The structure around the thioester bond in bovine α_2-macroglobulin

Possible implications for the conformational stability of the inhibitor on thioester cleavage

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The residues contributing to the thioester bonds in bovine α_2-macroglobulin were differentially labelled by modification of the Glu moiety with [^{14}C]methylamine and of the Cys moiety with iodo[^{3}H]acetate. The labelled region was identified and analyzed in a tryptic peptide. Two amino acid replacements between human and bovine α_2-macroglobulin were found at positions +3 (Val/Ala) and +4 (Leu/Arg) from the Glu moiety of the thioester. Thus, marked differences exist between the human and bovine proteins in side chain size and charge close to the thioester bonds. These differences may explain the greater conformational stability of bovine α_2-macroglobulin, compared with that of the human inhibitor, after cleavage of the thioester bonds.

α_2-Macroglobulin *Protease inhibitor* *Thioester* *Sequence homology* *Conformational change*

1. INTRODUCTION

α_2-Macroglobulin is a high-molecular-mass tetrameric plasma protease inhibitor with wide specificity [1,2]. Its inactivation of proteases is initiated by restricted proteolysis of a specific 'bait' region of the inhibitor polypeptide chain. This triggering event leads to cleavage of a thioester bond, present in each subunit of α_2-macroglobulin, and to a conformational change that is involved in binding of the protease [1,3–11]. The inactivated enzyme is bound in a manner that abolishes its activity only against macromolecular substrates. The protease may thus be physically entrapped by the inhibitor [1]. Maximally two enzyme molecules can be bound per α_2-macroglobulin tetramer [4–6,12,13].

Nucleophilic cleavage of the thioester bonds of human α_2-macroglobulin by amines leads to a conformational change similar to that induced by proteases and is associated with inactivation of the inhibitor [4,6,8–11]. In contrast, no analogous conformational change occurs on cleavage of the thioester bonds of bovine α_2-macroglobulin, and this inhibitor also retains activity after the thioester bond cleavage [14,15]. Recent studies have shown that cyanylation of the liberated sulfhydryl group of human α_2-macroglobulin concomitant with the cleavage of the thioester bond largely prevents the change of conformation and loss of activity of this protein [16,17]. Thus, the extent of the conformational change can be affected by minor structural changes in the thioester bond region. Consequently, the greater conformational stability of bovine α_2-macroglobulin than of the human inhibitor, in the absence of an intact thioester bond, could arise from residue replacements close to this bond. To test this possibility, we have determined the amino

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acid sequence of the thioester bond region of bovine α_2-macroglobulin and compared it with the known structure of this region of the human inhibitor [18].

2. MATERIALS AND METHODS

Bovine α_2-macroglobulin was purified and its concentration was determined as described [15]. The glutamic acid moiety of the thioester bond was labelled by reacting the protein (4 mg/ml) with 10 mM [^{14}C]methylamine (spec. act. 53 GBq/mol; Amersham, England) for 16 h (i.e. about 5 × $t_{1/2}$ for the thioester bond cleavage [15]) at 25°C in 0.1 M Hepes /NaOH, 0.1 M NaCl, pH 8.2. Unlabelled methylamine was then added to a concentration of 100 mM, and the reaction was continued for 1 h. The cysteine moiety of the thioester bond was labelled in another batch of α_2-macroglobulin by treatment with 100 mM unlabelled methylamine in the presence of 1 mM iodo[2-^{3}H]acetate (spec. act. 2150 GBq/mol; Amersham) for 3 h (i.e. about 10 × $t_{1/2}$) at 25°C in the Hepes buffer. In both cases, excess reagents were removed by exclusion chromatography on Sephadex G-50 (Pharmacia, Sweden).

A mixture of the separately labelled batches of α_2-macroglobulin (total conc. 5 mg/ml) was reduced for 4 h with 10 mM dithiothreitol in 0.4 M Tris/HCl, 6 M guanidine/HCl, 10 mM EDTA, pH 8.2, and then carboxymethylated by the addition of unlabelled iodoacetate to a concentration of 25 mM. After 30 min, mercaptoethanol was added to 50 mM, and the material was dialyzed extensively against 1 mM HCl. Trypsin (treated with *p*-tosyl-L-phenylalanine chloromethyl ketone) was added in an amount corresponding to a trypsin/protein ratio of 1/100 by weight, pH was raised to 8.0, and the solution was made 0.1 M in ammonium bicarbonate. Digestion was allowed to proceed for 4 h at 37°C with continuous stirring.

The tryptic peptides obtained were immediately fractionated by chromatography (25 ml/h) on a column (2.5 × 90 cm) of Sephadex G-75 Superfine (Pharmacia) in 0.1 M ammonium bicarbonate. Further separation was achieved by gel permeation HPLC at 2.4 ml/h in 0.1 M ammonium acetate on an UltroPac TSK G-2000SW column (0.75 × 60 cm; LKB, Sweden). Final isolation of the labelled peptide was carried out by two reverse-phase HPLC steps on a μBondapak C18 column (0.39 × 30 cm; Waters). In the first step, the column was eluted at 1 ml/min with a linear gradient of acetonitrile (0–70% in 70 ml) in 0.1% trifluoroacetic acid. The second step involved rechromatography with a more shallow gradient (20–35% in 30 ml, starting after an isocratic elution of about 10 ml at 20%). The pure, labelled peptide was analyzed for total composition after acid hydrolysis and subsequent phenylthiocarbamyl conversion [19], and for amino acid sequence by degradation in a liquid-phase sequencer (Beckman 890D), supplemented by HPLC (Hewlett Packard 1090) identification of thiohydantoin derivatives [20].

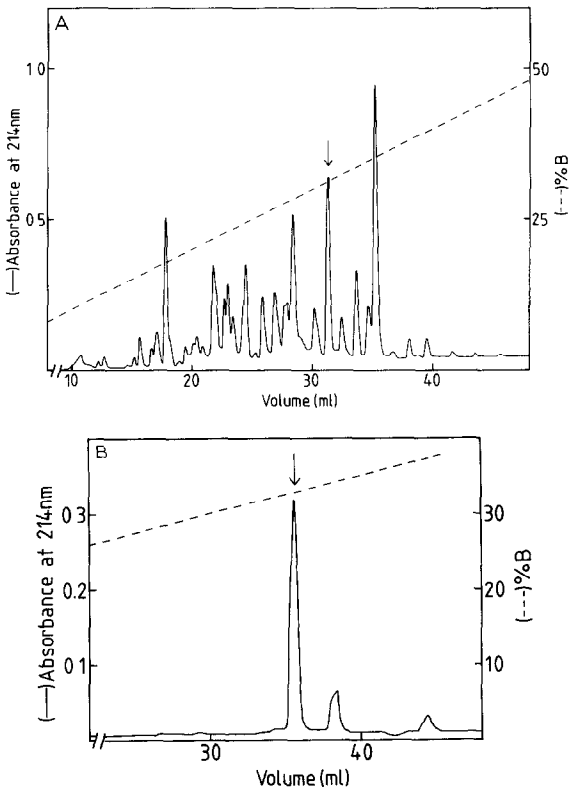

Fig.1. Final purification by reverse-phase HPLC of the labelled peptide derived from the segment of bovine α_2-macroglobulin originally containing the thioester bond. (A) Chromatography of the radioactive fraction from the preceding exclusion chromatography step. (B) Re-purification of the radioactive material from A, giving final separation of two peptides. The positions of the labelled peptide are indicated by arrows. The column was developed with a gradient of acetonitrile (%B) in 0.1% trifluoroacetic acid.

3. RESULTS

The glutamic acid and cysteine moieties forming the thioester bond of bovine α_2-macroglobulin were labelled in separate batches of the protein with [^{14}C]methylamine and iodo[2-^{3}H]acetic acid. Incorporations of 0.85 and 1.1 mol of the two labelled reagents per mol of protein monomer were obtained, consistent with one thioester bond per subunit [14,15]. The two separately labelled protein preparations were mixed to given a ^{3}H:^{14}C dpm ratio of 9.7.

Table 1

Analytical data for the labelled peptide obtained from the second reverse-phase HPLC step. (A) Total composition, derived from analysis after phenylthiocarbamyl conversion [19]. A low recovery of methionine, as in this case, is sometimes observed. Values from the sequence analysis are given within parentheses. (B) Results of liquid-phase sequencer degradations. Values below the residues indicate nmol recovered. The two bottom lines show radioactivity in all extracts (expressed in % of maximal recovery, which is about 12000 cpm ^{3}H in extract 12 and 3000 cpm ^{14}C in extract 15), establishing the positions of the ^{3}H and ^{14}C labels. The modified glutamine derivative in cycle 15 behaved almost indistinguishably from the phenylthiohydantoin derivative of glutamine in the HPLC analysis

A

Residue	mol/mol
Cys(Cm)	0.7 (1)
Asx	2.8 (3)
Thr	1.1 (1)
Ser	0.4 (–)
Glx	3.7 (4)
Pro	1.0 (1)
Gly	2.1 (2)
Ala	1.3 (1)
Met	1.4 (2)
Leu	1.6 (2)
Tyr	0.8 (1)
Arg	1.0 (1)

B

	Asn-	Thr-	Gln-	Asn-	Leu-	Leu-	Gln-	Met-	Pro-	Tyr-	Gly-	Cys-	Gly-	Glu-	Glx-	Asn-	Met-	Ala-	Arg
	2.3	2.2	1.9	2.1	3.9	3.9	1.4	3.2	1.8	2.7	2.3	1.6	1.9	1.7	1.3	0.9	1.3	1.9	0.2
^{3}H:		1	1	1	1	2	2	1	2	2	2	100	57	17	5	3	3	2	2
^{14}C:		4	4	5	3	4	5	5	4	4	3	8	6	6	100	60	33	14	13

The double-labelled protein (50 nmol tetramer) was reduced, carboxymethylated, dialyzed, digested with trypsin, and subjected to exclusion chromatography on Sephadex G-75 (see section 2). One symmetric peak of radioactivity, eluting at $K_d \sim 0.70$, was obtained, accounting for ~60% of the original radioactivity measured before reduction. This fraction was further separated by gel permeation HPLC on TSK G2000SW, producing only one, symmetric peak of radioactivity, eluting at $K_d \sim 0.72$ and accounting for ~40% of the original radioactivity. The material in this peak was further purified by reverse-phase HPLC (fig.1A). One radioactive peak was recovered in a yield of ~13% of the radioactivity in the original starting material. The ^{3}H:^{14}C dpm ratio for this peptide was 9.7, identical to that of the original material, indicating no preferential loss of either labelled group. An attempt at sequence analysis at this stage showed that the material corresponding to the radioactivity peak contained two peptides in an approximate ratio of 2:1. Although two residues were obtained in each cycle, both structures could be identified because of the difference in recovery between the two peptides. The structures deduced were homologous with two different regions in the known primary structure of the human form of the protein [18]. Thus, the minor peptide was tentatively identified as Ser-49–Lys-58, containing no radioactivity, and the major peptide as Asn-938–Arg-956, containing all the radioactivity.

To prove these identifications, the peptide mixture was resolved by a second step of HPLC (fig.1B), with a more shallow gradient of acetonitrile than in the initial step (fig.1A). As expected, the radioactivity was recovered in the major peptide, and the structure tentatively assigned to this peptide was completely verified (table 1). These results establish the amino acid sequence around the thioester bond in bovine α_2-macroglobulin.

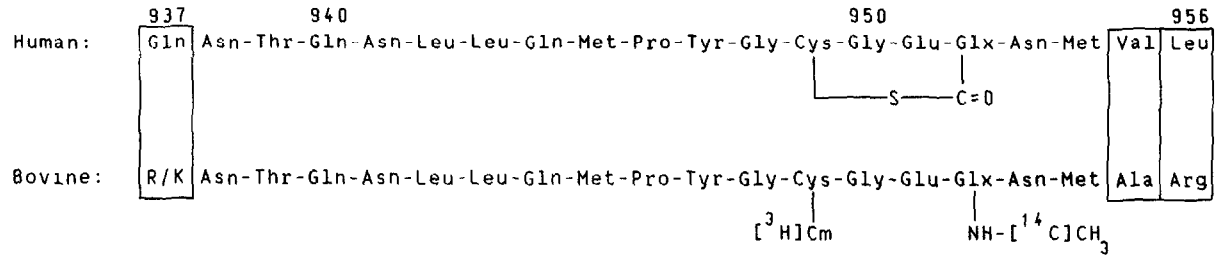

Fig.2. Comparison of the structures around the thioester bond in the subunits of human (top) and bovine (bottom) α_2-macroglobulin. The bovine protein structure is from the present analysis (table 1), the human one and the numbering system (above the top line) are from [18]. The two established amino acid exchanges are boxed, as well as the probable difference at position 937, where an Arg or Lys (indicated by R/K) in the bovine inhibitor is indirectly deduced from the major tryptic cleavage that has occurred after this site. The position of the thioester bond is indicated in the top structure, the differential labelling used to detect the reactive residues in the present work is indicated in the bottom structure.

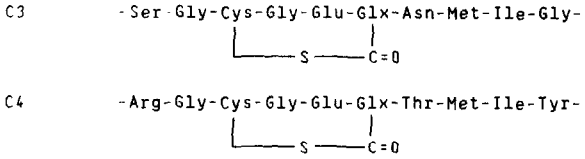

Fig.3. Comparison of the structures around the thioester bond in the human complement factors C3 (top) and C4 (bottom). The two structures are from [21,22].

4. DISCUSSION

A comparison of the structures around the thioester bonds in human and bovine α_2-macroglobulin (fig.2) shows two amino acid exchanges, the sequences of the remainder of the segments being identical. Thus, Val-955 and Leu-956 in the human protein have been replaced by Ala and Arg, respectively, in the bovine form. Both these exchanges are compatible with one-base mutations. The two replacements, together with those in the minor peptide (not shown), and that deduced from the presence of a tryptic cleavage site before the labelled major peptide (fig.2), account for six residue differences in 31 positions. Consequently, the human and bovine α_2-macroglobulin structures appear to have a homology with about 80% positional identities, a degree of identity similar to that between other proteins from the two species.

The sequence around the thioester bond is highly conserved in the human thioester-containing proteins α_2-macroglobulin and the complement factors C3 and C4 ([18,21,22]; figs 2 and 3). The structure of this region therefore must be of paramount importance for the functional properties of the thioester bond. The three human proteins, which all undergo a major conformational change and are inactivated on thioester cleavage by amines [4,9–11,23,24], all have a large hydrophobic amino acid in position +3 from the Glu moiety of the thioester and a neutral amino acid in position +4. In bovine α_2-macroglobulin, which does not undergo a corresponding conformational change and which remains active on amine-treatment [14,15], the two amino acid replacements at these positions markedly alter the properties of the segment of the chain just C-terminal of the thioester bond. The Arg/Leu exchange thus introduces an extra positive charge in this region of the bovine inhibitor, and the adjacent Ala/Val exchange leads to a difference in the size of the side chain. Previous work has shown that relatively subtle structural changes in the thioester bond region are sufficient to stabilize considerably the conformation of human α_2-macroglobulin after thioester cleavage [16,17]. It is therefore likely that the different side chain properties close to the thioester bond confer additional conformational stability to bovine α_2-macroglobulin, such that this protein remains in its near-native state also in the absence of the stabilizing thioester bonds.

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