Different N-terminal forms of α_2 -plasmin inhibitor in human plasma

Kristian BANGERT,* Anders H. JOHNSEN,* Ulla CHRISTENSEN† and Sixtus THORSEN*‡

*Department of Clinical Biochemistry KB 3-01-1, Rigshospitalet, and †Department of Chemistry, University of Copenhagen, DK-2100 Copenhagen, Denmark

Mature α_2 -plasmin inhibitor in human plasma has 12 more N-terminal residues than hitherto anticipated. The first residue is the methionine at position 28, downstream from the N-terminus of the pre-protein. The cDNA sequence predicts that the site cleaved upon formation of the mature inhibitor is a typical signal-peptidase recognition site. The mature inhibitor (464 residues) and the previously reported, and presumably degraded,

form with N-terminal asparagine (452 residues), are present in plasma in about equal amounts. They both form a stable complex with plasmin. Recent studies on a recombinant α_2 -plasmin inhibitor suggest that the 12 additional residues have functional implications [Sumi, Ichikawa, Nakamura, Miura and Aoki (1989) *J. Biochem.* **106**, 703–707].

INTRODUCTION

α_2 -Plasmin inhibitor (α_2 -PI) belongs to the α_1 -proteinase-inhibitor class of the serine-proteinase-inhibitor superfamily (serpins) [1]. It is synthesized by the liver [2]. Its target proteinase is plasmin, which is the key enzyme in fibrinolysis. α_2 -PI contains three regions of importance for efficient inhibition of plasmin-catalysed fibrin degradation. From the N-terminus they are as follows: (1) the region containing the Gln residue which is cross-linked to fibrin in an activated coagulation factor XIII-catalysed reaction; (2) the region with the reactive-centre loop; and (3) the region with the secondary binding site for the plasmin(ogen) kringle structures 1–3 (K1+2+3). The last-mentioned region is located in a C-terminal extension, which among serpins is unique for α_2 -PI.

The complete primary structure of human α_2 -PI has been deduced from the cDNA sequence [3,4] and from amino-acid-sequence analysis of α_2 -PI purified from plasma [5]. The combined sequencing data suggested that the pre-form of α_2 -PI is composed of 491 amino-acid residues, where the first 39 N-terminal residues constitute the signal peptide and the rest the mature protein with an N-terminal asparagine residue (452 residues). Recently, human α_2 -PI was expressed in a baby-hamster-kidney (BHK) cell line [6]. Amino-acid-sequence analysis of the N-terminus of the recombinant protein revealed that it contained an additional 12 residues, starting with the methionine at position 28 downstream from the N-terminus of pre- α_2 -PI. Evidence was provided that these additional residues had to be cleaved off in order to achieve optimal cross-linking of α_2 -PI to fibrin, and thereby efficient inhibition of plasmin generated on the fibrin surface [6].

The above-mentioned difference in the N-terminus of plasma and recombinant α_2 -PI led us to re-investigate the structure of the inhibitor in human plasma. It is demonstrated that α_2 -PI with each of the N-termini is indeed present in human plasma.

MATERIALS AND METHODS

Buffers and reagents

Tris/HCl buffer (50 mM Tris/HCl/100 mM NaCl) was adjusted to pH 7.70 at 20 °C with NaOH. *trans*-4-(Aminomethyl)-

cyclohexane-1-carboxylic acid (tranexamic acid) and D-Val-Leu-Lys-4-nitroanilide (S-2251) were from Chromogenix, Mölndal, Sweden. Plasminogens with N-terminal glutamic acid (Glu¹-plasminogen, i.e. residues 1–791) and N-terminal lysine (Lys⁷⁸-plasminogen, i.e. residues 78–791) and plasmin were those used previously [7]. The K1+2+3 fragment of plasminogen was prepared from Glu¹-plasminogen as described by Sottrup-Jensen et al. [8]. K1+2+3-Sepharose (3.5 mg of K1+2+3/ml of packed gel) was prepared by coupling K1+2+3 to CNBr-activated Sepharose 4B (Pharmacia LKB, Uppsala, Sweden) as described by the manufacturer. Rabbit immunoglobulin raised against human α_2 -PI (anti- α_2 -PI) was from DAKO, Glostrup, Denmark.

Purification of human α_2 -PI

The source of α_2 -PI was plasma obtained from blood stabilized by 0.123 vol. of 24.7 mM citric acid/141.9 mM trisodium citrate/25.5 mM NaH₂PO₄/204.4 mM glucose (CPD-solution). Plasma was frozen within 4 h of blood collection, stored at –40 °C and used within 6 months. α_2 -PI was purified at 4 °C from four portions of plasma. Each portion (500–1000 ml) originated from three to five donors. The purification procedure involved three steps: first removal of plasminogen by adsorption to lysine-Sepharose 4B, and then fractional (NH₄)₂SO₄ precipitation of the plasminogen-depleted plasma as described previously [9]; finally affinity chromatography on K1+2+3-Sepharose [10]. The precipitate obtained between 0.8 M and 2.7 M (NH₄)₂SO₄ in step 2 was dissolved in 250 ml of 0.04 M sodium phosphate, pH 7.4, and dialysed against the same buffer. Then the dialysate was incubated for 60 min with 80 ml of K1+2+3-Sepharose. The gel was filtered and washed on a Buchner funnel with 0.04 M sodium phosphate, pH 7.4, until $A_{1\text{cm},280}^1\%$ of the effluent was less than 0.05. The washed gel was poured into a XK 16/40 column (Pharmacia LKB, Uppsala, Sweden) and α_2 -PI was eluted at a flow rate of 50 ml/h with the same buffer containing 1 mM tranexamic acid. Fractions containing α_2 -PI were pooled and dialysed against Tris/HCl buffer. The purified inhibitor was analysed by modified crossed immunoelectrophoresis with Lys⁷⁸-plasminogen in the first-dimension

Abbreviations used: Glu¹-plasminogen, plasminogen with N-terminal glutamic acid (residues 1–791); Lys⁷⁸-plasminogen, plasminogen with mainly N-terminal lysine (residues 78–791); K1+2+3, kringle structures 1–3 (residues 73/79–354) prepared by elastase cleavage of plasminogen; α_2 -PI, α_2 -plasmin inhibitor; Met¹- α_2 -PI, α_2 -PI with N-terminal methionine (residues 1–464); Asn¹³- α_2 -PI, α_2 -PI with N-terminal asparagine (residues 13–464); tranexamic acid, *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid; BHK, baby-hamster kidney.

‡To whom correspondence should be addressed.

gel (retards migration of α_2 -PI with an intact C-terminus) and anti- α_2 -PI in the second-dimension gel as described by Kluft and Los [11]. It showed that the C-terminal extension containing the secondary binding site for the plasmin(ogen) K1 + 2 + 3 structures was intact in the purified α_2 -PI preparations.

Analysis of protein concentration

The concentration of plasmin was determined by measuring the initial rate of hydrolysis of D-Val-Leu-Lys-4-nitroanilide (390 mM) in Tris/HCl buffer at 25 °C [12], using values for K_m of 150 mM and k_{cat} of 15.2 s⁻¹. The concentration of α_2 -PI was determined by active-site titration of a known amount of plasmin with the inhibitor as described [9,13].

N-terminal-sequence analysis

The purified α_2 -PI was contaminated with Glu¹-plasminogen and other minor impurities. They were removed by reversed-phase h.p.l.c. on a 2.1 mm × 150 mm 5 μ m particles C₄ column (Vydac, Hesperia, CA, U.S.A.), which was eluted by a gradient from 0.1% (v/v) trifluoroacetic acid in water to 0.1% (v/v) trifluoroacetic acid in acetonitrile. The instrument was a 1090 h.p.l.c. from Hewlett-Packard (Waldbronn, Germany). The amino-acid sequences of the h.p.l.c.-purified protein were determined by using an automatic protein sequencer (475A; Applied Biosystems, Foster City, CA, U.S.A.) equipped with an on-line h.p.l.c. system for detection of the amino acid phenylthiohydantoin derivatives. These derivatives were separated on a C18-DB column (5-7943; Supelco, PA, U.S.A.). The chemicals and solvents were sequence- or h.p.l.c.-grade delivered by Applied Biosystems.

SDS/PAGE analysis

SDS/PAGE in a 6% (w/v) acrylamide gel without urea was performed as described previously [9].

RESULTS AND DISCUSSION

Reduced SDS/PAGE of preparations of purified plasma α_2 -PI showed an homogeneous band with an M_r of approx. 67000 (results not shown). N-terminal-sequence analysis of the purified inhibitor revealed two parallel sequences (Table 1). One (sequence A) was identical with that reported earlier for plasma α_2 -PI [5], starting with the asparagine residue at position 40 downstream from the N-terminus of pre- α_2 -PI [3,4] (Figure 1). The other (sequence B) started with the methionine residue at position 28 downstream from the N-terminus of pre- α_2 -PI. From these results it is concluded that the liver secretes mature α_2 -PI with 12 more N-terminal residues than hitherto anticipated [3–5], and that the previously reported inhibitor with N-terminal asparagine (Asn¹³- α_2 -PI) is a proteolytically modified form. For this reason the N-terminal methionine is assigned as residue number 1, and not number –12 [3,4], throughout the paper. About equal amounts of Met¹- α_2 -PI (residues 1–464) and Asn¹³- α_2 -PI (residues 13–464) were found in each of four different inhibitor preparations (Table 2). Our results agree with the finding that Met¹- α_2 -PI is expressed by BHK cell line transfected with genomic material coding for the entire inhibitor [6]. Furthermore, they strongly support the proposal that the signal peptide is composed of 27 amino acids (residues Met^{–27}–Ala^{–1}) [6], and not of 39

amino acids (residues Met^{–27}–Pro¹²) as previously suggested [3,4]. This is compatible with the fact that the scissile Ala^{–1}–Met¹ peptide bond is a much more likely signal-peptidase cleavage site than the scissile Pro¹²–Asn¹³ peptide bond [6,14]. The most

Table 1 N-terminal sequences of α_2 -PI purified from human plasma

This illustrates sequencing results obtained from preparation 1 (Table 2). Two parallel sequences were obtained one (sequence A) was identical with the earlier reported sequence of α_2 -PI [5]. By subtraction a second sequence could be identified, which is shown as sequence B. Values given are the yields obtained in the individual cycle corrected for background levels in the previous cycle(s) and lag in the following cycle.

Cycle no.	Sequence A		Sequence B	
	Amino acid	Content (pmol)	Amino acid	Content (pmol)
1	Asn	69	Met	51
2	Gln	56	Glu	43
3	Glu	62	Pro	48
4	Gln	63	Leu	49
5	Val	63	Gly	35
6	Ser	34	Arg	80
7	Pro	43	Gln	28
8	Leu	(54)*	Leu	(54)*
9	Thr	(76)*	Thr	(76)*
10	Leu	45	Ser	17
11	Leu	50	Gly	19
12	Lys	36	Pro	26
13	Leu	27	Asn	20
14	Gly	24	Gln	18
15	Asn	30	Glu	14
16	Gln	(46)*	Gln	(46)*
17	Glu	19	Val	18
18	Pro	22	Ser	7
19	Gly	24	Pro	14

* Due to identical amino acids no values could be assigned to the individual sequences.

Figure 1 Alignment of N-terminal sequences of α_2 -PI in plasma with the amino-acid sequence deduced from the cDNA sequence

(a) Part of the amino-acid sequence deduced from cDNA ([3] and [4]). (b and c) These sequences were found by N-terminal sequence analysis of purified human α_2 -PI. The sequence in (b) is identical with the N-terminal sequence of recombinant Met¹- α_2 -PI [6]. The sequence in (c) is identical with the N-terminal sequence originally found in α_2 -PI [5]. (↑) indicates the signal-peptidase-cleavage site deduced from the data in this paper and from the results of Sumi et al. [6]; (↑) indicates the signal-peptidase-cleavage site originally proposed [3,4]. The protein numbering is based on the α_2 -PI form with an N-terminal methionine residue.

Table 2 Relative amounts of the α_2 -PI forms in four preparations

The total amounts of Met¹- α_2 -PI and Asn¹³- α_2 -PI were calculated as the mean of the yields of the five first amino-acid residues of each sequence. The yields were calculated as described for preparation no. 1 in Table 1.

Preparation no. ...	1	2	3	4	Mean
Amount of Met ¹ - α_2 -PI (%)	42	49	32	28	38
Amount of Asn ¹³ - α_2 -PI (%)	58	51	68	72	62

important structural determinants for cleavage of the signal peptide from the mature protein seem to reside in the P_3 and P_1 residues flanking the scissile peptide bond [14]. The preferred P_1 residue mentioned in the order of decreasing frequency is Ala, Gly, Ser, Cys or Thr; the P_3 residue cannot be aromatic, charged or large and polar. Furthermore, the P_3 to P_1' residues cannot be proline. In the case of the scissile Ala¹-Met¹ peptide bond all these criteria are fulfilled (P_3 = Val; P_1 = Ala). This is not the case with the scissile Pro¹²-Asn¹³ peptide bond (P_3 = Ser; P_1 = Pro), since P_1 is not allowed to be Pro.

It remains to be determined whether the Pro¹²-Asn¹³ peptide bond is cleaved *in vivo* or *in vitro* after blood collection. An intracellular prolyl endopeptidase with high specificity towards Pro-Xaa peptide bonds is present in various tissues [15]. It is unlikely that this enzyme cleaves the Pro¹²-Asn¹³ peptide bond in α_2 -PI, since its function appears to be limited to proteolytic modification of oligopeptides (hormones and neuropeptides). The extracellular metalloproteinases stromelysin (MMP-3) and human polymorphonuclear leucocyte collagenase cleave a Pro³⁵⁷-Met³⁵⁸ peptide bond in the reactive-centre loop of α_1 -proteinase inhibitor, leading to inactivation of the inhibitor [16–18]. These proteinases may be candidates for the cleavage of the Pro¹²-Asn¹³ peptide bond in α_2 -PI.

In order to investigate if plasma Met¹- α_2 -PI reacted with its target enzyme, purified α_2 -PI (containing both N-terminal forms) and plasmin (each at a concentration of 2 μ M) in Tris/HCl buffer were incubated for 15 min at room temperature. Then a non-reduced sample of the reaction mixture was subjected to SDS/PAGE. Inspection of the gel showed that all α_2 -PI and plasmin were converted into enzyme-inhibitor complex (results not shown), thus demonstrating that both N-terminal forms of the inhibitor form stable complexes with plasmin. This agrees with the observation that the rates of inhibition of plasmin by recombinant Met¹- α_2 -PI and plasma Asn¹³- α_2 -PI were very similar [6].

It has been reported that recombinant Met¹- α_2 -PI, as well as a recombinant inhibitor form with a tripeptide extension on the N-terminal side of Asn¹³, is significantly less cross-linked to fibrin than is plasma Asn¹³- α_2 -PI by activated coagulation factor XIII [6,19]. This suggests that N-terminal modification of Met¹- α_2 -PI, forming Asn¹³- α_2 -PI, is a physiological regulatory mechanism of importance in fibrinolysis, since cross-linking of the inhibitor to fibrin markedly reduces plasmin-catalysed fibrin resolution [20]. Proteolytic cleavage of the reactive-centre loop of α_2 -PI leading to inactivation of the inhibitor may be another physiological regulatory mechanism [9,21,22]. This also applies to the proteolytic release of the C-terminal secondary plasmin(ogen) binding site of α_2 -PI, the loss of which markedly decreases the rate of

inhibition of plasmin [9]. Significant amounts of α_2 -PI lacking this binding site are present in plasma [9,11]. C-terminal proteolytic modification changing inhibitor function has also been reported for α_1 -anti-chymotrypsin, α_1 -proteinase inhibitor and anti-thrombin III [16–18]. α_1 -Anti-chymotrypsin, α_1 -proteinase inhibitor and plasminogen activator inhibitor 1 exhibit heterogeneity of the N-terminus (for references see Lindmark et al. [23]). The functional implication of this heterogeneity is unknown.

In conclusion, Met¹- α_2 -PI is the mature inhibitor of plasmin-catalysed fibrinolysis in the blood, the reactivity of which may be both positively and negatively regulated by N- and C-terminal proteolytic modification respectively.

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