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N^{τ} - AND N^{π}-HISTIDINOALANINE: NATURALLY OCCURRING CROSS-LINKING AMINO ACIDS IN CALCIUM-BINDING PHOSPHOPROTEINS

Ronald L. Sass and Mary E. Marsh

Department of Biology
Rice University
Houston, Texas 77251

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SUMMARY: Two isomeric amino acid cross-links, N^{τ}-histidinoalanine and N^{π}-histidinoalanine have been isolated from calcium-binding phosphoprotein particles derived from the extrapallial fluid of the estuarine clam Rangia cuneata. The cross-links were identified and compared by ^{13}C and ^{1}H NMR spectroscopy and mass spectroscopy. In the phosphoprotein particles, 6% of the amino acid residues are involved in cross-linkages. This is the first demonstration of the occurrence of the isomer N^{π}-histidinoalanine.

INTRODUCTION: Calcium-binding phosphoprotein particles approximately 40 nm in diameter are components of the extrapallial fluid and developing shell lamella of the estuarine clam Rangia cuneata (1). Particles isolated from the extrapallial fluid have a molecular weight in excess of 30 million and cannot be reduced to smaller subunits by treatment with common dissociating agents such as 8M urea and 1% sodium dodecyl sulfate or by demineralization with EDTA. The phosphoprotein contains no disulfide bridges and is very polar; 90% of the amino acid residues are phosphoserine (29%), aspartic acid (22%), histidine (35%) and lysine (4.6%). In this report we describe the isolation and characterization of two bifunctional amino acids, N^{τ}- and N^{π}-histidinoalanine, which can function as covalent cross-links between monomeric subunits within the particles.

MATERIALS AND METHODS: Phosphoprotein particles were isolated from the extrapallial fluid of Rangia cuneata as described previously (1). One gram of the particles was hydrolyzed in 25 ml of 6N HCl in sealed tubes for 24 hr at 105°C. HCl was removed under reduced pressure at room temperature. The residue was suspended in 10 ml of water and filtered through a 0.45 μm filter. The filtrate was applied to a 0.8 x 25 cm column of Dowex 50W-X8 (200-400 mesh; H^+ form). The column was washed with 50 ml 3N HCl to elute the monofunctional amino acids. Then two unidentified compounds (I and II) were eluted with 30 ml of 4N HCl. The HCl was removed as before, then I and II were separated from each other by chromatography on the same resin previously equilibrated with 0.2N sodium citrate, pH 3.25. The 0.8 x 25 cm column was eluted with 0.38N sodium citrate pH 5.35 at 50°C, and 1.0 ml fractions were collected. I was eluted in fractions 21 through 30, and II was eluted in fractions 32 through 48. The appropriate fractions were pooled and desalted on 0.8 x 25 cm columns of Dowex 50W-X8 (H^+ form). Citrate ions were eluted with 25 ml water, and sodium ions with 35 ml 2N HCl. The unknowns were eluted with 4N HCl, evaporated to dryness and taken up in 0.5 ml water. Contaminates derived from ion exchange resins were removed by gel filtration chromatography on Sephadex G-10 columns (1.6 x 100 cm) equilibrated and eluted with water.

Analytical amounts of phosphoprotein particles derived from the extrapallial fluid of Rangia cuneata and Mercenaria mercenaria were hydrolyzed at 105°C in a 4N methanesulfonic acid solution (2) for 24 to 72 hrs.

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Amino acid analyses were performed on a Glenco amino acid analyzer equipped with a 0.32 x 30 cm column of DC-4A resin and Pico-Buffer system II (Pierce Chemical Co., Rockford, IL).

I and II were characterized by ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy on a JEOL FX90Q at the Department of Chemistry, Rice University. All spectra were recorded in D_2O at pD 7; chemical shifts are referenced to sodium 3-trimethylsilylpropionate (TSP) internal standard. Mass spectra of underivatized and methyl ester-trifluoracetyl derivatives of I and II were recorded on a Finnigan Model 1015 GC-MS instrument modified for desorption chemical ionization (DCI) at the Institute for Lipid Research, Baylor College of Medicine, Houston, Texas (3).

RESULTS: NMR and mass spectra demonstrate that the unidentified residues I and II are the N^{τ} and N^{π} isomers of the bifunctional amino acid N-(2-amino-2-carboxyethyl)-histidine (Fig. 1). For the purpose of explanation, the imidazole ring atoms are numbered according to IUPAC convention, while the atoms on the side chains are labeled according to common convention for amino acids. The N^{τ} isomer, given the trivial name histidinoalanine, was recently discovered in bone and dentin collagen where it represents less than 0.04 mole % of the amino acid residues (4). This is the first report of a naturally occurring N^{π}-histidinoalanine cross-link in proteins. The ^{13}C proton decoupled NMR chemical shifts and proton coupled multiplicities for I and II are presented in Table I. The published chemical shifts for histidine (5) are included for comparison. The spectral assignments are straightforward. The chemical shifts of I and II are similar and comparable to those for equivalent nuclei of histidine. The downfield shift of Cβ' is greater than that of Cβ in both I and II because of the deshielding effect of the bonded imidazole nitrogen. The peak multiplicities observed in proton coupled spectra are consistent with the structure in Figure 1. The multiplet structure of C2 was obscured by slow proton exchange during the experiment. Effects from this exchange were also evident in proton spectra and have been reported in previous studies of imidazole derivatives (6). The ^{1}H NMR spectra of I and II are shown in Figure 2 and compared with that of 1-methylhistidine. Side chain hydrogens of both I and II show evidence of higher order ABX interaction. In I (Fig. 2a) the imidazole protons occur at 7.71(1H,s,C2) and 7.09(1H,s,C5). The β'-methylene of the nitrogen bonded side chain is at 4.55(2H,d,J=5Hz) while the β-methylene of the carbon bonded side chain gives two signals at 3.18(1H,d,J=5Hz) and 3.16(1H,d,J=9Hz). The alpha hydrogens from the two side chains

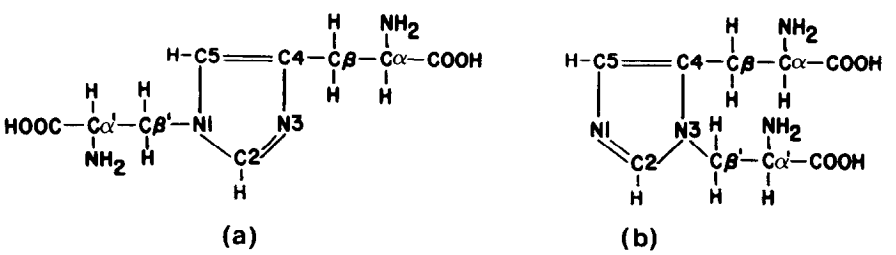

Figure 1. a) N^{τ}-histidinoalanine and b) N^{π}-histidinoalanine.

TABLE I - ^{13}C NMR SPECTRA OF COMPOUNDS I AND II

Atom	Compound I δppm	Compound II δppm	H Coupled Multiplicity	Histidine[b] δppm
Cβ	32.39	29.80	3	35.9
$C\beta^1$	50.78	51.46	3	
$C\alpha$ ($C\alpha^1$)	58.44	57.46	2	61.4
$C\alpha^1$ ($C\alpha$)	58.73	58.68	2	
C5	122.28	124.87	2	126.8
C4	139.45	134.98	1	137.3
C2	142.44	142.28	a	143.0
COOH	175.41	174.92	1	
COOH	177.64	176.48	1	180.0

a. Multiplet structure of C2 was obscured due to H-D exchange while the spectra were obtained.

b. Comparable reported values for histidine (5).

give a multiplet of peaks from 3.96 to 4.19(2H,m). In II (Fig. 2b) the imidazole protons are observed at 7.87(1H,s,C2) and 7.09(1H,s,C5). The nitrogen bonded β'-methylene is at 4.55(2H,d,J=7Hz) and the β-methylene is at 3.32(2H,d,J=6Hz). The alpha hydrogens are

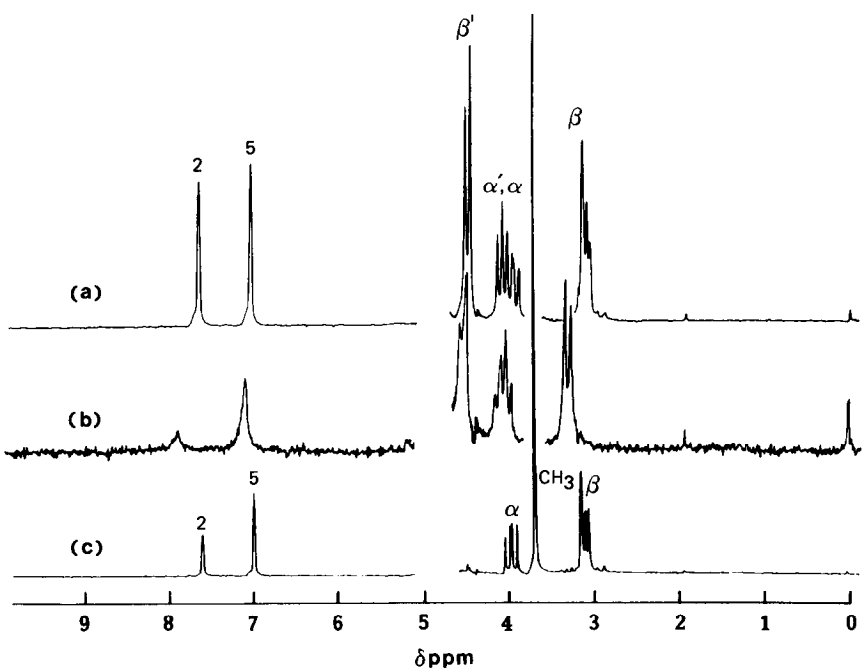

Figure 2. ^1H NMR spectra of a) Compound I, b) Compound II, c) N^{τ}-methylhistidine for comparison. Water resonance is removed for clarity.

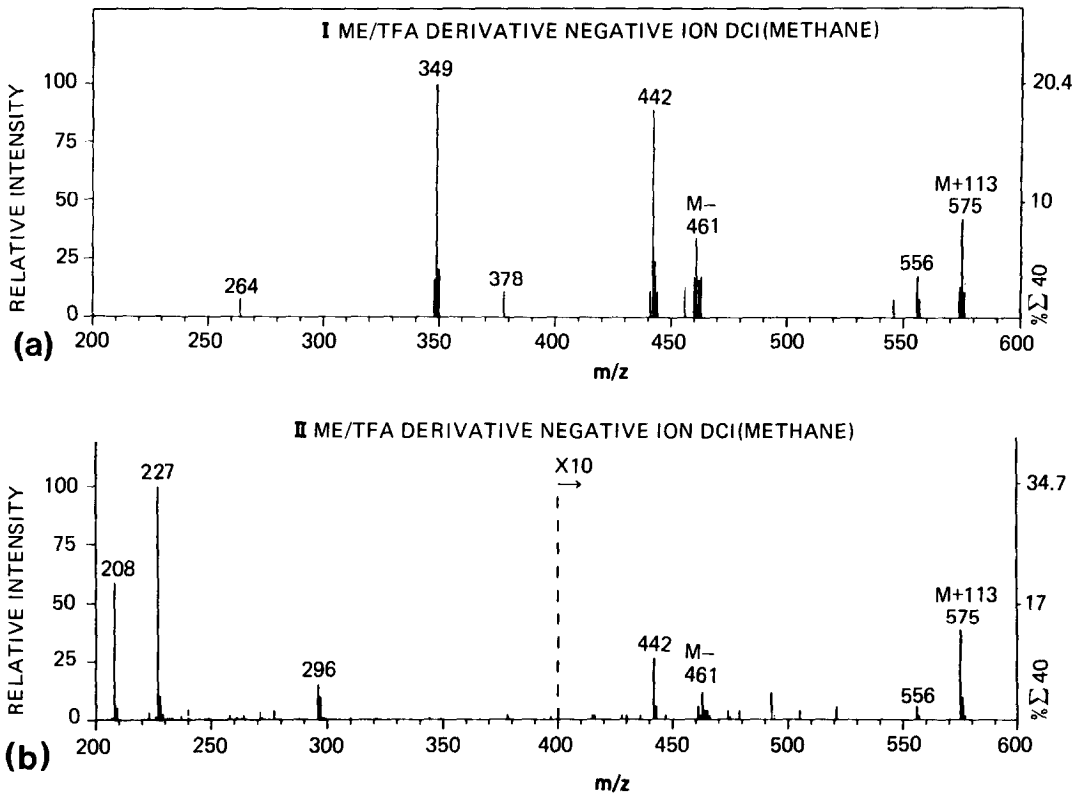

Figure 3. The negative ion desorption chemical ionization mass spectra of the methyl ester-trifluoroacetyl derivative of a) Compound I and b) Compound II.

again not resolved, showing a multiplet of peaks from 3.96 to 4.16(2H,m). At pD 5 (not shown) the α and α' hydrogens separate into two triplets in both I and II.

The underivatized positive ion DCI mass spectra of both I and II exhibited a molecular ion at m/e 243 (M+H) consistent with an elemental composition of $C_9H_{14}N_4O_4$ for each. A fragment at m/e 156 is accounted for by the loss of a side chain, and one at m/e 199 corresponds to the loss of CO_2 which probably arises from thermal decomposition on the probe. The negative ion DCI mass spectra of the methyl ester-trifluoracetyl derivatives of I and II are presented in Figure 3. The molecular ion peak (M-H) is at m/e 461, and the reagent adduct ($M+CF_3COO$) is at m/e 575. Peaks at m/e 442 and 556 correspond to the loss of fluoride atom from each of the above ions. The identification of m/e 575 as the trifluoracetyl adduct ion was made by selectively subjecting it to collision induced dissociation in a triple quadrapole mass spectrometer system at the Institute for Lipid Research, Baylor College of Medicine. The resulting spectrum (Fig. 4) shows only the fragments (M-H) at m/e 461 and the trifluoracetyl adduct anion at m/e 113.

I and II occur at a relative abundance of 3:1 and are designated as the N^{τ} and N^{π} isomers, respectively, of histidinoalanine. Isomeric assignment was made on the following bases: 1. N substitution of histidine occurs predominately at N^{τ} (7) and would account for

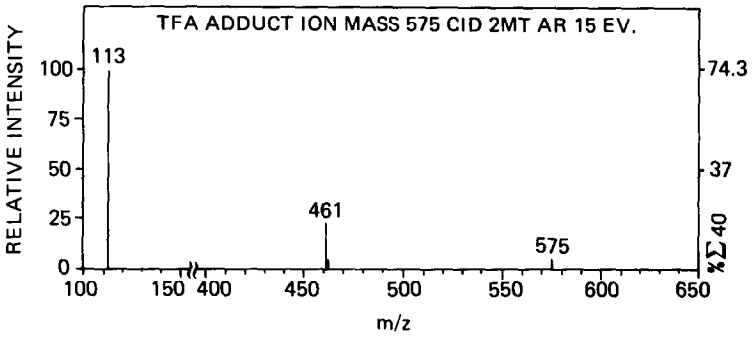

Figure 4. The collision induced dissociation negative ion mass spectrum of the m/e 575 ion of the methyl ester-trifluoracetyl derivative of Compound I.

the larger quantity of compound I observed; 2. The ^{13}C NMR chemical shift differences between equivalent imidazole ring atoms of I and II correlate with shift differences observed in N^{τ} - and N^{π}-methyl-histidine (8).

Under the conditions of amino acid analysis described here, N^{τ}-histidinoalanine elutes in 71 min, the N^{π} isomer in 72 min, and lysine in 75 min. The histidinoalanines represent 6.0 mole % of the residues (based on leucine equivalents) in the phosphoprotein particles isolated from Rangia cuneata and 1.0 mole % in particles from Mercenaria mercenaria. The concentration of the cross-links is independent of 1. the size (age) of the animals, 2. the duration of hydrolysis, and 3. the hydrolytic agent (HCl or methanesulfonic acid). The cross-link concentration is, however, species dependent.

DISCUSSION: Histidinoalanine is a member of a class of cross-linking compounds postulated to form when proteins are treated with strong alkali or high temperature (9). Under these conditions dehydroalanine is derived from phosphoserine, serine or cysteine by β-elimination. Dehydroalanine reacts further with an available nucleophile to complete the cross-link. Of the several possible diamino acid cross-linking compounds formed in this manner, the one most often reported is lysinoalanine. The formation of histidinoalanine by these methods has not been demonstrated. N^{τ}-histidinoalanine occurs in mammalian connective tissue. Its content increases with age, and its formation is thought to be non-enzymatic, non-specific and slow to proceed (10). However, the formation of histidinoalanine in phosphoprotein particles is apparently under strict biological control and is species specific. The absence of detectable lysinoalanine in the particles suggests that the more nucleophilic lysine residues are not available as a reactant either because of geometric constraints or as a result of an enzymatic specificity for histidine.

In the Rangia particles, 6% of the amino acid residues are involved in cross-linkages. The particles contain a protected pool of mineral ions (unpublished results), and the cross-links may be functional requirements for maintaining the structural integrity of the particles as they transport the mineral to the developing shell surface (1). If the large

histidine content (35 mole %) and cross-links of the phosphoprotein particles are ignored, then the amino acid composition of the particles is essentially identical to the composition of phosphophoryn, a protein of average molecular weight and a postulated intermediate in vertebrate dentin mineralization (11). In the particles, histidinoalanine may cross-link domains with a high aspartic acid and phosphoserine content, perhaps compositionally and functionally similar to phosphophoryn, to domains which are essentially polyhistidine.

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