Collagen fragments in urine derived from bone resorption are highly racemized and isomerized: a biological clock of protein aging with clinical potential

Paul A. C. CLOOS¹ and Christian FLEDELIUS²
Osteometer BioTech A/S, Osteopark, Herlev Hovedgade 207, DK-2730 Herlev, Denmark

INTRODUCTION

Aging affects intra- and extra-cellular proteins, which are subject to a variety of spontaneous chemical degradation reactions under physiological conditions [1]. These reactions include (i) racemization, i.e., conversion of naturally occurring L-amino acids to the biologically rare D-isomers; and (ii) isomerization, i.e., transfer of the peptide backbone from the α-carboxy group to a side-chain β-carboxy group in susceptible Asp or Asn residues.

Racemization of Asp is generally believed to be associated with aging of proteins and peptides [1–8]. Thus an age-dependent accumulation of the ε-enantiomeric form of Asp has been demonstrated in hydrolysates of various proteins in different tissues [2–8], and a large number of studies have established that the chronological age of an individual can be estimated with high precision from the degree of racemization in tissues with no turnover, e.g. enamel and dentin [3–5]. Correspondingly, isomerization of Asp or Asn residues has been shown to occur during aging of susceptible proteins in vitro and in vivo [9–13].

Most experimental evidence currently suggests that the processes of Asp racemization and isomerization (RI) are interdependent reactions, and that the accumulation of D-Asp in polypeptides is determined primarily by the rate of succinimide formation [12–14]. The initial event in these non-enzymic reactions of Asp is believed to be the nucleophilic attack of the side-chain carbonyl group by the peptide-bond nitrogen, to yield an unstable five-membered t-succinimide ring [12] (Scheme 1, A → B). This succinimide ring is prone to hydrolysis and racemization, yielding peptides and β-Asp peptides in both the D and L configurations. The reaction thus leads to the formation of four end-products: the native peptide form (denoted αL, where all amino acids are of the L form and linked through normal peptide bonds; Scheme 1A); an isomerized form (βL, where the Asp residue is linked to the adjacent residue through the side-chain β-carboxy group, forming a so-called β-Asp bond; Scheme 1C); a racemized form (αD, containing a D-Asp residue; Scheme 1D); and a racemized/isomerized form (βD; Scheme 1F).

The accumulation of the age-related forms (βL, βD and αD) in proteins is the result of two opposing reactions: RI on the one hand and the turnover of the protein/tissue on the other. Hence the processes of RI constitute a potential biochronological tool reflecting the metabolic activity and rate of remodelling of the protein or tissue in question.

The present study focuses on RI of the residue Asp¹²¹¹ contained within the sequence AHDGGR¹²⁰⁹–¹²¹¹ specific for the αL C-telopeptide of type I collagen. Molecular fragments containing this sequence are termed CTx molecules. The applicability of RI to determining tissue turnover is assessed using CTx as a model system. We have developed two highly specific ELISAs measuring the βD and αD forms of CTx, and use these to

Abbreviations used: αL, native peptide; βL, isomerized peptide containing a β-Asp bond; αD, native peptide containing a D-Asp residue; βD, isomerized peptide containing a D-Asp residue; RI, racemization and isomerization, CTx, molecule comprising the sequence AHDGGR; TMB, 3,3',5,5'-tetramethylbenzidine.

¹ To whom correspondence should be addressed (e-mail paul-cloos@osteobio.dk).
² Present address: Department of Pharmacological Research 2, NOVO Nordisk A/S, DK-2760 Målev, Denmark.
Scheme 1  Pathways for RI of aspartic acid

The attack by the peptide backbone nitrogen on the side-chain carbonyl group of an adjacent aspartic residue can result in the formation of a succinimide ring (A → B). The spontaneous hydrolysis of the succinimide produces either peptides (A and D) or β-Asp peptides (C and F). Studies have shown that the formation of β-Asp occurs primarily via the succinimide intermediates (B and E), due to the increased acidity of the α-carbons of these residues [12,14]. Other pathways, such as direct proton abstraction (A → D or C → F), may, however, also contribute to racemization [14]. The peptide backbone is shown as a bold line throughout.

demonstrate the presence of D-Asp residues at this specific site in bone collagen. Applying these assays in conjunction with two previously developed immunoassays (measuring the βL and αL forms of CTx), we determine the kinetics of RI in CTx molecules in vitro, and show that RI of these molecules varies in vivo depending on tissue age and turnover. This has permitted us not only to assess the various isomeric forms of Asp\(^{2+11}\) in tissue extracts, but also to monitor RI of bone collagen non-invasively in living individuals through measurements of CTx isoforms in urine. Our findings suggest that an assessment of RI in urinary CTx molecules may provide an estimate of bone turnover, aiding in the diagnosis of metabolic bone diseases.

In traditional racemization studies [2–8] the degree of racemization is described by the D/L ratio. In the following we have chosen to describe RI by the relative contents of each isomer (\(\%\)αL, \(\%\)βL, \(\%\)βD and \(\%\)αD) or using the ratios between the native and age-related forms of CTx (αL/βL, αL/βD and αL/αD).

EXPERIMENTAL

Materials

All chemicals were of analytical grade from either Sigma (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany), unless otherwise stated. Acetonitrile was from Rathburn (Walkerburn, Scotland, U.K.), and trifluoroacetic acid was from Applied Biosystems (Foster City, CA, U.S.A.). Protease inhibitor cocktail tablets (BM 1836153) were from Boehringer Mannheim (Mannheim, Germany). Spectra/Por\(^\text{®}\) dialysis membranes with a molecular-mass cut-off of 3500 Da were from Spectrum Medical Industries (Houston, TX, U.S.A.). The reverse-phase C18 HPLC column (250 mm × 4.6 mm internal diam.; 5 μm particle size) used for chromatographic separation of CTx isoforms was from Vydac (Hesperia, CA, U.S.A.; cat. no. 218TP54). Peroxidase-conjugated goat anti-(rabbit IgG) was from Jackson Immuno-Research Laboratories (West Grove, PA, U.S.A.). Bronidox L5 was from Henkel (Düsseldorf, Germany). 3,3',5,5'-Tetramethylbenzidine (TMB) solution was from Kierkegaard & Perry (Gaithersburg, MD, U.S.A.). The immunoassays z-CrossLaps\(^\text{®}\) RIA and CrossLaps\(^\text{®}\) ELISA were from Osteometer Biotech A/S (Herlev Denmark).

Buffers used were as follows. PBS comprised 1.5 mM KH\(_2\)PO\(_4\), 8.5 mM Na\(_2\)HPO\(_4\); 2H\(_2\)O, 2.7 mM KCl and 137 mM NaCl, pH 7.4. Assay buffer contained 300 mM Tris(hydroxymethyl)aminomethane, 0.5% (w/v) BSA, 0.2% (w/v) Tween-20, 0.36% (w/v) Bronidox L5 and 15 mg/l Phenol Red, pH 7.5. Washing buffer consisted of 25 mM Tris, 50 mM NaCl and 0.1% (w/v) Tween-20, pH 7.2.

Synthetic peptides included αL-AHDGGR, βL-AHDGGR, αD-AHDGGR, βD-AHDGGR, αD-EKAHDGGR and βD-EKAHDGGR, and were synthesized by CHIMEX Ltd. (St. Petersburg, Russia).

Urine samples

Urine samples were collected as the first morning void from 32 healthy children (males and females; age 7.6 ± 4.2 years), 82 healthy adults (males and pre- and post-menopausal women; age 49.9 ± 17.0 years), 11 patients with Paget’s disease (six women and five men; age 67.1 ± 12.3 years) and 30 women with post-menopausal osteoporosis (otherwise healthy women, at least 10 years post-menopause; bone mineral density at the distal forearm more than 2.5 S.D.s below the pre-menopausal mean; mean age 64.6 ± 4.6 years). At entry into the study, none of the participants were taking any drugs known to influence bone metabolism. Informed consent was obtained from all participants, in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Preparation of bone powder

Bone tissue was obtained from femurs of a foetus (7 months in utero) and an adult animal. Trabecular bone was obtained from the femoral neck and cortical bone from the midshaft of the bones. Adhering muscle and cartilage tissue was removed with a scalpel, and the bone was washed in water. Bone was cut to
pieces with a saw, washed extensively at 4 °C with 0.14 M NaCl/0.05 M Na₂SO₄, pH 7.4, and subsequently extracted overnight at 4 °C with 4 M guanidine hydrochloride/0.05 M Tris, pH 7.4, to remove marrow. After rinsing with water, the bone was lyophilized and processed into fine powder under liquid nitrogen in a Spex Impact grinder (Spex Freezer-Mill model 6700; Spex Industries). To ensure a homogeneous product, bone was lyophilized and processed into fine powder under

**Preparation of immunoreactive CTx from bone powder**

Bone powder was de-fatted by extraction with acetone, lyophilized and demineralized in Spectra/Por*-4 dialysis tubing against large volumes of 0.5 M EDTA/0.05 M Tris/1 % (w/v) Na₂SO₄, pH 7.4, for 72 h. The sample was centrifuged at 3000 g for 30 min. The pellet was rinsed in cold water and lyophilized. The pellet was then reconstituted in 100 mM NaH₂PO₄, pH 7.6 (1 ml of buffer/10 mg of pellet), a freshly prepared trypsin solution [20 mg/ml trypsin XIII (EC 3.4.21.4; Sigma) in cold 1 mM HCl] was added (final trypsin concentration 1 mg/ml), and the mixture was incubated overnight at 37 °C. Trypsin digestion was terminated by adding protease inhibitor to the mixture. The trypsin digest was centrifuged for 30 min at 3000 g. The supernatant, containing immunoreactive CTx, was collected and stored at −80 °C until analysis.

**Separation and quantification of CTx isoforms by reverse-phase HPLC**

Synthetic CTx isoforms were separated using reverse-phase HPLC on a Vydac C18 column. Chromatography was performed at room temperature employing isocratic elution with 0.1 % trifluoroacetic acid over 15 min at a flow rate of 1.0 ml/min. Peptides were quantified by measuring peak areas. Areas were determined as the mean of two independent HPLC runs.

**Quantification of CTx isoforms by immunoassay analysis**

The α₁ and β₁ forms of CTx was measured using the α-CrossLaps® RIA and CrossLaps® ELISA respectively, according to the manufacturer’s instructions. Two new assays (αβ- and βd-CrossLaps® ELISA) were developed for the measurement of the αD and βd forms of CTx respectively. Antiserum were raised against peptide conjugates prepared by coupling of αD-EKAHDGGR or βd-EKAHDGGR to thyroglobulin using a carbodi-imide method [15]. Adult female (New Zealand White) rabbits were immunized subcutaneously with 125 µg of either of these antigens in 1.25 ml of PBS emulsified with an equal volume of Freund’s incomplete adjuvant. Immunizations were repeated every 2 weeks. Blood was collected 1 week after the third, fourth and fifth immunizations.

Assays were performed as follows [all incubations are carried out at 20 °C on a mixing apparatus (300 rev./min)]. For βd-CrossLaps® ELISA, plates were coated with PBS containing collagen treated with 100 ng/ml collagenase (100 µl/well). Collagenase-treated collagen was prepared as described by Rosenquist et al. [16]. Portions of 15 µl of standards, diluted controls or unknown samples were pipetted into appropriate wells in the microtitre plate, followed by 100 µl of anti-βd-CTx antiserum diluted 1:7000 in assay buffer. The wells were covered with sealing tape and incubated for 1 h. Wells were then emptied and washed five times using the washing solution. Then 100 µl of peroxidase-conjugated goat anti-(rabbit IgG) (diluted 1:1000 in a protein stabilized buffer) was added to all wells, which were subsequently incubated for 1 h. After another washing step, 100 µl of TMB solution was added to each well and the plate was incubated in the dark. The enzyme reaction was stopped after 15 min by adding 100 µl of 0.18 M H₂SO₄ per well, and the absorbance was measured at 450 nm (with 650 nm as the reference wavelength) using a microtitre plate reader (E₆₅₀ Easy Microtitre Reader; Molecular Devices, Menlo Park, CA, U.S.A.). Finally, a standard curve was constructed on the basis of six standards, and the concentration of βd-CTx antigen in each unknown sample was determined by interpolation on this curve.

For the αβ-CrossLaps® ELISA, 15 µl of sample was applied to a microtitre plate precoated with αβ-EKAHDGGR conjugated to BSA by a glutaraldehyde linker [20] (at a concentration of 100 ng/ml in PBS; 100 µl/well). All wells received 100 µl of anti-αD antisera (diluted 1:60000 in assay buffer), and were then incubated for 1 h and washed as described above. Following a second incubation with peroxidase-conjugated goat anti-(rabbit IgG), plates were developed with TMB substrate and read as described above for the βd assay.

The four CrossLaps® immunoassays were calibrated using synthetic CTx molecules of the respective isoforms, the concentration of which had been determined by amino acid analysis. Amino acid analysis was performed after acid hydrolysis by ion-exchange chromatography combined with post-column derivatization, as described in [17].

The specificity of the four CrossLaps® assays towards the various CTx isoforms was assessed by chromatographic analysis. A mixture of the different CTx isoforms (each at a concentration of approx. 100 µg/ml) was separated by reverse-phase HPLC as described above. The effluent was monitored for peptide bonds at 214 nm, collected in 100 µl fractions, lyophilized, redissolved in assay buffer, and analysed using the four CrossLaps® assays.

**RI of CTx in vitro**

The RI kinetics of CTx were investigated by studies on the different conformations (αA, βT, βD and αD) of the synthetic hexapeptide AHGDGGR at physiological pH and temperature. A 0.2 M sodium phosphate buffer, pH 7.4 (at 22 °C), was prepared and heated to 100 °C for 1 h in order to sterilize the solution. Pure preparations of each of the four CTx isoforms were dissolved in this buffer (at 4 °C) at a concentration of 100 µg/ml, and 0.1 % (v/v) chloroform was added to each solution as a bacteriostatic agent. Aliquots of 400 µl were transferred to borosilicate tubes (6 mm × 50 mm) that had been pyrolysed to destroy all contaminating peptides and amino acids. The tubes were flame-sealed at the top and incubated in a water bath pre-equilibrated to 37 °C. At various time points, ampoules were removed and stored at −80 °C to prevent any further reaction. The content of the various CTx isoforms was determined immediately after thawing by reverse-phase HPLC and immunoassay analysis. The relative content of each of the isoforms was plotted against time, and kinetic curves were constructed by a computer-aided iteration procedure. Here the mechanism of RI was described by six coupled differential equations with 14 rate constants and six mass-action terms; 4 curves were fitted simultaneously to the 4 × 4 sets of data points using a non-linear squares-fit procedure employing Excel’s Problem Solver (Excel for Windows 95®; Microsoft Inc.). The concentrations of the succinimide intermediates (Scheme 1, B and E) and the 14 rate constants were modulated (by Problem Solver) to obtain the best possible fit to data obtained from the incubation of synthetic CTx.
To assess the RI kinetics of CTx from mineralized bone, eight ampoules were prepared, each containing 50 mg of trabecular bone powder (from a foetal calf) suspended in 400 µl of sterilized 0.2 M sodium phosphate, pH 7.4, containing 0.1% (v/v) chloroform. Ampoules were flame-sealed and incubated at 37 °C. Following incubation, samples were immediately frozen at −80 °C and lyophilized. Immunoreactive CTx was subsequently prepared from the bone powder as described, and the various isoforms were determined using immunoassay analysis. The relative concentrations of CTx isoforms were plotted against incubation time and compared with the curves obtained by incubation of synthetic αL-AHDGGR.

**RI of CTx in bone and urine in vivo**

The various isoforms of CTx were determined in urine from an age series of healthy humans (0.2–74 years; n = 114). The relative contents of the isoforms (% of total CTx) were calculated and plotted against age. In addition, the contents of the isoforms were determined in ‘young’ and ‘old’ trabecular and cortical bone by immunoassay analysis of immunoreactive CTx prepared from bovine bone. The corresponding CTx ratios were calculated and compared with those found in urine from adults and children.

**RESULTS**

**Specificity and technical performance of CrossLaps® immunoassays**

A mixture containing equal amounts of the four AHDGGR isoforms (αL, βL, αD and βD) was subjected to reverse-phase HPLC (Figure 1A). Immunoassay measurement of the eluted fractions demonstrated that only the αL form of the CTx epitope was recognized in the α-CrossLaps® RIA (Figure 1B). Correspondingly, only βL, αD and βD were reactive in the CrossLaps®, αD-CrossLaps® and βD-CrossLaps® ELISAs respectively (Figure 1B). The cross-reactivity towards the ‘non-reactive’ isoforms was below 1.2% in all assays. The technical performance of the newly developed αD- and βD-CrossLaps® ELISAs was evaluated, and results are compiled in Table 1.

**RI of CTx in vitro**

The time course of RI for each of the four AHDGGR isoforms is shown in Figure 2. Incubation of pure preparations of each of the four AHDGGR isoforms at physiological pH and temperature resulted in the appearance of four products, as detected by elution from the HPLC column. The products were identified as the βD, βL, αL, and αD forms of AHDGGR by co-injecting synthetic peptide standards. The elution conditions employed resulted in complete separation of the four different isoforms of AHDGGR. The imide intermediates (Scheme 1, B and E) could not be detected in any of the chromatograms.

A computer iteration procedure was used to predict the equilibrium concentrations of the various CTx isoforms. Although Asp racemization is believed to occur primarily through the succinimide intermediate, the inclusion of the direct proton abstraction pathways in the kinetic model increased the goodness of fit. The relative concentrations of the different
Figure 2  Time course of RI of the different CTx isoforms

Pure preparations of the various CTx isoforms were incubated in aqueous solution at pH 7.4 and 37 °C: (A) αL-AHDGGR; (B) βL-AHDGGR; (C) αD-AHDGGR; (D) βD-AHDGGR. The composition of the incubated solutions was determined at different time points by HPLC analysis. Data are plotted as means of the two independent HPLC determinations, overlaid with curves obtained by computer-aided iterations. ●, αL; ■, βL; ○, αD; □, βD.

Figure 3  Comparison of the kinetics of RI in synthetic CTx and CTx in mineralized bone

Trabecular bone powder (from a foetal calf) was incubated in aqueous solution at pH 7.4 and 37 °C. The composition of the incubated solutions was determined at different time points by immunoassay analysis. Data are plotted as means of the two independent duplicate measurements, superimposed with curves obtained from the incubation of synthetic αL-CTx (from Figure 2). ●, αL; ■, βL; ○, αD; □, βD.

The kinetic data for RI of native CTx in mineralized bone collagen did not deviate significantly from those found for synthetic AHDGGR (Figure 3).

RI of CTx in bone and urine in vivo

The age-related development of RI in urinary CTx was analysed in 114 humans aged 0.2–74 years (Figure 4). The relative content of the age-related CTx forms is low in children, increases gradually through adolescence and is relatively constant from the age of 20 onwards. The αL/βL, αL/αD and αL/βD CTx ratios determined in urine and in enzyme digests of bone are given in Table 2. Evaluated on a group basis, the CTx ratios were 2.3–4.8-fold higher in urine from children as compared with adults (Table 2).

In bone, the highest ratios were found in foetal trabecular bone, followed by foetal cortical bone, ‘old’ trabecular and cortical bovine bone (Table 2). The CTx ratios measured in urine from adults were similar to those in ‘old’ cortical and trabecular bovine bone. Correspondingly, CTx ratios in children’s urine were in the same range as those found in trabecular and cortical bone from a bovine foetus (Table 2).

RI of CTx in metabolic bone disease

The urinary concentrations of the four CTx isoforms were measured by immunoassay analysis, and the αL/βL, αL/αD and αL/βD CTx ratios were determined. Two different patient groups were analysed: patients with Paget’s disease and patients with post-menopausal osteoporosis. CTx ratios were increased in the disease groups, as compared with healthy controls. The best overall discriminatory power between patients and controls was obtained using the αL/αD CTx ratio. Thus all patients with...
Figure 4 Relative contents of CTx isoforms in urine from healthy humans aged 0.2–74 years

The concentrations of the different CTx isoforms were determined in 114 healthy humans by use of the CrossLaps® immunoassays. The relative content of each isoform (% of total CTx) and corresponding CTx ratios were calculated. 

(A) Age-dependent variation of \( \alpha_L \); (B) age-dependent variation of \( \beta_L \) (inset shows \( \alpha_L / \beta_L \) ratio); (C) age-dependent variation of \( \beta_D \) (inset shows \( \alpha_L / \beta_D \) ratio); (D) age-dependent variation of \( \alpha_D \) (inset shows \( \alpha_L / \alpha_D \) ratio).

Table 2 RI of CTx in bovine bone and human urine in vivo

Bone extracts were analysed in each immunoassay by two independent quadruplicate measurements. CTx isoforms in urine were assessed by one quadruplicate measurement in each assay. Subsequently CTx ratios were calculated.

<table>
<thead>
<tr>
<th>Source</th>
<th>CTx ratio</th>
<th>( \alpha_L / \beta_L )</th>
<th>( \alpha_L / \beta_D )</th>
<th>( \alpha_L / \alpha_D )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine from 32 humans aged &lt; 16 years (mean ± S.D. 7.6 ± 4.2 years)</td>
<td>( \text{mean} \pm \text{S.D.} )</td>
<td>1.62 ± 0.45</td>
<td>5.35 ± 1.32</td>
<td>10.20 ± 2.56</td>
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<tr>
<td></td>
<td>Absolute range</td>
<td>0.76– 2.63</td>
<td>2.43– 8.45</td>
<td>4.57–14.42</td>
</tr>
<tr>
<td></td>
<td>Trabecular*</td>
<td>1.77</td>
<td>11.06</td>
<td>25.42</td>
</tr>
<tr>
<td></td>
<td>Cortical*</td>
<td>0.95</td>
<td>1.63</td>
<td>7.88</td>
</tr>
<tr>
<td>Fetal bovine bone (7 months in utero)</td>
<td>( \text{mean} \pm \text{S.D.} )</td>
<td>0.69 ± 0.20</td>
<td>1.10 ± 0.35</td>
<td>2.29 ± 0.61</td>
</tr>
<tr>
<td></td>
<td>Absolute range</td>
<td>0.28– 1.16</td>
<td>0.39– 1.87</td>
<td>0.76–4.21</td>
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<tr>
<td></td>
<td>Trabecular*</td>
<td>0.23</td>
<td>4.12</td>
<td>2.47</td>
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<tr>
<td></td>
<td>Cortical*</td>
<td>0.13</td>
<td>0.92</td>
<td>2.20</td>
</tr>
<tr>
<td>Urine from 73 humans aged &gt; 25 years (mean ± S.D. 53.3 ± 14.7 years)</td>
<td>( \text{mean} \pm \text{S.D.} )</td>
<td>0.23</td>
<td>4.12</td>
<td>2.47</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Cortical*</td>
<td>0.13</td>
<td>0.92</td>
<td>2.20</td>
</tr>
<tr>
<td>Bovine bone (full-grown cow)</td>
<td>( \text{mean} \pm \text{S.D.} )</td>
<td>0.69 ± 0.20</td>
<td>1.10 ± 0.35</td>
<td>2.29 ± 0.61</td>
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* Ratios in bone are given as means of two independent measurements.

Paget’s disease and 20% of patients with osteoporosis displayed \( \alpha_L / \alpha_D \) ratios more than three S.D.s above the mean of healthy adults, with \( T \)-scores of 23.2 (\( P < 0.0001 \)) and 1.5 (\( P < 0.0001 \)) respectively (Figure 5). The \( \alpha_L / \beta_L \) and \( \alpha_L / \beta_D \) CTx ratios yielded \( T \)-scores of 11.5 (\( P < 0.0001 \)) and 16.4 (\( P < 0.0001 \)) for Paget’s disease, and 0.06 (not significant) and 0.26 (\( P = 0.01 \)) for osteoporosis, respectively. Comparing the patient groups with sex- and age-matched healthy controls only further increased the \( T \)-score and the differentiation between patients and healthy controls (results not shown).

**DISCUSSION**

The quantification of Asp racemization in peptides and proteins is traditionally accomplished by chromatographic analysis [2–8]. The analysis is carried out by hydrolysis of the sample followed by chromatographic enantiomer analysis of
is strongly mineralized, which may pose additional sterical restraints on RI. For this reason, we also studied RI of CTx in mineralized bone in vitro. The time course for these reactions in bone collagen was quite similar to that found for synthetic AHDGGR hexapeptides, suggesting that cross-linking and mineralization do not significantly influence the reaction rates (Figure 3). The time course of RI determined for synthetic CTx may therefore be very close to that taking place in vivo in bone.

Bone collagen is degraded extensively by proteolytic enzymes during resorption. The resultant release of CTx fragments through the osteoclasts into the extracellular space [19,20] and their subsequent detection in serum or urine thus provides an index of bone resorption [21–23]. It is pertinent to note that degradation products of type I collagen in urine might also be derived from non-skeletal sources, as type I collagen is widely distributed in most connective tissues throughout the body. An explanation for the clear bone-resorption specificity of CTx markers in urine may be that specific post-translational modifications (i.e., cross-linking, glycosylation, etc.) protect CTx fragments derived from resorption of bone against degradation, securing their survival into urine [22]. Conversely, CTx fragments derived from bone formation or non-skeletal sources may lack such modifications and may consequently be unprotected against proteolysis, resulting in complete metabolism of such fragments [22].

Turnover comprises the cellular processes that secure the replacement of old tissue with new. The so-called bone-turnover markers comprise various bone-related molecules measured in urine or blood, and have been applied in the diagnosis and management of bone metabolic diseases [24]. The term ‘bone-turnover markers’ is somewhat misleading, as none of these markers estimate bone turnover, but reflect either bone formation or bone resorption.

As argued above, the assessment of each CTx isoform in urine per se provides a dynamic index of the rate at which bone is resorbed. In contrast, RI of urinary CTx may reflect the ‘age of bone resorbed’ and provide an index of bone turnover. The rationale is as follows. The accumulation of the age-related molecular species (βL, αD and βD forms) depends on the rate constants for the inversion of the different isomers on the one hand, and of that of protein turnover on the other. If the turnover is essentially zero, conditions are favourable for the accumulation of the age-related species, and reactions may essentially reach equilibrium within a short time. In contrast, when the rate of tissue/protein turnover is very high compared with the inversion reactions, racemized and isomerized species cannot accumulate, since proteins containing βL-Asp bonds or L-amino acids are rapidly metabolized. Between these cases, situations may occur where age-related species build up at different rates or reach a steady state reflecting the metabolic activity of the protein in question. In all cases, when the RI kinetics are known for the particular protein, this relationship can potentially be used to estimate the turnover rates of the analysed protein or tissue.

The present work shows that RI in bone CTx reflects the biological age of the tissue and the rate of tissue turnover (Table 2). The close relationship between CTx ratios in urine and bone turnover is very high compared with the inversion reactions, racemized and isomerized species cannot accumulate, since proteins containing βL-Asp bonds or L-amino acids are rapidly metabolized. Between these cases, situations may occur where age-related species build up at different rates or reach a steady state reflecting the metabolic activity of the protein in question. In all cases, when the RI kinetics are known for the particular protein, this relationship can potentially be used to estimate the turnover rates of the analysed protein or tissue.
Combining our remodelling cycle lasts 4–8 months, and that the bone extent of RI in CTx may be altered in high-turnover conditions, patients with increased aggressive form of the condition [28]. It is possible that the high bone turnover osteoporosis, the latter being the most variants according to the turnover of bone: low normal and high-turnover osteoporosis, which is in reasonable agreement with a previous study [29].

Healthy adults had $\alpha_{1} / \alpha_{2}$ CTx ratios in a narrow, well defined range (Figure 5, Table 2). Conversely, all patients with Paget’s disease displayed $\alpha_{1} / \alpha_{2}$ CTx ratios more than three S.D.s above the normal mean. This is in accordance with previous findings indicating increased urinary $\alpha_{1} / \beta$ CTx ratios in patients with Paget’s disease [15,26]. On a group basis, patients with osteoporosis, and the corresponding ‘age of bone’ was calculated (arrows).

Histological studies of bone from osteoporotic individuals have displayed $\alpha_{1} / \alpha_{2}$ CTx ratios in patients with Paget’s disease more than three S.D.s above the normal range. This is in accordance with previous findings indicating increased urinary $\alpha_{1} / \beta$ CTx ratios in patients with Paget’s disease [15,26]. On a group basis, patients with osteoporosis, and the corresponding ‘age of bone’ was calculated (arrows).

In summary, we have shown that the residue Asp$^{211}$ in the $\alpha_{1}$(I) collagen chain is prone to RI, resulting in four isomeric end-products ($\alpha_{1}$, $\alpha_{2}$, $\beta_{1}$ and $\beta_{2}$). Using immunoassay analyses and chromatographic techniques, we have demonstrated that these reactions reflect the biological age of collagen, producing an age-dependent increase in racemized/isomerized isoforms in vivo. Finally, and most importantly, we present evidence showing that an assessment of R1 in urinary CTx constitutes a non-invasive approach to determine bone turnover rates, thus aiding the diagnosis of metabolic bone diseases.

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Figure 6 Time- and turnover-dependent variation of CTx ratios

The time-dependent variation of CTx ratios was calculated from the in vitro kinetic analysis of synthetic peptides: $\alpha_{1}$/CTx ratio; $\alpha_{1}$/dCTx ratio; $\alpha_{1}$/p CTx ratio. The absolute ranges for the urinary $\alpha_{1}$/dCTx ratios were determined for children, healthy adults, patients with Paget’s disease and patients with osteoporosis, and the corresponding ‘age of bone’ was calculated (arrows).

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25. Reference deleted