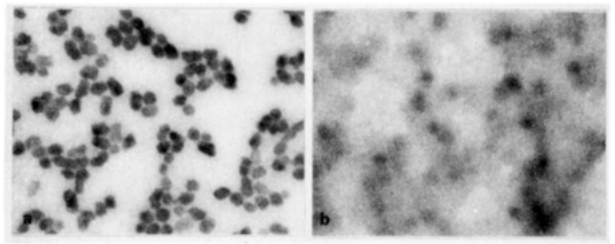

FIGURE 1: Electron micrographs of native (a) and demineralized (b) phosphoprotein particles. Magnification 40500×.

FIGURE 2: (a) Electrophoresis of the native phosphoprotein particles on 1% agarose gels in Tris–glycine buffer, pH 8.3, containing 10 mM $CaCl_2$. (b) Electrophoresis of EDTA–demineralized phosphoprotein particles under the same conditions but in the absence of $CaCl_2$.

which does penetrate the gel migrates as a streak of low mobility and discrete bands of higher mobility (Figure 2b).

The demineralized phosphoprotein was centrifuged at 400000*g* to yield a low molecular weight fraction in the supernatant fluid, which represented only 8% of the total protein. Figure 3 is an agarose gel to which the unfractionated protein and the high and low molecular weight fractions were applied in equal amounts. The low molecular weight fraction is dominated by a high-mobility subunit (I) and contains progressively lesser amounts of the lower mobility subunits II, III, and IV. In addition to the discrete subunits, this fraction contains material which streaks from the origin to the end of the gel. The high-mobility subunits are absent in the high molecular weight fraction. Comparison of lanes 1, 2, and 3 in Figure 3 demonstrates that very little of the high molecular weight fraction (lane 2), which contains the majority of the protein, is capable of penetrating the gel.

The native phosphoprotein particles derived from *Macrocallista* are characterized by a high content of aspartic acid, phosphoserine, phosphothreonine, histidine, and histidinoalanine residues (Table I). Serine and threonine are probably fully phosphorylated because the number of phosphate residues is equal to the sum of the serine and threonine residues. Except for the cross-linking residues, the amino acid compositions of the native protein and its low molecular weight fraction are similar. Per 1000 amino acid residues, the native protein contains 78 more histidinoalanine residues and correspondingly 73, 82, and 79 fewer serine, phosphate, and histidine residues, respectively. This result demonstrates that the histidinoalanine cross-links are derived from phosphoserine and histidine residues.

When the low molecular weight fraction is applied to a DEAE-cellulose column and eluted with a shallow NaCl gradient, all subunits are eluted in a single band (B) at 0.33

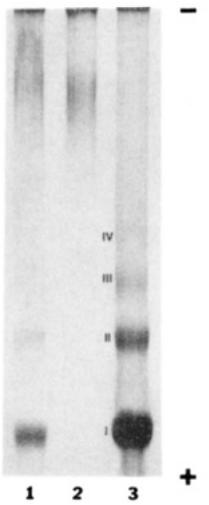

FIGURE 3: Agarose gel of the demineralized phosphoprotein. Lane 1 is the total protein, lane 2 is the high molecular weight fraction, and lane 3 is the low molecular weight fraction. Equal amounts of protein were applied to each lane. Roman numerals refer to the discrete low molecular weight subunits. Electrophoretic conditions are given in Figure 1b.

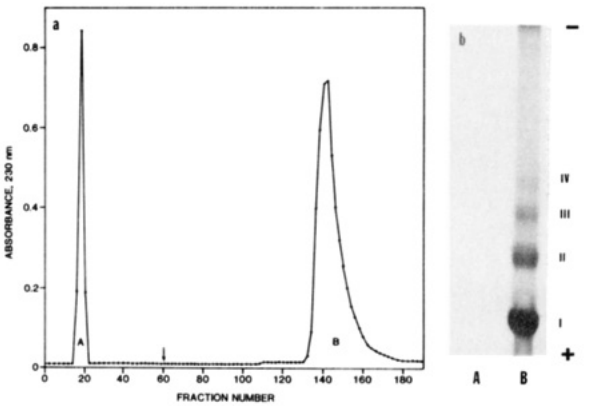

FIGURE 4: (a) Chromatography of the low molecular weight fraction on a DEAE-cellulose column (1.6 × 22 cm). Eluted with 100 mL of 0.2 M NaCl in 50 mM Tris, pH 8.3, and then with a linear gradient from 0.2 to 0.4 M NaCl in the same buffer. The arrow indicates the beginning of the gradient. Band B was eluted in 0.33 M NaCl. (b) Agarose gel of the combined fractions of bands A and B. Conditions given in Figure 1b.

Table I: Amino Acid Composition of the *Macrocallista* Phosphoprotein[a]

residue	native protein	low mol wt fraction
aspartic acid	197	200
threonine	109	106
serine	171	244
glutamic acid	13	15
glycine	77	77
alanine	1.3	1.5
valine	3.9	3.5
isoleucine	1.5	1.5
leucine	1.5	1.5
tyrosine	1.5	1.0
phenylalanine	1.0	1.0
half-N^{τ}-histidinoalanine	129	1.0
half-N^{π}-histidinoalanine	27	0.3
histidine	264	343
arginine	2.8	3.9
phosphate	283	365

[a] Units are residues per 1000 amino acid residues.

M NaCl (Figure 4). An additional component (A) eluting in 0.2 M NaCl contained no protein and was probably residual EDTA (Dimuzio & Veis, 1978). When the subunits contained

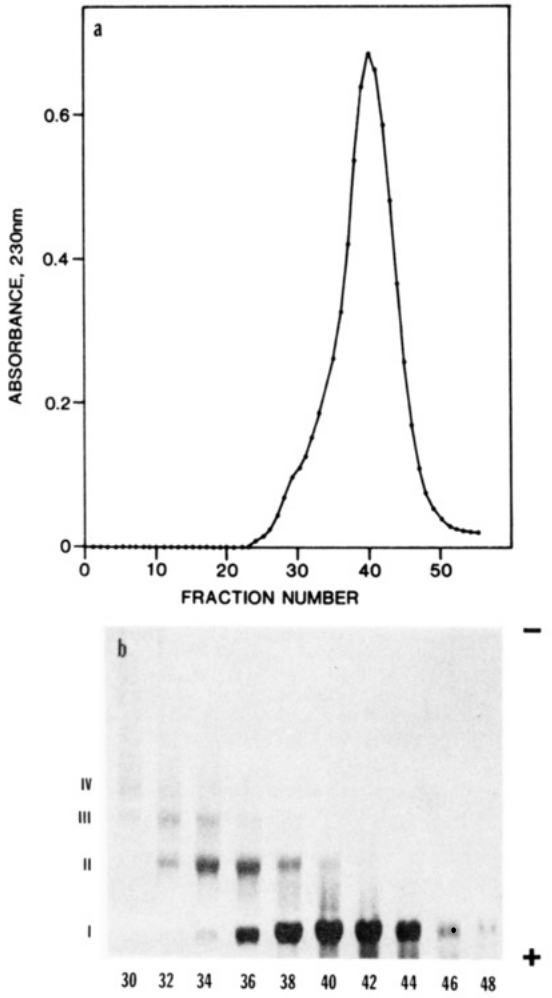

FIGURE 5: (a) Chromatography of band B (Figure 3) on a Sephacryl S-500 column (1.6 × 100 cm). Eluted with 50 mM NaCl. (b) Agarose gel of individual fractions from the same column. Conditions given in Figure 1b.

in band B were subjected to molecular sieve chromatography on Sephacryl S-500, the protein eluted in a broad peak with a leading shoulder (Figure 5). Agarose gel electrophoresis of fractions collected through the peak demonstrated that subunits I, II, III, and IV were sequentially eluted in reverse order. Thus, the subunits have a similar charge density but vary in molecular weight. There is a linear relationship between the relative mobility of the subunits on agarose gels and log (nM), where M is an unspecified molecular weight and n is 1, 2, 3, and 4 for subunits I, II, III, and IV, respectively (Figure 6). Therefore, subunits II, III, and IV are probably the dimer, trimer, and tetramer, respectively, of subunit I. The major subunit (I) may be the phosphoprotein monomer since the histidinoalanine content of the low molecular weight fraction is small. Assuming one phenylalanine residue per molecule, the minimum molecular weight of the monomer is 139 000 based on the amino acid composition of the low molecular weight fraction. The low molecular weight components which streak from the origin to the end of the gel during electrophoresis may represent subunit fragments produced by random peptide bond cleavage at phosphoserine residues (see below). The subunits continued in the high molecular weight fraction are too large to be resolved by either molecular sieve chromatography or gel electrophoresis.

Discussion

The native phosphoprotein particles of *Macrocallista nimbosa* can be dissociated into subunits of different molecular

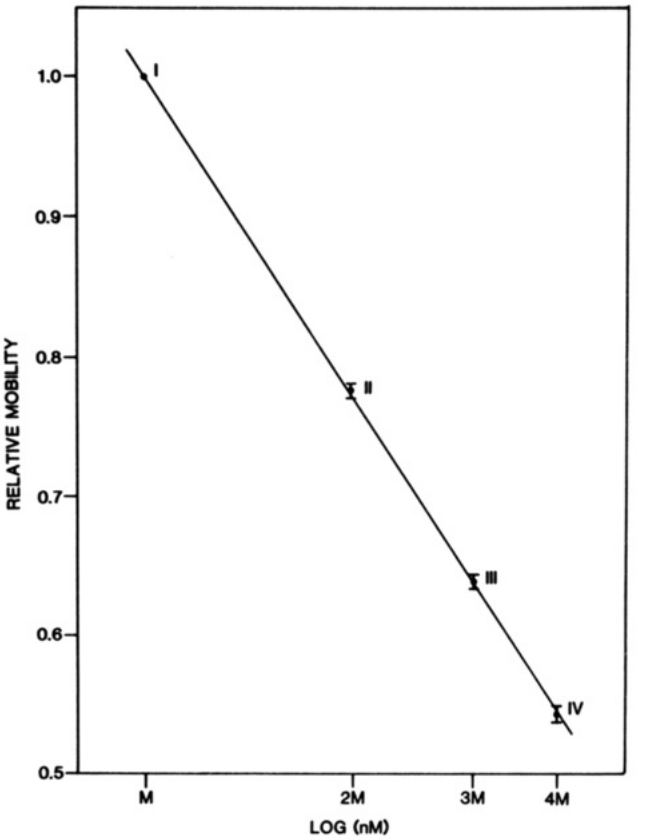

FIGURE 6: Relative mobility of the low molecular weight phosphoprotein subunits on agarose gels as a function of log (nM) where M is an unspecified molecular weight and n is 1, 2, 3, and 4 for subunits I, II, III, and IV, respectively. Mobilities were measured relative to subunit I. Electrophoretic conditions given in Figure 1b.

weight by treatment with EDTA to remove the mineral ions. The low molecular weight subunits represent 8% of the total protein and have the same amino acid composition as the native particles except for the absence of histidinoalanine and a corresponding increase in phosphoserine and histidine residues from which the cross-links are derived. The histidinoalanine was not formed during acid hydrolysis for amino acid analysis, since both the low molecular weight subunits and the unfractionated phosphoprotein were hydrolyzed under the same conditions. Cross-linking could not have occurred during the isolation of the phosphoprotein particles since the conditions used were very mild; i.e., only filtration and differential centrifugation methods were used, and the entire procedure required only 4 h. Therefore, histidinoalanine is a naturally occurring cross-link in the phosphoprotein particles. The low molecular weight fraction contains very little histidinoalanine and is dominated by a single subunit (roughtly 75% of the fraction) which may be the phosphoprotein monomer. On the basis of the amino acid composition, the minimum molecular weight of the monomer is about 140 000. The native phosphoprotein particles are composed of subunits ionically cross-linked via divalent cations. The subunits in turn are variable molecular weight aggregates of a single phosphoprotein molecule covalently cross-linked via histidinoalanine residues.

The mineral ions also affect the conformation of the polypeptide chains. The native protein forms discrete compact particles about 40 nm in diameter, has a low solution viscosity, and readily penetrates 1% agarose gels. The demineralized protein is not compact and has a high solution viscosity, and its larger subunits are too voluminous to penetrate a 1% agarose gel. These properties indicate that in the absence of

divalent mineral ions the phosphoprotein chains with their high negative charge density are probably fully extended at least to the degree permitted by the covalent cross-links. In the native mineralized state, the phosphoprotein chains are collapsed in a compact structure. The conformation of the compact structure is unknown.

Formation of the histidinoalanine cross-links may not be an enzymatic process. Enzyme-catalyzed reactions characteristically produce only a single isomer while the histidinoalanine in the phosphoprotein particles occurs as both the N^{τ} and N^{π} isomers. Cross-link formation may be initiated by elimination of phosphate from phosphoserine to yield dehydroalanine residues which then react with available histidine residues. In addition to cross-linking, dehydroalanine can further decompose into pyruvate and NH_3, causing cleavage of the peptide chain. In this connection, it should be noted that both cross-linking and molecular weight polydispersity in the dentin phosphophoryns have been attributed to the nonenzymatic degradation of phosphoserine residues (Masters, 1985; Fujisawa, 1985). Fujisawa (1985) has identified pyruvate in phosphophoryn, so the streaking observed in agarose gels of the bivalve phosphoprotein may also be due to molecular weight polydispersity caused by random chain cleavage at phosphoserine residues.

The histidinoalanine content of vertebrate connective tissues has been correlated with both aging and calcification (Fujimoto, 1982a,b; Fujimoto & Yu, 1984). The elevated level of histidinoalanine in calcified tissues may indicate the presence of highly phosphorylated proteins since they are postulated intermediates in the calcification process (Veis, 1978; Marsh & Sass, 1984). An increase in the histidinoalanine content with age probably reflects a slow degradation of phosphoserine residues in those proteins which are retained indefinitely in the extracellular matrix without turnover.

The histidinoalanine content is 1 or 2 orders of magnitude greater in the bivalve phosphoprotein particles than in any vertebrate protein examined to date, undoubtedly due to the very high level of phosphoserine and histidine residues in the former. The histidinoalanine content can vary more than 10-fold in phosphoprotein particles isolated from different species (Marsh & Sass, 1985). Although the amino acid composition of the particles is different in each species, aspartic acid, phosphoserine, and histidine represent at least 80% of the residues in all of them. Conditions regulating the extent of cross-linking in the phosphoprotein particles have not been identified.

Registry No. L-Histidine, 71-00-1; L-phosphoserine, 407-41-0; N^{τ}-histidinoalanine, 65428-77-5; N^{π}-histidinoalanine, 87047-17-4.

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