Thermal Instability of the Trimeric Structure of the N-terminal Propeptide of Human Procollagen Type I in Relation to Assay Technology

Jette Brandt, Thomas N. Krogh, Charlotte H. Jensen, Jette K. Frederiksen, and Børge Teisner

The N-terminal propeptide of procollagen type I (PINP) appeared in two peaks after size chromatography. The high-molecular weight form was transformed to the low-molecular weight form during incubation at 37 °C, whereas the low-molecular weight form remained unchanged. The PINP concentrations in amniotic fluid and sera remained unchanged during 37 °C incubation when measured using an ELISA; however, concentrations decreased by 89–93% when measured using an RIA. The ELISA:RIA ratio varied from 1.1 to 2.9 in these fluids because of different size distributions and the inability of the RIA to measure the low-molecular weight form. Thermal transition of the high-molecular weight form caused a change in its elution volume but did not change its migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Mass spectrometry revealed identical results for both forms. We reached the following conclusions: (a) the trimeric structure of PINP is unstable at 37 °C; (b) the two molecular forms represent intact α1 chains in trimeric and monomeric forms; (c) thermal transition is an ongoing in vivo process; and (d) this is important in the choice of assay technology.

Collagen type I is present in soft connective tissue and bone, where it constitutes ~90% of the organic matrix (1). Collagen type I is derived from procollagen type I by enzymatic cleavage, which releases the N- and C-terminal propeptides from the procollagen (2). Analysis of the serum N-terminal propeptide of procollagen type I (PINP) in patients with hypovitaminosis D-induced hyperparathyroidism, (3) Paget disease (4), and postmenopausal osteoporosis (5) suggests PINP as a useful marker of bone metabolism.

PINP was first isolated from amniotic fluid under the name fetal antigen 2 (6), and amino acid sequencing identified the high-molecular weight form as a homomer of the α1 chains of PINP (7). Antibodies against the α1 chain of PINP have formed the basis for immunoassays for quantification of PINP, i.e., electroimmunoassay and ELISA (3, 6, 8), as well as RIA techniques (9, 10).

These antibodies recognize two molecular forms of PINP, which appear as distinct peaks after size chromatography (3, 6). These molecular forms of PINP are readily analyzed by electroimmunoassay and sandwich ELISA techniques (3, 6, 9, 11), using antibodies specific for the α1 chain of PINP. Moreover, the two molecular forms of PINP interact with the reaction of immunological identity, and both forms migrate at an apparent molecular mass of 27 kDa when analyzed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (3, 11). However, when the same antibodies were used in RIA with 125I-PINP as tracer, only the high-molecular weight form of PINP could be detected (9, 11, 12). A commercial RIA for quantification of PINP became available recently, and this assay is also based on α1-chain-specific antibodies. Like the RIA described previously by Price and co-workers (9, 12), the commercial RIA fails to detect the low-molecular weight form of PINP (10, 11).

The anti-PINP antibodies used for ELISA development (3), the antibodies applied in the RIA described by Price et al. (9), and the antibodies from the commercial RIA detect both molecular forms of PINP (10, 11). Thus, the difference in the ability of these assays to detect the low-molecular weight form of PINP is most likely caused by different functional affinities of the antibodies for the high- and low-molecular weight forms, which is revealed by the diminished reactivity in the competitive (antibody-limiting) RIA system but not in the ELISA (excess reagent) (11).

Departments of 1 Medical Microbiology and 2 Molecular Biology, University of Odense, Winsløwparken 19, DK-5000 Odense C, Denmark.

*Author for correspondence. Fax 45 65 915267; e-mail b.teisner@imbmed.ou.dk.

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The trimeric structure of PINP is achieved via the noncovalent forces within the collagenous part of the chains. The helical form of the collagens is a consequence of many reinforcing bonds, each of which is relatively weak, and it is well known that the thermal stability of soluble collagens is low; e.g., soluble calf skin collagen has a melting temperature of 39 °C (13). The collagen region of PINP is short compared with skin collagen; hence, the thermal stability of PINP should be lower.

The aim of the present study was to analyze the thermal stability and structures of the two molecular forms of PINP and to relate those observations to the choice of assay technology.

**Materials and Methods**

**BIOLOGICAL SAMPLES**

Second trimester human amniotic fluid was obtained from the Department of Clinical Genetics, Vejle County Hospital, Vejle, Denmark. Serum samples were donated by staff members and their children at the Department of Medical Microbiology, University of Odense, Odense, Denmark. Cord serum samples from routine term deliveries were made available from the Department of Obstetrics and Gynecology, Odense University Hospital, Odense, Denmark. All collections of biological samples were approved by the ethics committees of the counties of Funen and Vejle, Denmark.

**IMMUNOASSAYS**

*Electroimmunoassay.* Rocket immunoelectrophoresis was performed in 1.5 mm thick, 1% agarose (Litex HSA; Litex Industries) in 0.02 mol/L Tris-barbital buffer, pH 8.6, containing monospecific rabbit anti-PINP prepared as described previously (6).

*ELISA.* The sandwich ELISA technique using immunospecifically purified rabbit anti-PINP as the capture antibody and the same antibody conjugated with biotin as indicator has been described in detail previously (3).

*RIA.* The procollagen, intact $^{125}$I-PINP RIA kit was purchased from Orion Diagnostica and used in accordance with the manufacturer's recommendations. This RIA is identical to that described by Melkko et al. (10) in 1996.

**PINP PURIFICATION**

PINP was purified from amniotic fluid on a CNBr-activated Sepharose (Pharmacia) matrix to which immunospecifically purified rabbit anti-PINP antibodies were coupled. This matrix (3.5 mL) was packed in a XK16 column (Pharmacia). The column was connected to a Fast Performance Liquid Chromatography system (Pharmacia) and equilibrated with phosphate-buffered saline, pH 7.3, containing extra 1 mol/L NaCl. The capacity of the column was 80 mL of amniotic fluid (25 mg PINP/L). The bound PINP was eluted with 5 g/L citric acid, pH 2.8, and the PINP-containing fractions were identified by rocket immunoelectrophoresis.

**ANALYSIS OF THERMAL STABILITY**

Samples of purified PINP, second trimester amniotic fluid, cord serum, and human serum were exposed to 37 °C for up to 72 h in a water-jacketed incubator (Forma Scientific). The control samples were kept at 4 °C for the same period. After incubation, the samples were subjected to size chromatography, and the distribution of PINP was analyzed by rocket immunoelectrophoresis, ELISA, and RIA.

**SIZE CHROMATOGRAPHY**

Aliquots (200 μL) of second trimester amniotic fluid, human serum, cord serum, or purified PINP were applied to a Superose 12 HR 10/30 column connected to a Fast Performance Liquid Chromatography system. The chromatography was performed with phosphate-buffered saline, pH 7.3, at a flow rate of 0.5 mL/min, and 0.5-mL fractions were collected.

**SDS-PAGE**

SDS-PAGE analysis of fractions from the size chromatography was performed under reducing conditions on 4–20% gradient gels with discontinuous buffers. Protein bands were detected by Coomassie staining.

**MASS SPECTROMETRY**

Matrix-assisted laser desorption/ionization mass spectrometric analysis of samples (5–20 pmol) was performed on a PerSeptive Biosystems Voyager STR instrument (PerSeptive Biosystems). Protein samples were desalted using Poros R1 column material. The desalted protein was eluted in 800 mL/L acetonitrile containing 2.0 g/L trifluoroacetic acid, and a small fraction was mixed with an
equal volume of sinapinic acid (20 g/L in 700 mL/L acetonitrile).

Mass spectra were acquired in the linear mode, using an acceleration voltage of 20 kV and a delay time of 300 ns. Single-shot (n = 128) spectra were added, and the final spectrum was calibrated externally.

**Results**

**Thermal Instability of Purified PINP**

PINP purified from amniotic fluid by immunospecific affinity chromatography was subjected to size chromatography six times in succession, using pooled fraction collection. Fig. 1 shows the absorbance profiles at 280 nm and the results of analytical rocket immunoelectrophoresis of the fractions before and after incubation at 37 °C for 72 h. From the results shown in Fig. 1, fractions 22 and 27 were chosen to represent the high- and low-molecular weight forms of PINP, respectively. Fraction 22 was subjected to rechromatography before and after incubation at 37 °C for 72 h, and as seen in Fig. 2, incubation at 37 °C caused a pronounced change from the high- to the low-molecular weight form of PINP. This change was revealed by both the change in the absorbance profile at 280 nm and the result of rocket immunoelectrophoretic analysis of the PINP. Size chromatography of the high-molecular weight form of PINP after 0, 18, and 24 h at 37 °C revealed a half-life of ~10 h.

**Table 1. The influence of exposure to 37 °C on the result of PINP quantification using ELISA and RIA.**

<table>
<thead>
<tr>
<th>Time at 37 °C</th>
<th>PINP, μg/L</th>
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<tbody>
<tr>
<td></td>
<td>Amniotic fluid</td>
</tr>
<tr>
<td>0 h</td>
<td>ELISA</td>
</tr>
<tr>
<td>24 h</td>
<td>24 840</td>
</tr>
<tr>
<td>48 h</td>
<td>23 422</td>
</tr>
<tr>
<td>72 h</td>
<td>24 101</td>
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<td></td>
<td>23 730</td>
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In contrast, incubation of fraction 27 (the low-molecular weight form) at 37 °C for 72 h did not produce marked changes in either the absorbance profile at 280 nm or the distribution of PINP as analyzed by rocket immunoelectrophoresis (Fig. 3).

The concentration of PINP in amniotic fluid, cord serum, and nondiseased human serum was measured by ELISA and RIA before and after incubation for 24, 48, and 72 h at 37 °C (Table 1). Whereas only minor fluctuations were observed in the ELISA result, the apparent PINP concentration as measured by RIA decreased to 6.4% (amniotic fluid), 11% (cord serum), and 6.6% (nondiseased human serum) of the values obtained for control samples over a 72-h incubation period at 37 °C. The other obvious difference between the two assays is reflected in that the ratio [PINP] \(_{\text{ELISA}}\)/[PINP] \(_{\text{RIA}}\) differed markedly in different biological fluids: the ratio was 2.0 for amniotic fluid, 2.9 for cord serum, and 1.1 for nondiseased human serum. To find an explanation for these differences, we analyzed the size distribution of PINP in amniotic fluid, cord serum, and nondiseased human serum and compared the ELISA and RIA results for fractions (size chromatography) before and after treatment at 37 °C for 72 h.

The size distribution of PINP as analyzed by ELISA in amniotic fluid, cord serum, and nondiseased human serum without 37 °C treatment is shown in Fig. 4. As seen from Fig. 4, the ratios between the high- and low-molecular weight forms differed between the biological fluids, and calculation revealed the ratios to be 1.64 for amniotic fluid, 0.67 for cord serum, and 3.1 for nondiseased human serum.

ASSAY TECHNOLOGY AND THERMAL INSTABILITY

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The RIA measures mainly the high-molecular weight form of PINP (9–11); therefore, the differences in size distribution between the biological fluids seem to explain the differences in the concentration ratio measured using the ELISA and the RIA in amniotic fluid, cord serum, and nondiseased human serum. Moreover, the calibrator supplied by the commercial RIA contains only the high-molecular weight form, whereas the calibrator used in the ELISA contains both forms of PINP (11).

The ELISA and RIA results for fractions from size chromatography of nondiseased human serum with and without treatment at 37 °C are shown in Fig. 5. Before treatment, the RIA detected eluted PINP antigen specific for the high-molecular weight form of PINP, whereas the ELISA detected both forms. After incubation at 37 °C for 72 h, the RIA failed to detect PINP in the fractions (detection threshold, 5 μg/L), whereas the results of the ELISA revealed a dramatic loss of the high-molecular weight form and an increase in the low-molecular weight antigen. Similar results were obtained with amniotic fluid and cord serum as antigen sources.

**Analysis by SDS-PAGE and Mass Spectrometry**

Fraction 22 (Fig. 1) was incubated at 37 °C for 72 h (control at 4 °C), and analytical size chromatography revealed a shift in distribution identical to that shown in Fig. 2. The high-molecular weight form of PINP with or without 37 °C treatment for 72 h was analyzed by SDS-PAGE, and the result is shown in Fig. 6. Both the treated and untreated samples migrate in single bands corresponding to a molecular mass of 27 kDa, and this result indicates that the α1 chain remains intact in spite of the dramatic shift in the size chromatography profiles observed in samples after treatment at 37 °C.

Mass spectrometric analysis of fractions 22 and 27 (Fig. 7) was performed to verify the presence of intact PINP α1 chains in both fractions. Mixing of the protein with matrix solution, necessary for mass spectrometry, causes dissociation of noncovalent multimers into monomers. The resulting mass spectrum of fraction 22 is shown in Fig. 7A. The PINP monomeric form of the α1 chain was identified by this analysis as several peaks appearing with a mass difference of 16 Da. The difference between the calculated molecular mass of the α1 chain (14 213.62 Da) and the measured value corresponds to the presence of six to nine hydroxyprolines in the collagenous C-terminal part of the intact α1 chain. Exactly the same peak pattern was seen by mass analysis of fraction 27 (Fig. 7B), indicating intact α1 chain in both fractions.

Peptide mapping of the proteins present in the two fractions, using endoproteinase Lys-C digestions (data not shown), further confirmed the presence of intact α1 chain in both fractions.

**Discussion**

PINP, as defined antigenically by antibodies against the α1 chain, appears in two distinct molecular forms when subjected to size chromatography (3, 6, 11). These forms interact with the reaction of immunological identity, and...
after SDS-PAGE both forms migrate in single bands corresponding to an apparent molecular mass of 27 kDa (11). These observations suggest that the two molecular forms of PINP represent different (monomeric or multimeric) structures of the intact α1 chains.

The ratio between the two molecular forms varies in biological fluids, as demonstrated here in amniotic fluid, cord serum, and pooled nondiseased human serum. We have also observed major differences in the ratio of the two molecular forms in sera obtained from apparently healthy individuals, patients treated with continuous peritoneal dialysis, and the dialysis fluid. The amount of PINP recovered in the 24-h dialysis fluid exceeded the circulating amount by >100%, suggesting local synthesis of PINP in the peritoneal cavity induced by an inflammatory reaction to the applied treatment (11). The hypothesis tested in the present study is that the PINP is released from the procollagen molecule as a trimer represented by the high-molecular weight form and that thermal transition at 37 °C produces monomeric α1 chains.

The incubation of the high-molecular weight form of PINP purified from amniotic fluid at 37 °C for 72 h followed by size chromatography produced a pronounced shift from the high- to the low-molecular weight form. The half-life of the high-molecular weight form at 37 °C was calculated to be ~10 h. In contrast, the low-molecular weight form remained unchanged after incubation at 37 °C for 72 h. Could the thermal instability of the high-molecular weight form be caused by the use of pH 2.8 citric acid buffer in the purification procedure and the lack of protecting proteins in the purified state?

This question was addressed by analysis of the thermal stability of native PINP in serum and amniotic fluid. Treatment of amniotic fluid and serum at 37 °C for 72 h produced a change of PINP antigen similar to that observed when purified PINP was used. These data indicate that PINP is unstable at body temperature and that a continuous shift in PINP size distribution takes place in body fluids. This finding underlines the importance of knowing the specificity of the assays used to assess collagen type I metabolism.

It was demonstrated previously that antibodies recognizing both molecular forms of PINP fail to detect the low-molecular-weight weight form of PINP when applied in an RIA that uses 125I-labeled PINP as the tracer (9–11). In contrast, the sandwich ELISA technique has been demonstrated to detect the α1 chain independent of molecular form (3, 11).

The present study also demonstrates that quantification of PINP in amniotic fluid, cord serum, and nondiseased human serum, using the ELISA technique, revealed no marked change in the results during incubation of samples at 37 °C for up to 72 h. In contrast, in the RIA, the apparent PINP concentration decreased up to 93% after incubation at 37 °C. The decrease in the apparent concentration in RIA during the first 24 h (Table 1) is in good agreement with the estimated half-life of the purified high-molecular weight form of PINP, i.e., ~10 h. Moreover, the ratio between the results of PINP quantification obtained using ELISA and RIA differed markedly between these three biological fluids, with the ratio being 2.9 in cord serum and 1.1 in nondiseased human serum. These differences seem to be explained by the difference in size distribution of the PINP antigen between the biological fluids and by the fact that RIA fails to detect the low-molecular weight form of PINP. This was further substantiated by the results obtained by ELISA and RIA on fractions (from size chromatography) of nondiseased human serum, where 37 °C treatment led to the disappearance of the signal in the RIA, whereas the ELISA signal simply moved from the high- to the low-molecular weight fractions.

It has been suggested that the low-molecular weight form of PINP is identical to the Col 1 fragment that has been produced by digestion of procollagen by bacterial collagenase (10). However, this artificial fragment is unrelated to the two molecular weight forms of PINP because Col 1 migrates in a band corresponding to 14 kDa in contrast to the 27-kDa band observed for both molecular forms of native PINP (10, 11). The RIA detects only the high-molecular weight form of PINP, which in accordance with our observations is eluted in a volume corresponding to a molecular mass of ~100 kDa from size chromatography and migrates in a band corresponding to 27 kDa in SDS-PAGE (10, 11). Thus we find no evidence for the hypothesis that the low-molecular weight form of PINP is related to the Col 1 fragment (11).

Analysis of the two molecular forms by mass spectrometry showed identical spectra and a mass in accordance with the theoretical mass of the intact α1 chain of PINP when the proline hydroxylations are taken into account. These data clearly show that both molecular forms of PINP contain intact α1 chains and further substantiate our hypothesis that the high-molecular weight form represents the trimeric structure of PINP and the low-molecular weight form represents the monomeric form of intact α1 chain. The apparent difference in molecular mass estimated by SDS-PAGE (27 kDa) and mass spectrometry (~14 kDa) is attributable to aberrant migration in SDS-PAGE caused by the collagenous stretch in PINP compared with the globular molecules used as molecular markers.

The observations described in the present study using thermal transition, SDS-PAGE, and mass spectrometry strongly support our hypothesis that the high-molecular weight form of PINP represents the unstable trimeric structure and that the low-molecular weight form represents the monomeric α1 chain released from the trimeric structure at body temperature.

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References


