Characterization of Urinary Degradation Products Derived from Type I Collagen

IDENTIFICATION OF A β-ISOMERIZED ASP-GLY SEQUENCE WITHIN THE C-TERMINAL TEOPEPTIDE (α1) REGION

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The heterogeneity of urinary degradation products of C-terminal telopeptides derived from the α1 chain of human type I collagen was investigated and characterized. The urinary fragments characterized in this study consisted of two cross-linked (X) amino acid sequences derived from the C-terminal telopeptide (α1) of type I collagen. Fragments containing the sequence EXAH-DGGR, with a DG site being either nonisomerized (Asp-Gly) or β-isomerized (βAsp-Gly), were identified. Pyridinoline was detected among the pyridinium cross-links, but there was a dominance of deoxypyridinoline and a cross-link containing pyridinoline having a molecular weight identical with that of galactosyl pyridinoline. A nonfluorescent cross-link was also found. The concentration of fragments derived from the C-terminal telopeptide region of type I collagen containing the sequence Asp-Gly (αCTX) and βAsp-Gly (βCTX) was measured by enzyme-linked immunosorbent assays in urine and in collagenase digests of trabecular and cortical bone of young and old origin. It was shown that the urinary ratio between such fragments, αCTX/βCTX, was higher in children compared with adults and that the ratio decreased with increasing age of bone. The results indicated that the C-terminal telopeptide fragments derived from type I collagen excreted into urine originated mainly from bone. In conclusion, it is demonstrated for the first time that the C-terminal telopeptide α1 chain of type I collagen contains an Asp-Gly site prone to undergo β-isomerization and that the degree of β-isomerization of this linkage apparently increases with increasing age of bone. These findings indicate that the ratio αCTX/βCTX might be clinically important in diagnosing metabolic bone diseases.

The rate of bone turnover has for years been estimated by measurement of different markers reflecting bone formation and bone resorption in urine and blood (1). The traditional bone-resorption markers are urinary calcium and hydroxyproline. Hydroxyproline, however, is not specific for bone (2), and bone resorption markers are urinary calcium and hydroxyproline. However, little is known about these fragments, their size, and their possible relation to the degree of osteoclastic activity. So far, it is uncertain whether the fragments are further metabolized in the circulation, in the liver, or in the kidneys. Neither is it clear to what extent the fragments are cross-linked, what types of cross-links are dominant, and if certain fragments can be related to degradation of different connective tissues containing type I collagen. Finally, it is unknown whether the C-terminal telopeptide Asp-Gly sequence is prone to undergo β-isomerization in proteins/peptides prone to β-isomerize (11, 12). In the case of β-isomerization, the peptide backbone is transferred from the aspartyl α-carboxyl group to the side chain β-carboxyl group, resulting in a perturbation of the peptide backbone (11) (Fig. 1). β-Isomerization is generally believed to be associated with aging of proteins and peptides (13, 14).

Recently, new assays measuring urinary degradation products derived from the C-terminal telopeptide region of type I collagen have been developed (15–17). It has been shown that measurements of such fragments in urine reflects the rate of bone resorption in a number of metabolic bone diseases (15–19). However, little is known about these fragments, their size, and their possible relation to the degree of osteoclastic activity. So far, it is uncertain whether the fragments are further metabolized in the circulation, in the liver, or in the kidneys. Neither is it clear to what extent the fragments are cross-linked, what types of cross-links are dominant, and if certain fragments can be related to degradation of different connective tissues containing type I collagen. Finally, it is unknown whether the C-terminal telopeptide Asp-Gly sequence is prone to undergo β-isomerization in type I collagen from bone and other connective tissues.

The aim of the present study was to investigate the nature of degradation products derived from the C-terminal telopeptide region of type I collagen excreted into urine.

αCTX, amino acid involved in peptide cross-linking; PBS, phosphate-buffered saline.

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The abbreviations used are: Pyr, pyridinoline; Dpyr, deoxypyridinoline; αCTX, nonisomerized C-terminal telopeptide (α1) fragments reactive in CrossLaps ELISA; CTX, αCTX and βCTX; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; MAbA7, monoclonal antibody from hybridoma A7; X, amino acid involved in peptide cross-linking; PBS, phosphate-buffered saline.
A/S (Herlev, Denmark), CNBr-activated Sepharose® 4B was from Applied Biosystems (Foster City, CA). Synthetic peptides were from Schäfer-N (Copenhagen, Denmark). The CrossLaps™ ELISA was from Osteomet BioTech A/S (Herlev, Denmark), CNBr-activated Sepharose™ 4B was from Pharmacia Biotech Inc., and the Sep-Pak® C18 cartridges were from Waters (Milford, MA).

EXPERIMENTAL PROCEDURES

Materials

All chemicals were of analytical grade and obtained from Sigma or Merck. Acetonitrile was from Rathburn, heptafluorobutyric acid was from Aldrich, and trifluoroacetic acid was from Applied Biosystems (Foster City, CA). Synthetic peptides were from Schäfer-N (Copenhagen, Denmark). The CrossLaps™ ELISA was from Osteomet BioTech A/S (Herlev, Denmark), CNBr-activated Sepharose™ 4B was from Pharmacia Biotech Inc., and the Sep-Pak® C18 cartridges were from Waters (Milford, MA).

Subjects

Urine samples were collected as second morning void between 8 and 10 a.m. from 36 children and 42 postmenopausal women aged 6–11 and 45–54 years of age, respectively. The urine used for immunoaffinity purification was first morning void from an 11-year-old boy. Informed consent was obtained from all participants according to the Helsinki Declaration of 1975, as revised in 1983, and the investigation was approved by the ethical committee of Copenhagen County.

MAbA7 ELISA

The MAbA7 ELISA is a competitive assay employing a peroxidase-labeled monoclonal antibody (MAbA7) reactive with peptide fragments derived from degradation of the C-terminal telopeptide (α1) region of human type I collagen. The assay was performed as described previously (18).

CrossLaps ELISA

The CrossLaps ELISA is a competitive assay based on a polyclonal antibody reactive with degradation products derived from the C-terminal telopeptide region (α1) of human type I collagen. The assay was performed as recommended by the manufacturer.

Amino Acid Analysis

Amino acid composition analysis of EKAHDGGR, EKAHβDGGR, and urinary fragments derived from type I collagen was performed after acid hydrolysis by ion exchange chromatography combined with post-column derivatization as described previously (20).

Peptide Sequence Analysis

N-terminal sequencing was performed on a 494A protein sequencer (Applied Biosystems) and chemicals recommended by the manufacturer.

Mass Spectrometry

Freeze-dried material was redissolved in 20 μl of 30% acetonitrile (v/v) containing 0.1% trifluoroacetic acid (v/v). A 0.5-μl aliquot was mixed with 0.5 μl of saturated o-cyano-4-hydroxycinnamic acid as matrix in the same solvent. The mixture (1 μl) was analyzed in a BiFlex instrument in linear mode at 15 kV (Bruker-Franzner Analytic). Spectra were averaged from 40–100 laser-beam shots. Using the synthetic peptide PYDRISNSAFSD-NH₂ (1517.6 Da) and melittin (2846.5 Da) as external calibrators, the inaccuracy of the method was lower than 0.1%. The molecular mass of each molecule was determined as the mean value of several measurements estimated using different external standard curves.

HPLC Equipment

The chromatographic system consisted of two 510 pumps, a 717 autosampler, a scanning fluorescence detector, and an extended wavelength module equipped with a 214-nm cutoff filter (all from Waters). Purification of synthetic EKAHDGGR, EKAHβDGGR, and urinary fragments derived from type I collagen was performed at room temperature using a reverse-phase Delta Pak C18 column (3.9 mm × 150 mm; particle size, 5 μm; pore size, 30 nm; Waters). Data were collected on a personal computer and evaluated using the Maxima software from Waters. In the following, the effluents were monitored for peptide bonds at 214 nm and for fluorescent molecules at 380 nm (emission) using 297-nm light for excitation.

Characterization of MAbA7 ELISA and CrossLaps ELISA

Preparations of synthetic EKAHDGGR were contaminated with its corresponding β-isomerized form, EKAHβDGGR. Prior to evaluation of the MAbA7 ELISA and the CrossLaps ELISA binding specificity, EKAHDGGR and EKAHβDGGR were separated by reverse-phase HPLC using an isocratic gradient of 0.5% acetonitrile (v/v) containing 0.1% trifluoroacetic acid (v/v) at a flow rate of 1 ml/min. The effluent was monitored for peptide bonds, collected in 1-mL fractions, freeze-dried, redissolved in PBS (1 ml), and measured in the two assays (Fig. 2).

Production of Monoclonal Antibody

The monoclonal antibody MAbA7, which is reactive with peptide fragments from the C-terminal telopeptide (α1) region of type I collagen, was produced, hybridomas were propagated, and monoclonal antibodies were purified as described previously (16).

Immunoaﬃnity Chromatography

Monoclonal antibody MAbA7 was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions. Urine was diluted 1:3 (v/v) in PBS, the pH was adjusted to 8.0 with 1 M NaOH, and 800 ml of diluted urine was recirculated on a MAbA7-Sepharose column (14 cm²) for 24 h at 4 °C at a flow rate of 0.8 ml/min. After washing the column with 200 ml of PBS, pH 8.0, bound material was eluted using 20 ml of 50% saturated (NH₄)₂SO₄ containing 1% trifluoroacetic acid (v/v). The eluted molecules were desalted using a C18 Sep-Pak cartridge conditioned with 20 ml of 80% methanol (v/v) and equilibrated with 20 ml of 1% trifluoroacetic acid (v/v). Bound material was washed with 20 ml of 1% trifluoroacetic acid (v/v) and eluted with 40% acetonitrile containing 0.1% trifluoroacetic acid (v/v), freeze-dried, and stored at −20 °C.

Purification of Immunoaﬃnity Purified Material

The purification of immunoaffinity purified material was carried out using three consecutive reverse-phase HPLC steps.

Step 1—Freeze-dried material extracted from urine by immunoaﬃnity chromatography was redissolved in 0.1% heptafluorobutyric acid (v/v) and eluted from a Delta Pak C18 column with a 16–24 acetonitrile gradient containing 0.1% heptafluorobutyric acid (v/v) over 90 min at a flow rate of 1 ml/min. Two-ml fractions were collected and stored at −20 °C. An aliquot (25 μl) from each fraction was freeze-dried, redissolved in PBS (200 μl), and assayed in the MAbA7 ELISA and the CrossLaps ELISA.

Step 2—Selected fractions from Step 1 containing high amounts of βCTX and αCTX as determined by CrossLaps ELISA and MAbA7 ELISA were freeze-dried, redissolved in 0.1% trifluoroacetic acid, and further purified using a Delta Pak C18 column. The fragments were eluted with a 0 to 12% acetonitrile gradient containing 0.1% trifluoroacetic acid (v/v) over 80 min at a flow rate of 1 ml/min. Two-ml fractions were collected and stored at −20 °C. From each fraction an aliquot of 50 μl was freeze-dried, redissolved in PBS (200 μl), and assayed in the MAbA7 ELISA and CrossLaps ELISA.

Step 3—Selected fractions from Step 2 containing high amounts of βCTX and αCTX as determined by CrossLaps ELISA and MAbA7 ELISA were purified once more on a Delta Pak C18 column. Freeze-
β-Isomerization of Type I Collagen

RESULTS

Purification of Urinary Fragments Derived from the C-terminal Telopeptide of Type I Collagen (CTX)—The monoclonal antibody MAbA7 was coupled to CNBr-activated Sepharose and used to purify urinary CTX fragments by immunoaffinity chromatography. All urinary molecules reactive in the MAbA7 ELISA and the CrossLaps ELISA were retained on the MAbA7-Sepharose column even though synthetic EKAHβDGGR could not be detected in the MAbA7 ELISA (Fig. 2 and data not shown). This indicates a relatively high cross-reactivity between the MAbA7 antibody and βCTX under the chromatographic conditions employed.

The urinary CTX fragments extracted by immunoaffinity chromatography were further separated by three consecutive reverse-phase HPLC steps, all employing acetonitrile as eluent. In HPLC Step 1, heptafluorobutyric acid was used as ion-pairing agent. The urinary extract consisted of a variety of different CTX molecules as shown by the elution profiles in Fig. 3. All fractions were assayed for αCTX and βCTX by MAbA7 ELISA and CrossLaps ELISA, respectively (Fig. 3). The immunological αCTX and βCTX profiles, however, were quite different. Only fractions from HPLC Step 1 containing high levels of αCTX and/or βCTX were subjected to HPLC Step 2 employing trifluoroacetic acid as ion-pairing agent. Fig. 4 and Fig. 5 show the reverse-phase and immunological elution profiles of fractions F1 and F4, respectively, from HPLC Step 1. Both fractions F1 and F4 contained several molecules as shown by the UV absorbance and fluorescence elution profiles. The molecules responsible for the immunological response given in fraction F1 and F4 were nonfluorescent (Fig. 4, F1*) and fluorescent (Fig. 5, F4*), respectively. In HPLC Step 3, selected fractions from HPLC Step 2 were subjected to a final purification prior to further analysis (data not shown). To ensure high purity of the CTX fragments, each peak was divided into three to five fractions. Again, fractions were assayed for their content of αCTX and βCTX fragments. The recovery was better than 95% in each of the three HPLC steps as evaluated by both MAbA7 ELISA and CrossLaps ELISA (data not shown).

Determination of Fragment Purity—The purity of the fragments separated by HPLC was evaluated by mass spectrometry and by determination of the cross-links Pyr and Dpyr after acid hydrolysis. Fractions containing only one detectable mass were regarded pure if they contained only Pyr, Dpyr, or none of the pyridinium cross-links. Mass spectra of the fragments P1 and P4 (Table I) responsible for the major immunological activity in fractions F1 and F4 (Fig. 3, Fig. 4, F1* and Fig. 5, F4*) are shown in Fig. 6. The molecular mass was 2039.8 Da for the nonfluorescent fragment P1 from fraction F1* (Fig. 4) and 2036.9 Da for the fluorescent fragment P4 from fraction F4* (Fig. 5). Consecutive fractions with the same molecular mass and an equal αCTX/βCTX ratio as determined by ELISA were pooled prior to analysis for amino acid composition and sequence analysis.

Characterization of Purified CTX Fragments—Table I summarizes the analytical results for some of the purified molecules. The fragments P4, P5, and P6 all contained Pyr and had a molecular mass of 2036.9 Da. However, the three fragments differed with respect to retention time and αCTX/βCTX ratio. The triplet pattern evident for P4, P5, and P6, showing increasing retention times followed by increasing αCTX/βCTX values, was also seen for the triplets P1, P2, and P3 and for the triplets P7, P8, and P9. The molecules P7, P8, and P9, with a molecular
mass of 1859.7 Da, all contained the pyridinium cross-link Dpyr. The fraction containing P8 was slightly contaminated with molecules having a molecular mass of 2036.9 Da containing Pyr. In accordance with their lack of fluorescence, the fragments P1, P2, and P3, all with a molecular mass of 2039.8 Da, did not contain pyridinium cross-links. These results indicate that the three fragments in each of the three triplets consisted of two cross-linked CTX fragments: either two βCTX fragments (P1, P4, and P7), one αCTX and one βCTX fragment (P2, P5, and P8), or two αCTX fragments (P3, P6, and P9).

Amino acid composition analysis of the fragments revealed that they apparently all consisted of the same six amino acids (Ala, Asp, Glu, Gly, His, and Arg) in the ratio 1:1:2:1:1:1. The amino acid compositions of P4, P5, P6, and synthetic EKAHDGGR and EKAβDGGR are given in Table II. Except for lysine (K), the amino acid composition was consistent with the sequence EKAHDGGR specific for a part of the C-terminal telopeptide α1 chain in type I collagen (10). Sequence analysis of the fragments P2, P3, P5, P6, P8, and P9 confirmed the identity of the fragments as being degradation products derived from the C-terminal telopeptide α1 chain of type I collagen. Sequence data for P4, P5, P6, EKAHDGGR, and EKAβDGGR are given in Table III. The blank run in the second cycle indicates the presence of a cross-link in P4, P5, and P6. This is consistent with previous findings showing that the C-terminal telopeptide lysine residue (K) is a site for inter-

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**FIG. 3.** Reverse-phase HPLC (Step 1) fractionation of urinary degradation products derived from the C-terminal telopeptide α1 chain of type I collagen. Heptafluorobutyric acid was used as ion-pairing agent in the HPLC assay. A, UV absorbance detecting peptide bonds; B, fluorescence, mainly detecting pyridinium cross-links; C, immunological profile of C-terminal telopeptides determined by MAbA7 ELISA; D, immunological profile of β-isomerized fragments determined by CrossLaps ELISA.

**FIG. 4.** Reverse-phase HPLC (Step 2) fractionation of urinary degradation products derived from the C-terminal telopeptide α1 chain of type I collagen. Trifluoroacetic acid was used as ion-pairing agent in the HPLC assay. A–D are as described in Fig. 3. Separation of peptides eluted between 32 and 34 min in HPLC Step 1 (see Fig. 3, fraction F1). Note that fraction F1* contains a major nonfluorescent peak reactive in the CrossLaps ELISA.
molecular cross-linking between mature type I collagen molecules (9, 22, 23).

N-terminal sequencing of the fragments P1, P4, and P7 stopped after the N-terminal His (H) residue, suggesting the presence of an alteration in the peptide backbone at the Asp-Gly (DG) site to preclude further steps in Edman degradation. As demonstrated by amino acid composition analysis using synthetic EKAHDGGR and EKAH\(^-\)DGGR, \(^-\)isomerization of the Asp-Gly (DG) linkage did not result in an altered amino acid composition (Table II), nor was the mass of the sequence altered. In the case of \(^-\)isomerization of the Asp-Gly sequence, however, the peptide backbone was transferred from the \(^-\)carboxyl to the side chain \(^-\)carboxyl group, resulting in a significant structural perturbation of the peptide backbone, making it resistant to Edman degradation (Table III) (11). Based on the immunological characterization (Table I), molecular weight, and the result of the Edman sequence analysis, we suggest that the fragments consist of two cross-linked \(^-\)CTX sequence and a \(^-\)CTX sequence. Sequence data for P5 and P6 supported this assumption, since the relative yield of Asp (D) and Gly (G) was significantly higher in P6 containing two \(^-\)CTX fragments than in P5 (Table III). It has been shown in several studies that \(^-\)isomerized peptides elute prior to their corresponding nonisomerized forms when separated by reverse-phase HPLC employing conditions similar to those used in this study (Fig. 2) (24, 25). The reverse-phase HPLC elution pattern seen for the triplets with \(^-\)CTX-X-\(^-\)CTX fragments eluting before \(^-\)CTX-X-\(^-\)CTX fragments, again followed by \(^-\)CTX-X-\(^-\)CTX fragments, is consistent with these findings.

The fluorescent fragments were shown to contain pyridoline (P4, P5, and P6) (Fig. 7) and deoxypyridinoline (P7, P8, and P9) after acid hydrolysis (Table I). The theoretical molecular mass of fragments consisting of two EXAHGDGR sequences cross-linked by Pyr or Dpyr at position X is 1874.9 and 1858.9 Da, respectively. P7, P8, and P9 cross-linked by Dpyr were shown to have a molecular mass of approximately 1859.7 Da, closely corresponding to the expected theoretical molecular mass of such a fragment.
The molecular mass of the fragments cross-linked by Pyr being 2036.9 Da, however, was not consistent with the theoretical molecular mass of such molecules (1874.0 Da), indicating that the Pyr cross-link has been further posttranslationally modified. Glycosylated Pyr derivatives can be present as galactosyl Pyr (Pyr-Gal) and glycosyl galactosyl Pyr (Pyr-Gal-Glu) (Fig. 7), dependent on whether the hydroxylysine residue from the triple helical domain involved in the Pyr cross-link originally was galactosyl hydroxylysine or glycosyl galactosyl hydroxylysine (26, 27). The theoretical molecular mass (2036.9 Da) of fragments consisting of two EKAXDGR sequences cross-linked by Pyr-Gal at position X is identical with the molecular mass of P4, P5, and P6, approximately 2036.9 Da.

The fragments cross-linked by a nonfluorescent cross-link (P1, P2, and P3) had a molecular mass of approximately 2039.8 Da. This molecular mass did not correspond to the molecular mass of any known fragment consisting of two EKAXDGR sequences cross-linked at position X by partly degraded nonfluorescent pyridinium cross-links or their glycosylated derivatives. A nonfluorescent cross-link, which is different from partly degraded pyridinium cross-links, containing a pyrrole ring has been described (22, 28, 29) (Fig. 7). The molecular mass of P1, P2, and P3, however, did not correspond to the theoretical molecular mass of any fragments cross-linked by intact pyrrole cross-links or their glycosylated counterparts.

Quantification of αCTX and βCTX in Bone and Urine—The concentration of urinary αCTX and βCTX fragments was evaluated in urine from children and adults aged 6–11 and 45–54 years of age, respectively, and in collagenase digests of young and old bovine bone as well, by MAba7 ELISA and CrossLaps ELISA. The αCTX/βCTX ratios found in urine and in collagenase digests of bone are given in Fig. 8. Evaluated on a group basis, the ratio αCTX/βCTX was approximately 3-fold higher in urine from children compared with adults evaluated on a group basis (p < 0.001). In bone, the highest αCTX/βCTX value was found in young trabecular bone followed by young cortical bone (both of fetal origin), old trabecular bone, and cortical bovine bone. The urinary ratio αCTX/βCTX from adults being approximately 0.4 was almost comparable to that found in old trabecular and cortical bone of bovine origin.

### DISCUSSION

In this study, we identified an Asp-Gly sequence within the C-terminal telopeptide α1 chain of type I collagen prone to undergo β-isomerization. The results presented here indicate that β-isomerization of this sequence takes place in vivo and that the degree of β-isomerization increases with increasing age of type I collagen molecules in bone.

Around 90% of the protein in bone is type I collagen (30). When bone is resorbed by osteoclasts, fragments of type I collagen are released into the circulation and to an unknown extent excreted into the urine. However, urinary degradation products of type I collagen might also be derived from nonkeletal sources, since type I collagen is widely distributed in most connective tissues throughout the body (30). So far, little is

### Table II

| AA   | P4  | P5  | P6  | EKAHDGGR | EKAHβDGGR |
|------|-----|-----|-----|----------|-----------|
| Asp  | 1.0 | 1.0 | 0.9 | 1        | 1         |
| Glu  | 1.3 | 1.2 | 1.3 | -        | -         |
| Ser  | 0.4 | 0.2 | 0.6 | -        | -         |
| Gly  | 2.2 | 2.0 | 2.4 | 2        | 2         |
| His  | 1.4 | 1.0 | 1.0 | 1        | 1         |
| Thr  | 0.1 | 0.0 | 0.2 | -        | -         |
| Ala  | 1.0 | 1.0 | 1.0 | 1        | 1         |
| Pro  | 0.0 | 0.0 | 0.0 | -        | -         |
| Tyr  | 0.0 | 0.0 | 0.0 | -        | -         |
| Val  | 0.1 | 0.1 | 0.1 | -        | -         |
| Met  | 0.0 | 0.0 | 0.0 | -        | -         |
| Ile  | 0.1 | 0.1 | 0.1 | -        | -         |
| Leu  | 0.1 | 0.1 | 0.2 | -        | -         |
| Lys  | 0.2 | 0.1 | 0.3 | 1        | 1         |
| Phe  | 0.0 | 0.0 | 0.0 | -        | -         |
| Arg  | 0.8 | 0.9 | 0.8 | 1        | 1         |
| Total| 8.8 | 7.6 | 9.0 | 8        | 8         |
known about the origin and biochemical structure of such urinary collagen-degradation products. In this context, it is interesting that the MAbA7 ELISA and the CrossLaps ELISA appear exclusively to measure urinary degradation products of type I collagen derived from osteoclastic resorption of bone (15–19). A biological explanation for this phenomenon could be that certain fragments derived from mature type I collagen are protected against complete degradation by the liver and/or the kidney due to posttranslational modifications.

The MAbA7 ELISA and the CrossLaps ELISA both measure urinary degradation products (CTX) derived from the C-terminal telopeptide of type I collagen found in urine and in collagenase digests of bone. The concentration of αCTX and βCTX was determined by MAbA7 ELISA and CrossLaps ELISA, respectively. The αCTX/βCTX was 3-fold higher in urine from children compared with adults. In collagenase digests of bone the ratio of αCTX/βCTX decreased in the following order: trabecular fetal calf bone (T<C,F), cortical fetal calf bone (C<F), trabecular bovine bone (T<B), and cortical bovine bone (C<B).

The concentration of αCTX and βCTX was determined by MAbA7 ELISA and CrossLaps ELISA, respectively. The ratio of αCTX/βCTX was 3-fold higher in urine from children compared with adults. In collagenase digests of bone the ratio of αCTX/βCTX decreased in the following order: trabecular fetal calf bone (T<C,F), cortical fetal calf bone (C<F), trabecular bovine bone (T<B), and cortical bovine bone (C<B).

The conversion of the Asp-Gly linkage to a β-isomerized Asp-Gly linkage is illustrated in Fig. 1. The attack by a peptide backbone nitrogen on the side chain carbonyl group of an adjacent Asp residue can result in the formation of a five-member succinimide ring (24, 31). The succinimide ring is prone to hydrolysis, producing Asp-Gly and βAsp-Gly sequences (24). Succinimide intermediates can form and undergo hydrolysis under physiological conditions (24). Several factors are known to influence the formation rate of such succinimide intermediates. Among these are the primary structure of the fragment, the secondary, tertiary, and quaternary structures of proteins (11, 32), pH (33) and temperature (32, 33), and the protein microenvironment.

The biological role of β-isomerization is not known yet. It is generally believed, however, that the degree of β-isomerization

\[ \text{Expected amino acid} \]

| Cycle no. | Expected amino acid | P4 | P5 | P6 | EKAHDGGR | EKAHDGGGR |
|---|---|---|---|---|---|---|
| 1 | Glu | 100 | 100 | 100 | 100 | 100 |
| 2 | – | – | – | – | 83 | 84 |
| 3 | Ala | 79 | 60.4 ± 1.6 | 49.2 ± 1.7 | 95 | 93 |
| 4 | His | (30) | (21.2 ± 3.8) | (14.9 ± 0.7) | (22) | (27) |
| 5 | Asp | – | 44.1 ± 6.7 | 64.9 ± 6.9 | 63 | – |
| 6 | Gly | – | 20.6 ± 0.3 | 35.3 ± 1.7 | 59 | – |
| 7 | Gly | – | 20.6 ± 0.3 | 35.3 ± 1.7 | 59 | – |
| 8 | Arg | – | (28.1 ± 7.5) | (45.4 ± 23.5) | (22) | – |
| 9 | – | – | – | – | – | – |

Fig. 7. Structure of pyridinium cross-links, pyrrole cross-links (Ehrlich Chromogens), and their glycosylated derivatives. Pyr and Dpyr contain –OH and –H, respectively, at position X. The glycosylated derivatives of the molecules contain the groups O-Gal-Glu and O-Gal at position X. The structure of the pyrrole cross-link was suggested by Kuypers et al. (22).

Fig. 8. Ratio between αCTX and βCTX fragments derived from the C-terminal telopeptide α1 chain of type I collagen found in urine and in collagenase digests of bone. The concentration of αCTX and βCTX was determined by MAbA7 ELISA and CrossLaps ELISA, respectively. The ratio of αCTX/βCTX was 3-fold higher in urine from children compared with adults. In collagenase digests of bone the ratio of αCTX/βCTX decreased in the following order: trabecular fetal calf bone (T<C,F), cortical fetal calf bone (C<F), trabecular bovine bone (T<B), and cortical bovine bone (C<B).

The conversion of the Asp-Gly linkage to a β-isomerized Asp-Gly linkage is illustrated in Fig. 1. The attack by a peptide backbone nitrogen on the side chain carbonyl group of an adjacent Asp residue can result in the formation of a five-member succinimide ring (24, 31). The succinimide ring is prone to hydrolysis, producing Asp-Gly and βAsp-Gly sequences (24). Succinimide intermediates can form and undergo hydrolysis under physiological conditions (24). Several factors are known to influence the formation rate of such succinimide intermediates. Among these are the primary structure of the fragment, the secondary, tertiary, and quaternary structures of proteins (11, 32), pH (33) and temperature (32, 33), and the protein microenvironment.

The biological role of β-isomerization is not known yet. It is generally believed, however, that the degree of β-isomerization

Table III

Sequence data for fragments P4, P5, and P6

| Cycle no. | Expected amino acid | P4 | P5 | P6 | EKAHDGGR | EKAHDGGGR |
|---|---|---|---|---|---|---|
| 1 | Glu | 100 | 100 | 100 | 100 | 100 |
| 2 | – | – | – | – | 83 | 84 |
| 3 | Ala | 79 | 60.4 ± 1.6 | 49.2 ± 1.7 | 95 | 93 |
| 4 | His | (30) | (21.2 ± 3.8) | (14.9 ± 0.7) | (22) | (27) |
| 5 | Asp | – | 44.1 ± 6.7 | 64.9 ± 6.9 | 63 | – |
| 6 | Gly | – | 20.6 ± 0.3 | 35.3 ± 1.7 | 59 | – |
| 7 | Gly | – | 20.6 ± 0.3 | 35.3 ± 1.7 | 59 | – |
| 8 | Arg | – | (28.1 ± 7.5) | (45.4 ± 23.5) | (22) | – |
| 9 | – | – | – | – | – | – |

FIG. 7. Structure of pyridinium cross-links, pyrrole cross-links (Ehrlich Chromogens), and their glycosylated derivatives. Pyr and Dpyr contain –OH and –H, respectively, at position X. The glycosylated derivatives of the molecules contain the groups O-Gal-Glu and O-Gal at position X. The structure of the pyrrole cross-link was suggested by Kuypers et al. (22).

FIG. 8. Ratio between αCTX and βCTX fragments derived from the C-terminal telopeptide α1 chain of type I collagen found in urine and in collagenase digests of bone. The concentration of αCTX and βCTX was determined by MAbA7 ELISA and CrossLaps ELISA, respectively. The ratio of αCTX/βCTX was 3-fold higher in urine from children compared with adults. In collagenase digests of bone the ratio of αCTX/βCTX decreased in the following order: trabecular fetal calf bone (T<C,F), cortical fetal calf bone (C<F), trabecular bovine bone (T<B), and cortical bovine bone (C<B).

The conversion of the Asp-Gly linkage to a β-isomerized Asp-Gly linkage is illustrated in Fig. 1. The attack by a peptide backbone nitrogen on the side chain carbonyl group of an adjacent Asp residue can result in the formation of a five-member succinimide ring (24, 31). The succinimide ring is prone to hydrolysis, producing Asp-Gly and βAsp-Gly sequences (24). Succinimide intermediates can form and undergo hydrolysis under physiological conditions (24). Several factors are known to influence the formation rate of such succinimide intermediates. Among these are the primary structure of the fragment, the secondary, tertiary, and quaternary structures of proteins (11, 32), pH (33) and temperature (32, 33), and the protein microenvironment.

The biological role of β-isomerization is not known yet. It is generally believed, however, that the degree of β-isomerization

is associated with aging of proteins (13). This hypothesis is based on results showing that the degree of \( \beta \)-isomerization in proteins and peptides prone to undergo this alteration increases with time (age) in vivo and in vitro (12, 34, 35). In addition, studies evaluating the function of proteins and peptides have revealed that increased \( \beta \)-isomerization is often accompanied by a decreased physiological protein/peptide activity (36). Furthermore, the presence of an intracellular methylytransferase repair system using \( \beta \)-isomerized molecules as substrate has been reported (25, 37), whereas extracellular repair systems, such as the methylytransferase system present in cells, has not (38). It is therefore improbable that a \( \beta \)-isomerized C-terminal telopeptide \( \beta \)Asp-Gly sequence will be enzymatically repaired in vivo, since type I collagen is an extracellular protein.

Our results seem to confirm the suggested relationship between \( \beta \)-isomerization and tissue age, since the ratio \( \alpha \)CTX/\( \beta \)CTX is high in collagenase digests of young bone compared with those of old bone. This relationship was further supported by our results showing that the relative amount of \( \alpha \)CTX in the above fractions (Fig. 8) was higher in trabecular bone compared with cortical bone, two bone tissues characterized by high and low bone turnover, respectively (39). We therefore suggest that the degree of \( \beta \)-isomerization of the C-terminal telopeptide \( \beta \)Asp-Gly site of type I collagen in bone is dependent on the age of the molecules in the bone tissue.

In a previous study, Haley et al. (40) were not able to show an increase in the number of \( \beta \)-isomerized Asp-Gly sequences from Achilles tendon per molecule of type I collagen with increasing age. In the same study, Haley et al. (40) estimated the content of \( \beta \)-isomerized Asp-Gly sites to be between 0.8 and 1.9 per collagen molecule. The \( \alpha 1 \) and \( \alpha 2 \) chains of type I collagen both contain several Asp-Gly sites. Combined with our results, however, the number of \( \beta \)-isomerized Asp-Gly sites as estimated by Haley et al. indicate that the C-terminal telopeptide \( \beta \)Asp-Gly sequence (\( \alpha 1 \)) probably is the only Asp-Gly sequence in type I collagen that is liable to undergo \( \beta \)-isomerization.

Using synthetic peptides containing an Asp-Gly sequence, Gieger and Clarke (24) showed that at equilibrium in the absence of the methylytransferase repair system approximately 75% of the Asp-Gly sequences were converted to their corresponding isofrom, \( \beta \)Asp-Gly. In our study 50% of the CTX Asp-Gly sequences were \( \beta \)-isomerized in urine from children, whereas 70–80% was \( \beta \)-isomerized in urine from adults and in collagenase digests from old trabecular and cortical bone. This seems to indicate that the C-terminal telopeptide \( \beta \)Asp-Gly sequence almost has reached equilibrium with respect to \( \beta \)-isomerization in old trabecular and cortical bone. Based on histological studies, it has been estimated that trabecular and cortical bone in adults are completely renewed within a period of 3 and 20 years, respectively (39). Accordingly, we suggest that the process of \( \beta \)-isomerization of the C-terminal telopeptide \( \beta \)Asp-Gly sequence of type I collagen in mineralized tissues such as bone almost has reached equilibrium within a period of 3 years.

Clinical results obtained using the MAbA7 ELISA and the CrossLaps ELISA strongly indicate that both assays measure urinary fragments derived from resorption of bone (15–19). This assumption was further supported by the close resemblance between the various \( \alpha \)CTX/\( \beta \)CTX levels found in urine and bone from young and old individuals, respectively. Therefore, it seems reasonable to assume that the urinary ratio of \( \alpha \)CTX/\( \beta \)CTX reflects the ratio in the resorbed bone. In this context, it is interesting that the urinary ratio found in patients with Paget’s disease is markedly elevated compared with that of age-matched healthy controls (16). Paget’s disease is characterized by very high bone turnover in restricted areas of the skeleton, resulting in a disorganized mosaic of woven and lamellar bone at the affected sites (41). If the hypothesis holds true that \( \alpha \)CTX and \( \beta \)CTX reflect degradation of young and old bone, respectively, apparently the contribution of \( \alpha \)CTX from the diseased areas of the skeleton is very high compared with \( \beta \)CTX. This is in keeping with the notion that the diseased areas in Paget’s disease are characterized by a highly increased rate of bone turnover, probably resulting in degradation of relatively newly formed (young) bone.

The urinary CTX fragments identified in this study were cross-linked by either pyridinium cross-links or an unknown nonfluorescent cross-link. The ratio between Pyr and Dpyr in bone is 3.5:1, whereas the ratio is higher than 10:1 in almost all other connective tissues containing these cross-links (8). The pyridinium cross-links are normally excreted into urine in a Pyr/Dpyr ratio of 3.8–6.0, which is almost comparable to that found in bone. This indicates that the majority of the pyridinium cross-links in urine are derived mainly from bone. In the present study, the Pyr/Dpyr ratio was 3.8 in the mixture of CTX fragments extracted from urine by immunooaffinity, indicating that at least the fragments cross-linked by pyridinium cross-links were derived from bone (data not shown).

In conclusion, we have identified an Asp-Gly sequence within the C-terminal telopeptide \( \alpha 1 \) chain of type I collagen prone to \( \beta \)-isomerization. The degree of \( \beta \)-isomerization at this site seems to increase with increasing age of type I collagen molecules in bone. The data further indicate that the degree of \( \beta \)-isomerization has almost reached equilibrium in old trabecular and cortical bone. In addition, our results seem to confirm previous clinical findings (15–19) showing that urinary \( \alpha \)CTX and \( \beta \)CTX are derived from bone and that the urinary ratio \( \alpha \)CTX/\( \beta \)CTX could be of clinical importance in assessing metabolic bone diseases.

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Characterization of Urinary Degradation Products Derived from Type I Collagen: IDENTIFICATION OF A β-ISOMERIZED ASP-GLY SEQUENCE WITHIN THE C-TERMINAL TEOPEPTIDE (α1) REGION

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