Limited Proteolysis of Human $\alpha_2$-HS Glycoprotein/Fetuin

EVIDENCE THAT A CHYMOTRYPTIC ACTIVITY CAN RELEASE THE CONNECTING PEPTIDE*

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$\alpha_2$-HS glycoprotein is a major protein of human plasma whose function is still obscure. A proteolytically processed form of $\alpha_2$-HS glycoprotein lacking a segment of 40 amino acid residues bridging its heavy and light chain portions ("connecting peptide") has been described suggesting that this peptide is released by post-translational processing to fulfill biological role(s) of $\alpha_2$-HS glycoprotein. To test this hypothesis we investigated how the connecting peptide is released from the parental molecule by limited proteolysis. We developed monoclonal antibodies to various portions of the connecting peptide and its NH$_2$-terminal flanking region which cross-react with the native $\alpha_2$-HS glycoprotein. Purified $\alpha_2$-HS glycoprotein from human plasma was subjected to limited proteolysis by proteinases including trypsin, chymotrypsin, elastase plasmin, kallikrein, thrombin, and renin. Immunoprint analysis of the proteolytic digests indicated that $\alpha_2$-HS glycoprotein is readily cleaved in its connecting peptide region. NH$_2$-terminal amino sequence analysis of the generated fragments demonstrated that a single proteinase, chymotrypsin, cleaves the critical Leu-Leu bond flanking the NH$_2$-terminal portion of the connecting peptide region. Most but not all of the other proteinase cleavage sites map to a short stretch of 9 residues located in the center portion of the connecting peptide region. Immunoprint analysis of plasma samples from patients with sepsis demonstrated that the connecting peptide region is cleaved under pathological conditions. Our results indicate that the connecting peptide and/or fragments thereof are readily releasable from $\alpha_2$-HS glycoprotein in vitro and in vivo.

Human $\alpha_2$-HS glycoprotein ($\alpha_2$-HS) belongs to the fetuin family of proteins that are collectively named after the bovine species homologue, fetuin (1). Fetuins are glycoproteins with both N-linked and O-linked carbohydrate side chains (2, 3).

Fetuins occur in large amounts in blood and cerebrospinal fluid and accumulate to high concentrations in calcified bone (4). The fetuins form a family within the superfamily of mammalian cystatins (5); they are characterized by a tripartite structure, i.e. two NH$_2$-terminally located cystatin-like domains (D1, D2) and a unique COOH-terminal domain (D3) not present in other mammalian cystatins (6). Although many biological functions have been attributed to fetuins, none has been unequivocally established to date (for review, see Ref. 7).

The human homologue of fetuin, $\alpha_2$-HS glycoprotein, has long been known as a major protein of the plasma and the cerebrospinal fluid. Early studies have demonstrated that $\alpha_2$-HS isolated from Cohn's fraction VI of human plasma is a two-chain molecule consisting of a A-chain of 282 amino acid residues and a B-chain of 27 residues (8, 9). Cloning of the $\alpha_2$-HS cDNA revealed that a single mRNA transcript encodes the A and the B chains, and that a sequence segment of 40 residues ("connecting peptide") bridges the A and B portions in the primary translation product, pre-$\alpha_2$-HS (10). Because this segment is absent in the $\alpha_2$-HS form of Cohn's fraction VI it has been hypothesized that the connecting peptide is released from the precursor protein by an unknown proteinase, and that the liberated peptide might serve a biological function (9). We isolated $\alpha_2$-HS from freshly drawn human plasma in the presence of proteinase inhibitors, and determined that the circulating form of $\alpha_2$-HS is a two-chain protein with a heavy chain of 321 residues and a light chain of 27 residues (11). This circulating form of $\alpha_2$-HS still contained the connecting peptide attached to the heavy chain, and only the terminal residue of arginine in position 322 was missing. These findings prompted us to investigate $\alpha_2$-HS proteolytic processing thereby addressing the following questions: (i) is the release of the connecting peptide a consequence of the isolation procedure or does it occur under (patho)physiological conditions; (ii) is the connecting peptide released in a single step, i.e. by cleavage of the Leu$^{262}$ Leu$^{263}$ bond, or by sequential processes; (iii) which proteinases (are) involved in the release of the connecting peptide or fragments thereof, and what is the nature of the enzyme(s), if any, that split(s) the critical Leu-Leu bond? Our experimental data indicate that the connecting peptide region is highly susceptible to proteolytic breakdown in vitro and in vivo, and that a chymotryptic activity can release the connecting peptide or fragments thereof from the circulating form of $\alpha_2$-HS.

EXPERIMENTAL PROCEDURES

Materials—Tosyl-phenylalanyl-chloromethane ketone-treated trypsin (from bovine pancreas) was obtained from Merck; tosyl-L-lysyl-chloromethane ketone-treated chymotrypsin (from bovine pancreas) from Serva; thrombin (from human plasma) and elastase (from human neutrophils) from Behringwerke; plasmin (from human plasma) from Kabivitrum; kallikrein (from porcine plasma) from Bayropharm; car-

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† The abbreviations used are: $\alpha_2$-HS, $\alpha_2$-HS glycoprotein; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
Purification of Antibodies—

Monoclonal antibodies were precipitated from mouse ascites with a saturated (NH₄)₂SO₄ solution followed by mixing 0.1% (v/v) trichloroacetic acid in H₂O and 0.1% trichloroacetic acid was monitored at 280 nm and fractions were pooled and rechromatographed by reverse-phase HPLC on a Hibar-Lichrospher 125-4 column. Low molecular weight proteins resolved by SDS-PAGE (cf. above) were electrotransferred to BA85 nitrocellulose sheets (Schleicher & Schuell) by the semi-dry blotting technique (23) at 0.65 mA/cm² for 90 min, and visualized by the colloid gold technique (24). To quench the residual protein binding capacity the sheets were incubated for 30 min with 0.2% (w/v) bovine serum albumin, 50 mM Tris, 200 mM NaCl, pH 7.4. Specific antibodies to α₂-HS (from rabbit or mouse) were diluted in 0.05% (w/v) Tween 20, 0.2% (w/v) bovine serum albumin, 50 mM Tris, 200 mM NaCl, pH 7.4.

Production of Antibodies to α₂-HS Glycoprotein—Production of α₂-HS was carried out in PHS, pH 7.2, at a protein concentration of 1 mg/ml. To optimize the conditions of limited proteolysis, a first set of experiments was performed on scale (10 μg of α₂-HS per experiment) where ratios of enzyme over substrate ranged from 1:100 to 1:10,000 (w/w). After 1 h of incubation at 37°C the reaction was stopped by addition of an equal volume of SDS sample buffer and boiling for 5 min at 95°C, followed by analytical SDS electrophoresis. In a second set of experiments the proteolytic digestion was optimized with respect to time at a fixed enzyme over substrate ratio. The experiments were carried out as above except that 100 μg of α₂-HS (total amount) was incubated with the optimized concentration of the proteinase in a final volume of 100 μl, and aliquots of 10 μl were withdrawn at fixed time intervals. The resultant protein mixtures were subjected to SDS-PAGE under reducing conditions and visualized by silver staining. For large-scale preparation of cleavage products, 50 mg of α₂-HS was cleaved at 37°C under optimized conditions. The proteolytic reactions were stopped by the addition of benzamidine-HCl (final concentration 1 mM) and stored at −20°C until analyzed.

Electrophoresis, Blotting, and Immunoprinting—Analytical SDS electrophoresis was performed in 10−20% or 15% (w/v) discontinuous polyacrylamide gradient gels according to Lanneau (22). Low molecular weight polypeptides were separated by electrophoresis in a continuous gradient of 2.5−12% (w/v) acrylamide gel in the presence of 0.125% (w/v) bis-acrylamide and 0.05% (w/v) methylene blue. Proteins resolved by SDS-PAGE (cf. above) were electrotransferred to BA85 nitrocellulose sheets (Schleicher & Schuell) by the semi-dry blotting technique (23) at 0.65 mA/cm² for 90 min, and visualized by the colloid gold technique (24). To quench the residual protein binding capacity the sheets were incubated for 30 min with 0.2% (w/v) Tween 20, 0.2% (w/v) bovine serum albumin, 50 mM Tris, 200 mM NaCl, pH 7.4. Specific antibodies to α₂-HS (from rabbit or mouse) were diluted in 0.05% (w/v) Tween 20, 50 mM Tris, 200 mM NaCl to a final concentration of 0.1−0.2 μg/ml, and incubated overnight at 4°C. The bound antibody was detected by incubation with the peroxidase-conjugated secondary antibody (diluted 1/12,000) for 30 min, and with 4-chloronapthol/H₂O₂ 1:400 in 0.05% (w/v) Tween 20, 50 mM Tris, 200 mM NaCl at 5 min room temperature.

Isolation of α₂-HS Fragments—Fifty mg of α₂-HS was subjected to limited proteolysis by chymotrypsin, elastase, kallikrein, plasmin, or thrombin, and the cleavage mixtures were fractionated by gel filtration on Superose 12 fast flow (Pharmacia) in a biotinylated avidin technique described by Chang et al. (26) using a Waters HPLC system with a linear gradient of 0−70% (v/v) acetonitrile formed by mixing 0.1% (v/v) trichloroacetic acid in H₂O and 0.1% trichloroacetic acid in acetonitrile as detailed earlier (11).
Proteolytic Processing of α2-HS Glycoprotein

MAHS7 identifies monoclonal antibodies, and AS5359 denotes the polyclonal antisera to human α2-HS. Peptides are identified by their three NH2-terminal residues (single-letter code) followed by the total number of residues. Pre-α2-HS denotes the single-chain precursor of 349 residues including the heavy chain (positions 1–321), the A-chain (1–282), the proline-rich region (235–282), the connecting peptide (283–322), and the light chain (323–348, identical with the B-chain) of human α2-HS. The domain composition lists the cystatin-like domains D1 (1–118) and D2 (119–234), and the fetuin-specific domain D3 (235–349).

To further map the target sequences of the antibodies to the A-chain, we expressed domain D3 of human α2-HS in Sf9 insect cells. The recombinant domain covers the COOH-terminal portion of the A-chain, the connecting peptide, and the light chain region of α2-HS. Purified α2-HS (Fig. 2, lane 1) and lyste of Sf9 cells overexpressing D3 (lane 2) were resolved by SDS-PAGE under reducing conditions; the total protein was visualized by silver staining (upper left panel). Antibodies MAHS1, MAHS2, and MAHS4 recognized the α2-HS heavy chain (lane 1) and domain D3 (lane 2). Furthermore, antibody MAHS1 decorated a degradation product of D3 that is not stained by antibodies MAHS2 or MAHS4. For comparison, antibody MAHS7 directed to the connecting peptide, and a polyclonal antibody raised against native α2-HS are included (Fig. 2, bottom panels). Because antibodies MAHS1, MAHS2, and MAHS4 recognize domain D3 (positions 235–349) but not the connecting peptide (283–322) or the light chain (323–349) they are likely to be directed to the COOH-terminal portion of 48 residues of the A-chain (235–282).

Epitope Mapping of MAHS Antibodies—To discriminate between epitopes recognized by the various MAHS antibodies a competitive ELISA was performed. In this assay the binding of a biotinylated antibody to its antigen is measured in the presence of increasing concentrations of unlabeled competitor antibodies; displacement of the biotinylated antibody by an unlabeled antibody indicates that the two antibodies recognize closely neighbored or even overlapping epitopes. The unlabeled cognate antibody and an unrelated antibody were used as the positive and negative controls, respectively. Of the antibodies to the A-chain, MAHS1 and MAHS2 bound to two distinct epitopes whereas MAHS4 competed with antibody MAHS2 for the same epitope (not shown). Antibodies to the connecting peptide, MAHS3 and MAHS7, did not displace each other and hence bind to two distinct epitopes (not shown). Hence the set

RESULTS

Isolation and Characterization of α2-HS from Human Plasma—α2-HS was isolated from freshly drawn human plasma by two successive immunofinity steps: capture of α2-HS on anti-α2-HS-Sepharose, and removal of remaining contaminating proteins on anti-albumin-Sepharose and anti-immunoglobulin-Sepharose, respectively. To prevent proteolytic modification the isolation procedure, proteinase inhibitors were added to the chromatography buffers. The characterization of the isolated protein by SDS-PAGE and immunoprinting showed a single band of 58 kDa reacting with polyclonal antibodies directed to α2-HS (not shown). The NH2-terminal amino acid sequence analysis revealed two sequences in equimolar amounts, Ala-Pro-His (heavy chain) and Thr-Val-Val (light chain). This form of α2-HS containing the heavy chain (residues 1–321) and light chain (323–349) was used throughout the study unless otherwise indicated.

Production of Monoclonal Antibodies to α2-HS—Mice were immunized with purified α2-HS (see above), their spleen cells were removed and fused with myeloma cells to obtain hybridomas secreting antibodies to α2-HS. Clones reacting positive in ELISA with intact α2-HS were further subtyped by immunoprint analysis for specific recognition of the heavy chain, the connecting peptide or the light chain (Fig. 1). Antibodies of 5 clones (MAHS1, MAHS2, MAHS3, MAHS4, and MAHS7) recognized the α2-HS heavy chain (53 kDa, lane 2) but failed to react with the light chain (5 kDa, lane 2). Antibodies MAHS3 and MAHS7 but not MAHS1, MAHS2, and MAHS4 reacted with the connecting peptide (4 kDa, lane 3), suggesting that the latter group of antibodies recognizes the A-chain portion of α2-HS. The MAHS antibodies cross-reacted well with plasma α2-HS except for MAHS4 which weakly recognized purified α2-HS but failed to detect α2-HS in plasma (lanes 1 and 2).
TABLE I

| Peptide
| Amino acid sequence | Positions |
|---|---|---|
| LAA 40<sup>a</sup> | LAAPPGHIQRHAYDLRHFATFGVWSLGSFSGEVSHPRKTR | 283–322 |
| LAA12 | LAAPPQHIQRHAYDLRHFATFGVWSLGSFSGEVSHPRKTR | 283–294 |
| HYD12 | HYDLRHFATFGVWSLGSFSGEVSHPRKTR | 295–306 |
| SLG16 | SLGSFSGEVSHPRKTR | 307–322 |
| TVV27 | TVVQSFVAAAGPVPFPCGRIRHKV | 323–349 |

<sup>a</sup> Peptides are named for their first three residues by the one-letter code followed by the total number of residues.

<sup>b</sup> Peptides covering the COOH-terminal portion of the connecting peptide region contain the residue of arginine at position 322 which is absent from the plasma form of α₂-HS (10).

Fig. 3. Fine mapping of the antibody epitopes of the connecting peptide region. Titer plates were coated with α₂-HS (●), the connecting peptide, LAA 40 (○), and conjugates of BSA and peptides LAA12 (▼), HYD12 (▲), SLG16 (□), respectively, 0.2 μg/ml each. Serial dilutions (2<sup>n</sup>) of monoclonal antibodies MAHS1 (panel A), MAHS3 (B), and MAHS7 (C) were added. Binding of monoclonal antibodies to the immobilized peptides and proteins was identified by incubation with biotinylated anti-mouse immunoglobulin (0.2 μg/ml) and the biotin-avidin-peroxidase detection method. The sequences of the peptides used for coating are listed in Table I.

which failed to process α₂-HS even at equimolar conditions (not shown). The optimum conditions varied among the different proteinases in that the enzyme over substrate ratios ranged from 1:100 (thrombin) to 1:10,000 (chymotrypsin), whereas the incubation periods varied from 5 min (trypsin at 1:1000) to 7 h (thrombin at 1:100). Under these conditions major cleavage products of 48–53 kDa were generated from 50 to 90% of the initial α₂-HS (Fig. 5, lanes 3–8). In the case of elastase, an additional major degradation product of about 40 kDa was formed (lane 6). For comparison α₂-HS preparations containing the heavy chain (lane 1) and the A-chain (heavy chain without connecting peptide), respectively (lane 2), were included in the analysis.

Immunoprint Analysis of the Proteolytic Digests—To study
the cleavage of the connecting peptide region, we analyzed the digests for the presence of the connecting peptide by immunoprinting with antibodies MAHS1, MAHS2, MAHS3, or MAHS7 (Fig. 6). Antibodies MAHS1 and MAHS2 directed to the A-chain portion of α₂-HS recognized the intact heavy chain of 58 kDa (upper panels, lane 1), the major cleavage products of 48–53 kDa (lanes 2–6), and the minor fragments of 18–35 kDa (e.g. lanes 4 and 5). In contrast, antibodies MAHS3 and MAHS7 directed to the COOH-terminal and the center part, respectively, of the connecting peptide region detected the intact heavy chain but none of the cleavage products. This finding indicates that the connecting peptide region is readily accessible and therefore a primary cleavage site for proteinases in the α₂-HS molecule. The failure of MAHS7 to detect any cleavage products of α₂-HS strongly suggests that the initial proteolytic cuts occur around the MAHS7 epitope, namely at positions 295–306 of the α₂-HS heavy chain. A time resolved analysis of the limited proteolysis indicated that the cleavage of the connecting peptide region whereas 4 fragments map to the flanking regions, i.e. the proline-rich region (single fragment) and the light chain region (three fragments). The remaining fragments are produced by the cleavage of the NH₂-terminal domain D1. These findings confirm that the connecting peptide region represents the major proteinase-sensitive site of α₂-HS.

**Isolation of α₂-HS Fragments**—To identify the proteolytic cleavage sites in α₂-HS the digests were size fractionated by preparative gel filtration. Fractions containing fragments of 10–20 kDa were pooled, concentrated, and subjected to reverse-phase HPLC (not shown). The separated peaks were collected manually and analyzed by quantitative amino acid analysis (not shown) and by manual NH₂-terminal amino acid sequence analysis performing three cycles per peptide. Table III presents 17 distinct fragments identified in the proteolytic digests generated with plasmin, kallikrein, elastase, or chymotrypsin.

Most of the fragments (13 out of 17) are derived from domain D3 of α₂-HS. As shown in the graphic representation (Fig. 7, top panel), 9 out of these 13 fragments originate from the connecting peptide region whereas 4 fragments map to the flanking regions, i.e. the proline-rich region (single fragment) and the light chain region (three fragments). The remaining fragments are produced by the cleavage of the NH₂-terminal domain D1. These findings confirm that the connecting peptide region represents the major proteinase-sensitive site of α₂-HS.

**In Vivo Processing of α₂-HS Probed by Monoclonal Antibodies**—Does proteolytic processing of α₂-HS also occur in vivo? To answer this question we examined human plasma samples by SDS-PAGE and Western blotting applying MAHS antibodies. Fifteen plasma samples from healthy donors and from patients suffering from polytrauma and sepsis were probed with monoclonal antibodies directed to the connecting peptide. A repre-
**Table III**

Major fragments from the proteolytic digests of α₂-HS

| Fragment | NH₂-terminal sequence | Positions |
|----------|----------------------|----------|
| K1       | Ala-Ile-Asp          | 25/26 (D1) |
| K2       | Asp-Tyr-Ile          | 27/28 (D1) |
| K3       | Gly-Tyr-Lys          | 36/37 (D1) |
| K4       | Asn-Gln-Ile          | 42/43 (D1) |
| E1       | Gly-Ala-Asn          | 244/245 (D3) |
| C1       | Leu-Ala-Ala          | 282/283 (D3) |
| C2       | Ala-Ala-Pro          | 283/284 (D3) |
| E2       | Ala-Ala-Pro          | 283/284 (D3) |
| P1       | Ala-His-Tyr          | 290/294 (D3) |
| C3       | Asp-Leu-Arg          | 296/297 (D3) |
| K5       | Arg-His-Thr          | 298/299 (D3) |
| P2       | His-Thr-Phe          | 299/300 (D3) |
| K6       | Phe-Met-Gly          | 301/302 (D3) |
| C4       | Met-Gly-Val          | 302/303 (D3) |
| E3       | Ala-Gly-Pro          | 332/333 (D3) |
| P3       | Ile-Arg-His          | 343/344 (D3) |
| P4       | His-Phe-Lys          | 345/346 (D3) |

*The prefix of the peptide designation identifies the processing enzyme: K, kallikrein; E, elastase; C, chymotrypsin; P, plasmin.

**FIG. 7.** Assignment of proteolytic cleavage sites in the α₂-HS molecule. Upper panel, this schematic presentation of α₂-HS structural organization shows two tandemly arranged cystatin-like domains (D1, D2) and a fetuin-specific domain 3 (D3) harboring the connecting peptide (CP) region, disulfide bonds (\(\beta\)), glycosylation sites (\(\alpha\)), phosphorylation sites (\(\gamma\)), and the proteolytic cleavage site separating heavy and light chain (\(\delta\)). The relative positions of 17 identified cleavage sites for chymotrypsin (C1 to C7), elastase (E1 to E6), kallikrein (K1 to K6), and plasmin (P1 to P6) are given. MAHS epitopes A and B are hatched. Lower panel, the amino acid sequence of the connecting peptide including its flanking residues: epitopes C and D of MAHS7 and MAHS3, respectively, are marked by thin lines, and protease cleavage sites are indicated by arrows. The processing sites for the unknown endoprotease (En) generating the circulating two-chain form of α₂-HS, and for the exopeptidase (Ex) releasing the terminal residue of Arg are also given.

**FIG. 8.** Immunoprinting of α₂-HS in pathological plasma. Whole normal plasma (lane 1), purified α₂-HS (lane 2), and plasma samples from three patients with sepsis (lanes 3–5) were separated by SDS-PAGE (10–20%) under reducing conditions followed by electroblotting onto a nitrocellulose membrane; 4 identical replicas were made. Left panel, total protein staining with colloidal gold. The center panels are immunoprints with monoclonal antibodies MAHS3 (epitope D) and MAHS7 (epitope C), and the right panel represents an immunoprint with polyclonal antiserum AS5359 to α₂-HS. Bound antibodies were detected by incubation with 1 μg/ml peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit immunoglobulin), followed by 4-chloronaphthol/H₂O₂.

**DISCUSSION**

Fetuins are abundant in mammalian plasma and tissues. Much knowledge has accumulated on the structure of these prototypic glycoproteins, however, their functional role is less well defined. Bovine fetuin has been implicated in the stimulation of cell growth (27) and the modulation of brain development (28). The rat homologue, first described as phosphoprotein pp63, was shown to be an inhibitor of the endogenous tyrosine kinase activity associated with the insulin receptor (29, 30). The fetuins have therefore been implicated in the regulation of the mitogenic response following insulin stimulation (29–31). The fact that fetuins bind transforming growth factor-β-like cytokines (32) and therefore can antagonize the action of transforming growth factor-β and bone morphogenic proteins similarly suggest a role for fetuins as modulators of growth and development.

Consistently, fetuins have been localized in mineralized bone tissue. Rat fetuin has also been described as bone sialic acid containing protein (33) or 60 K acidic glycoprotein (34), and both proteins were introduced as putative modulators of bone formation. A rabbit bone protein, hemonectin, which is closely associated or identical with rabbit fetuin (35) was originally defined as a homing factor for the granulocyte lineage (36). The human counterpart of fetuin, α₂-HS glycoprotein, is thought to play a major role in bone metabolism (37). To this end we have recently shown that domain D1 of fetuins mediates inhibition of apatite formation to inhibit unwanted mineralization in plasma and possibly during early stages of bone mineralization (18).

Although several functions have now been proposed for mammalian fetuins our knowledge about molecular mechanisms regulating these activities is still incomplete. Serine phosphorylation was shown to be critically required for rat fetuin inhibition of the insulin receptor tyrosine kinase (29) and multiple serine phosphorylation was also demonstrated in human fetuin (38). In contrast, the inhibition of apatite formation by fetuins was largely independent of secondary amino acid modifications (18). One intriguing structural feature of human fetuin with possible functional implication was revealed when the cDNA sequence of human α₂-HS was published (10) and compared to the known protein sequences from the A- and B-chains (8, 9). Namely, a stretch of 40 amino acids predicted by the cDNA sequence was conspicuously absent from the published protein sequence. This tempted us and
others to speculate that this connecting peptide served some unknown biological function after being proteolytically liberated. Subsequently we have isolated from freshly drawn plasma under the strict protection of protease inhibitors a two-chain form of \(\alpha_2\)-HS where the heavy chain still harbors the connecting peptide except for a single COOH-terminal residue of Arg at position 322 (11). This heavy chain (as opposed to A-chain) form of \(\alpha_2\)-HS is dominant in the plasma; we have failed to detect in plasma a single chain form or the two-chain form with the heavy chain including Arg

\[\text{desArg}^{40}\text{-CP.}
\]

Given the fact that the molar enzyme at positions 283/284 (Leu-Ala) is adjacent to the former site and over substrate ratio was 1:10,000, and that cleavage sites on a short segment indicates that this stretch is less conserved (2/12 and 8/20, respectively). Clustering of the COOH-terminal portion where we found no cleavage site are peptide in vitro and the exoproteolytic removal of Arg322 are closely associated cell line, HepG2, suggesting that the endoproteolytic cleavage and the notion that the connecting peptide (or at least major part of it) is released from the circulating two-chain form under patho-

logical conditions. We cannot, however, rule out that \(\alpha_2\)-HS, sequostered from the plasma compartment or locally synthesized, might release its connecting peptide in situ due to the action of chymotryptic activities. Future studies will focus on the proteolytic processing of \(\alpha_2\)-HS in peripheral organs such as the bone, and will directly address the potential biological roles of the connecting peptide and its major fragments.

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