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THE STRUCTURE OF PYRIDINOLINE, A COLLAGEN CROSSLINK

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SUMMARY Pyridinoline is an amino acid isolated from collagen and probably serves as a crosslink in collagen fiber. This compound was isolated on a large scale from bovine bone and investigated by ^{1}H-nmr and ^{13}C-nmr spectroscopy, mass spectroscopy and chemical degradation. The structure is proposed on the basis of these data.

The function of collagen is mainly mechanical and intermolecular crosslinking is necessary for tensile strength in collagen fiber. Structures of several crosslinks have been known (1). Recently we isolated a new amino acid from Achilles tendon collagen (2). The compound is a 3-hydroxypyridinium derivative with three amino acid side chains and has been named pyridinoline (2). Its structure suggests that it is a crosslink of novel type. However, the complete structure remains to be elucidated. This compound has now been isolated on a larger scale and in this communication the structure is proposed.

MATERIALS AND METHODS

Pyridinoline was isolated from bovine decalcified bone which was supplied by Dr. T. Fujii, Nippi Co., Tokyo. About 100 g of bone was refluxed with 600 ml of 6N HCl for 24 h. The solution was then dried up under reduced pressure and the hydrolysate was taken up in water (1000 ml), and applied on an Amberlite CG-120 column (H^+ form, 3.6 x 20 cm). The column was washed successively with 1000 ml each of water and 2N HCl and then pyridinoline was eluted with 4N HCl. The eluate was dried up *in vacuo*, taken up in water (1000 ml) and applied on a P-cellulose column (H^+ form, 3.6 x 25 cm). Elution was performed with a linear gradient, starting with 700 ml of water in the mixing chamber and 700 ml of 0.6N HCl in the reservoir. Fractions (18 ml each) were collected and ultraviolet absorption spectra were measured. The fractions showing the characteristic spectrum of pyridinoline (2) were combined, dried up *in vacuo* and further purified as described previously (2). About 30 mg of pyridinoline was obtained from total 300 g of bone. The preparation had the same chromatographic and spectral properties as those of pyridinoline isolated from purified bovine Achilles tendon collagen (2).

^{1}H-nmr spectrum was recorded in D_2O on a Jeol (PS-100)-(PFT-100)-(EC-100) spectrometer using sodium dimethylsilapentanesulfonate as an internal

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standard. ^{13}C-nmr spectrum was recorded in D_2O on the same instrument, using methanol as an external standard. Mass spectrum was recorded on an Jeol-JMS-01SG-2 mass spectrometer using perfluoroalkane 250(Pierce Chemical Co., U.S.A.) as a standard. Preparation of trifluoroacetyl methyl ester derivative was performed according to the method of Bailey et al (3). Degradation of pyridinoline was carried out by heating the sample (0.5 μmole) at 110°C for 24 h in 0.2 ml of water under air in a sealed tube.

RESULTS AND DISCUSSION

The ^{1}H-nmr spectrum of pyridinoline is shown in Fig.1A. The spectrum was similar to that obtained previously (2), however, since a large amount of the sample was available, higher resolution was attained and the number of protons could be estimated more accurately. The spectrum shows two singlets (7.61 and 7.66 ppm) in the aromatic region, indicating the presence of two ring protons rather than one ring proton (2). In the previous study, the position of the ring proton was assigned not to be α by comparison with the chemical shift of α proton of pyridoxine hydrochloride (8.13 ppm). However, the spectrum of pyridinoline was measured in the neutral solution and under the conditions, the hydroxyl group was dissociated (2). Therefore, the spectrum had to be compared with that of pyridoxine of the zwitterion form. The chemical shift of the ring proton of pyridoxine zwitterion is 7.67 ppm (4), which agrees with those of pyridinoline. The signals of β and γ protons are known to appear in higher field (5). Therefore, it can be concluded that there are two α protons in the ring of pyridinoline.

The spectrum also indicates the presence of two benzyl type methylene groups (2.84, d and 3.28, d, 4H) and three aliphatic methylene groups (1.5-2.2, m, 6H) (6). The other absorptions (3.7 - 4.6, m, total 6H) correspond to methylene attached to N^+, CHOH and α-hydrogens of the amino acid moieties (6,7). The spectrum excludes the possibility of the presence of CH_2OH attached to the ring (ca. 5 ppm) , which was postulated in the previous study (2).

The ^{13}C-nmr spectrum is shown in Fig.1B. The signals correspond to three carboxyl groups (164.8, 164.4, 163.3), five ring carbons (155.5, 132.7, 129.4, 121.9, 119.2), CHOH and CH_2-N^+ (60.1, 55.7), three α-carbons (44.8)

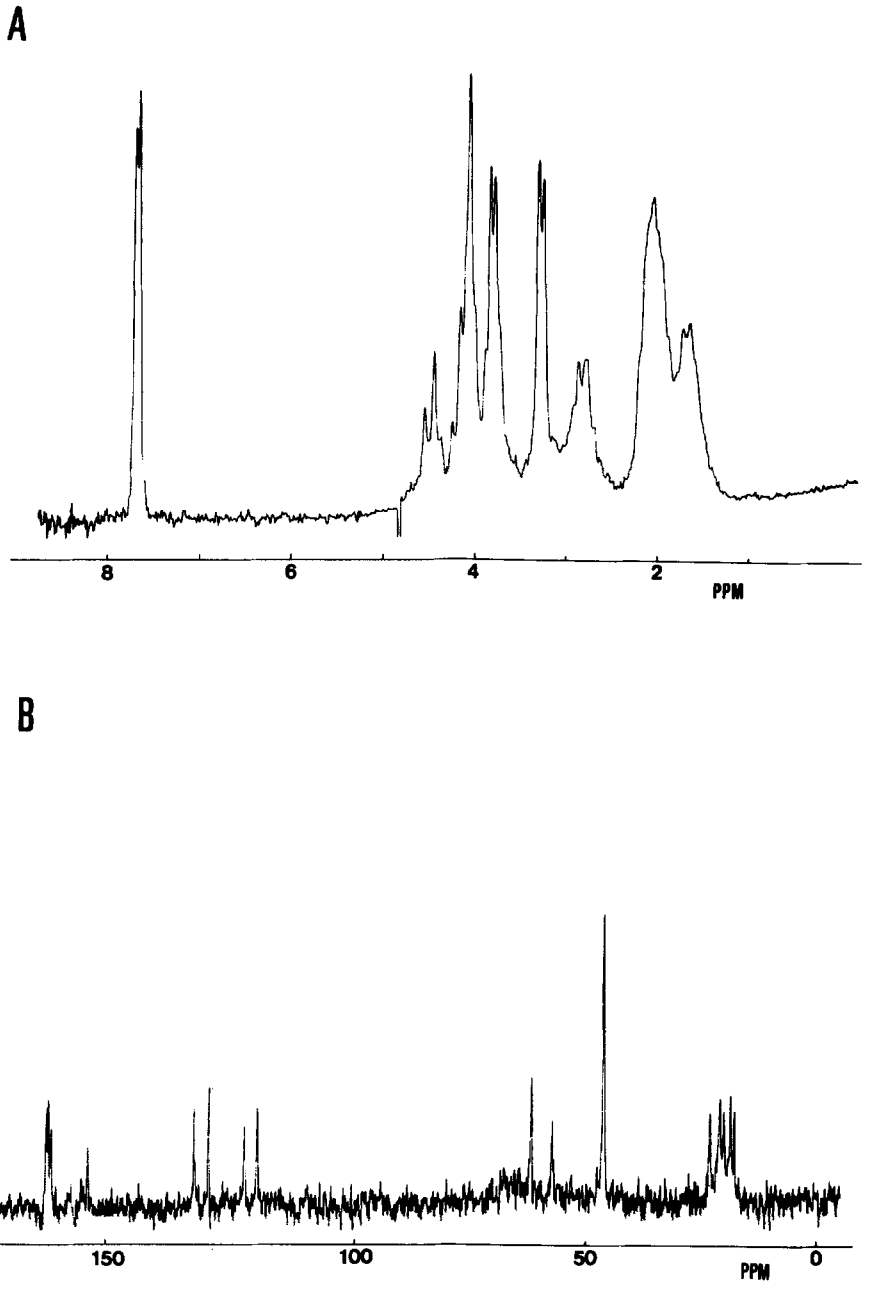
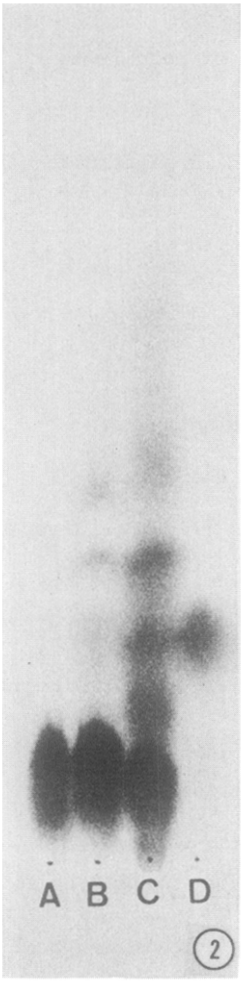


Fig.1. ^{1}H-nmr spectrum (A) and ^{13}C-nmr spectrum (B).

and five methylene groups (21.7, 19.5, 18.8, 17.3, 16.6).

Pyridinoline was converted to the trifluoroacetyl methyl ester derivative and subjected to electron ionization mass spectrometry. Although the peak corresponding to the molecular ion (pentatrifluoroacetyl pyridino-



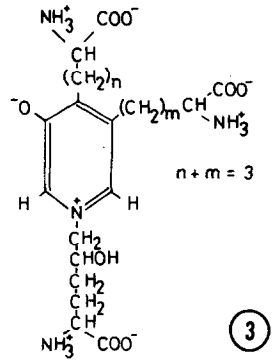


Fig.3. The structure of pyridinoline

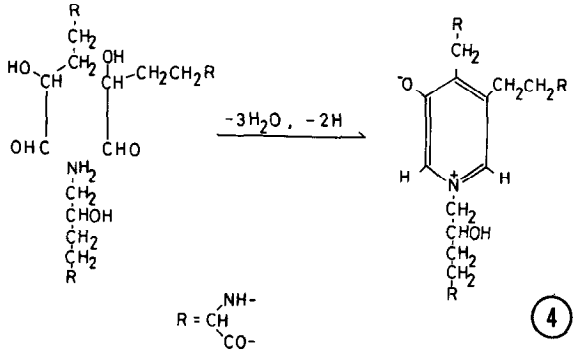


Fig.4. Postulated scheme of biosynthesis for pyridinoline.

Fig.2. Paper chromatogram of degradation products of pyridinoline. Pyridinoline (0.5 µmole) was heated at 110°C for 24 h in 0.2 ml of water or 6N HCl. The solution was then dried up in vacuo and the residue was taken up in 20 µl water. Three µl was applied on Toyo No.51 filter paper and developed in n-butanol-acetic acid-water (4:1:2). Amino acids were located with ninhydrin. A: pyridinoline, not heated., B: pyridinoline, heated in 6N HCl., C: pyridinoline, heated in water., D: hydroxylysine (Sigma).

line trimethyl ester, M/e=951) was very small, prominent peaks corresponding to $M^+ - CH_3$ (936) and $M^+ - CF_3CO$ (854) were observed.

Pyridinoline is fairly stable to acid hydrolysis. However, when it was heated in water under air, it was degraded appreciably, yielding several ninhydrin-reacting products (Fig.2). One of then was isolated by prepara-

tive paper chromatography and identified as hydroxylysine by amino acid analyzer and paper chromatography in the solvent systems of phenol-water (4:1), t-butanol-formic acid-water (14:3:3) and n-butanol-pyridine-water (1:1:1). This result indicates that the pyridine nitrogen is quaternated by δ-hydroxy-α-aminocaproic acid residue.

All these data lead to the structure proposed in Fig.3. The sum of n and m is three, but the exact values of n and m cannot be decided by chemical analyses.

It is known that various crosslinks of collagen and elastin are synthesized from lysine and/or hydroxylysine residues and the initial reaction of the synthesis is the formation of ε-aldehydes (1). For example, desmosine, a tetrafunctional crosslink containing pyridinium ring, has been shown to arise from the condensation of one lysine and three lysine-derived aldehydes (1). A crosslinked, triple-chained peptide containing pyridinoline has been isolated from bovine Achilles tendon collagen after the digestion with pronase (8). Its amino acid composition was characteristic: it contained His_2, Arg, Hyp, Asx_2, Glx_5, Pro_6, Gly_7, Ala_2, Met, Leu_2 and one residue of pyridinoline, and it lacked lysine and hydroxylysine (8). This suggests that one chain of the peptide may originate from the sequence ^{99}Leu-Hyp-Gly-Met-Hyl-Gly-His-Arg^{106} in the α1 chain and another chain may originate from the sequence ^{1036}Leu-Pro-Gln-Pro-Pro-Gln-Gln-Glx-Lys(or Hyl)-Ala-His-Asp-Gly-Gly^{1049} in the α1 chain (9,10), and that pyridinoline may arise from hydroxylysine (and lysine) residues. Considering the structure and the location of pyridinoline, it appears most probable that this compound is synthesized by the condensation of one hydroxylysine and two hydroxylysine-derived aldehydes as shown in Fig.4. Thus, n and m in Fig.3 may be 1 and 2, respectively.

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