SEQUENCE LOCATION OF THE REACTIVE THIOL ESTER IN HUMAN α_2-MACROGLOBULIN

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1. Introduction

It was shown recently that native α_2-macroglobulin (α_2M), M_r 725 000 contains one reactive, labile thiol ester in each of its 4 identical subunits of M_r 180 000, which is formed by a particular Glx-residue, whose γ-carboxyl group is esterified to the sulfhydryl group of a cysteinyl residue [1].

Complex formation with proteinases involves not only limited proteolysis in the 'bait' region but also the rapid cleavage of this thiol ester as evidenced by the concurrent appearance of the SH-groups (max. 4 mol/mol α_2M) [1]. Denaturation of α_2M or treatment with CH_3NH_2 also leads to cleavage of this thiol ester [1].

During 'inactivation' of the proteinase binding capacity with CH_3NH_2 the latter is incorporated covalently to form a γ-glutamyl methylamide [1,2] indicating that this site in α_2M may be a site for covalent binding of proteinase. Complement component C3 also reacts covalently with CH_3NH_2 again leading to the formation of γ-glutamyl methylamide [3–5].

The recent demonstration that the α-chain of C3 probably also contains a thiol ester [4,5] later shown [6] to involve the Glx-residue and the Cys-residue in the sequence:

–Gly–Cys–Gly–Glu–Glx–Asn–Met

which is identical to the sequence around the CH_3NH_2-reactive Glx-residue of α_2M [1,7,8] shows that α_2M and C3 are structurally and functionally related, at least with regard to this particular state.

Here, we show that the Cys-residue constituting one part of the thiol ester structure in α_2M is located in an identical sequence to that found in C3 [6].

Abbreviations: α_2M, α_2-macroglobulin; Quadrol, *N,N,N',N'*-tetrakis-(2-hydroxypropyl)ethylene diamine; polybrene, 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide; PTH, phenylthiohydantoin; HPLC, high performance liquid chromatography; DEAE, diethylaminoethyl; Glu(NHCH$_3$), γ-glutamyl methylamide; CmCys, *S*-carboxymethyl cysteine

2. Materials and methods

Human α_2M was prepared from plasma as in [1]. $CH_3NH_2 \cdot HCl$ was obtained from Sigma, St Louis, MO. Bovine α-chymotrypsin was from Worthington, Freehold, NJ. Iodo[1-^{14}C]acetamide (53 Ci/mol) was obtained from the Radiochemical Centre, Amersham, iodoacetic acid was from Merck, Darmstadt. Sephadex G-25, G-50 fine and DEAE-Sephacel were from Pharmacia, Uppsala.

Following initial separation of peptides on Sephadex G-50 fine and DEAE-Sephacel peptides were finally purified by high-voltage paper electrophoresis and by paper chromatography [9]. Samples for amino acid analysis were hydrolysed in 6 M HCl, 0.1% (v/v) phenol for 20 h at 110°C in 6 × 60 mm test tubes sealed at <1 Torr and the hydrolysates analysed on a Beckman 121MB amino acid analyzer using the standard single column hydrolyzate program. Automated Edman degradation was performed in a Beckman 890C Sequencer using 0.25 M Quadrol [10] and polybrene [11]. 25% trifluoroacetic acid was used for conversion (55°C, 30 min) [12]. After drying in vacuo the PTH derivatives were analysed and quantitated by HPLC using an ethanol based solvent system [13] on a Hewlett-Packard 1084B liquid chromatograph.

3. Experimental

α_2-Macroglobulin (450 mg, ~0.62 μmol) dissolved in 40 ml 0.05 M Na-phosphate, 0.1 M NaCl, 5 mM

Na_2-EDTA (pH 8.0) was treated with $CH_3NH_2 \cdot HCl$ (final conc. 50 mM) for 90 min at room temperature in order to achieve complete cleavage of the thiol ester group [1]. The thiol groups were then labelled with iodo[1-^{14}C]acetamide by adding 100 μCi, incubating for 120 min then followed by a 10 min incubation with 10 mM unlabelled iodoacetamide. The mixture was separated on a column of Sephadex G-25 (5 X 49 cm) in 0.1 M NH_4HCO_3 (pH 8.3). More than 95% of the ^{14}C-label co-eluted with the material appearing in the void volume. This fraction was freeze dried and redissolved in 40 ml 6 M guanidinium chloride, 0.1 M Na-phosphate (pH 8.0) reduced with dithiothreitol and alkylated with iodoacetic acid essentially as in [14]. After desalting on the G-25 column in 0.1 M NH_4HCO_3 (pH 8.0) all of the ^{14}C-label was associated with the protein peak. The reduced, alkylated α_2M (in 180 ml) was digested with chymotrypsin at 37°C for 24 h. Chymotrypsin (7 mg) was added to initiate digestion followed by 7 mg after 6 h. The digestion was terminated by addition of 10 mg phenylmethane sulfonyl fluoride in 1 ml ethanol and the solution was finally freeze dried.

The material was redissolved in 0.1 M NH_4HCO_3 (pH 8.3) and loaded on a column of G-50 fine (fig.1). The pool containing the bulk of the ^{14}C-label was

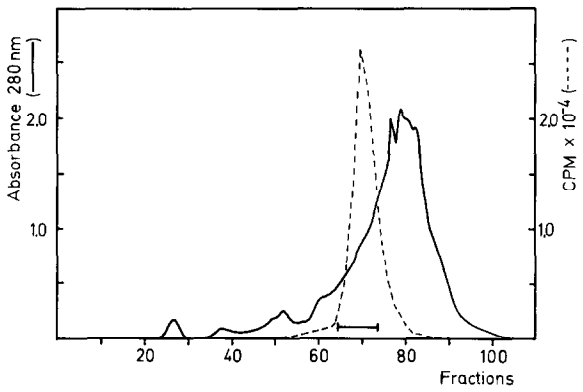

Fig.1. Separation of chymotryptic peptides from the [1-^{14}C]-carboxyamidomethylated, dithiothreitol, reduced, carboxymethylated α_2M on a column of Sephadex G-50 fine (5 X 42 cm) equilibrated and eluted with 0.1 M NH_4HCO_3 (pH 8.3) at a flowrate of 60 ml/h. Fractions of 10 ml were collected. The column effluent was monitored by measuring the A_{280}, by determining the radioactivity on 25 μl aliquots from every second tube and fingerprinting [9]. Due to the complexity of the digest the material in the trailing part of the ^{14}C-labelled peak was not included in the material that was pooled (horizontal bar).

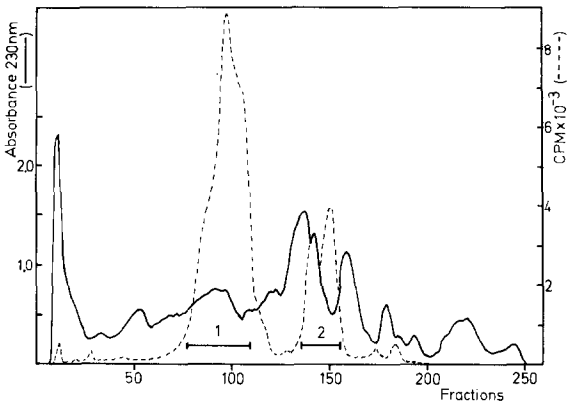

Fig.2. Separation of ^{14}C-labelled chymotryptic peptides from the G-50 pool (fig.1) on a column of DEAE-Sephacel (1.6 X 32 cm) equilibrated with 10 mM NH_4HCO_3 (pH 8.3) and eluted at a flowrate of 30 ml/h with two linear gradients of NH_4HCO_3, namely from 10–200 mM (300 ml + 300 ml) and from 200–600 mM (340 ml + 340 ml). Finally the column was eluted with 1 M NH_4HCO_3 (100 ml) (not shown). The separation was monitored by measuring the A_{230}, by determining the radioactivity on 25 μl aliquots and by fingerprinting. Two pools (1,2) were collected (horizontal bars).

freeze dried, redissolved in 20 ml 10 mM NH_4HCO_3 (pH 8.3) loaded on a column of DEAE-Sephacel which was eluated with gradients of NH_4HCO_3 (fig.2). Most of the ^{14}C-label (~75% and ~25%, respectively) appeared in two pools which were freeze dried and subjected to high-voltage paper electrophoresis at pH 6.5 and pH 2.1 followed by paper chromatography.

4. Results and discussion

Due to the large number of chymotryptic peptides in pool 1 (fig.2) it was found necessary to purify the peptides in 3 different systems, namely electrophoresis at pH 6.5 and pH 2.1 followed by paper chromatography in *n*-butanol–acetic acid–water–pyridine (15:3:10:2 by vol.). The yield of peptide material was consequently rather low. One strongly radioactive peptide was isolated almost pure in a yield of 130 nmol (5.2%) with the composition CmCys 0.7, Asx 1.1, Glx 2.0, Gly 1.8, Met 0.8, CH_3NH_2 1.0. Another slightly longer variant (+ Val, Leu) was recovered in a yield of ~150 nmol in mixture with other peptides.

On the Beckman 121MB analyzer CH_3NH_2 was

eluted 0.2–0.3 min after histidine as noted [6]. Subjecting a 30 nmol sample to automated sequence determination gave the sequence:
Gly–CmCys–Gly–Glu–Glu($NHCH_3$)–Asn–Met
(16.4, 4.2, 15.1, 22.3, 20.6, 15.9, 5.6 nmol, respectively)
The amount of ^{14}C-label released in each step of the sequence determination was measured on aliquots of the PTH-derivatives and was found only in steps 2 (27, 3×10^4 cpm) and 3 (1.7×10^4 cpm), respectively. This shows that the thiol group which becomes available to labelling after breaking the thiol ester by treating α_2M with CH_3NH_2 is in fact the SH-group of the Cys-residue located 3 steps before the CH_3NH_2-reactive Glx-residue in the sequence of α_2M (fig.3).

Thus, not only the CH_3NH_2-reactive Glx-residue [1,2,8] but also the Cys-residue involved in the thiol ester bond between Cys and Glx in native α_2M [1] are part of a heptapeptide sequence in α_2M [1,2,8] identical to the heptapeptide sequence of complement component C3 [6] which contains the thiol ester bond between Cys and Glx in the C3 α-chain [6].

The PTH-derivative of γ-glutamyl methylamide from step 5 of the chymotryptic peptide eluted ~1.35 min after PTH-Gly on HPLC using our ethanol-based solvent system [13]. When subjected to back hydrolysis [15] this derivative gave rise to two peaks on the amino acid analyzer corresponding to glutamic acid and methylamine. Furthermore, when the CNBr-fragment:
Pro–Tyr–Gly–Cys–Gly–Glu–Glu($NHCH_3$)–Asn–Hse
isolated from α_2M that had been treated with $^{14}CH_3NH_2$ was subjected to sequence determination the only radioactive PTH-derivative found was obtained in step 7 and eluted in the HPLC system in the same position as the derivative from the chymotryptic peptide.

Fig.3 shows a comparison between the sequences around the reactive thiol ester sites in α_2M and in complement component C3. Clearly the two sequences are highly homologous which leads one to expect that larger parts of α_2M and of the α_2'-chain of C3 will turn out to be homologous.

Since α_2M is a tetramer it is not clear whether its thiol ester groups form inter- or intra-chain bridges. Because of the homology with C3 and because C3 contains only 1 α-chain/molecule it seems a reasonable prediction that the 4 thiol ester bonds in α_2M form intra-chain bridges.

Of the estimated 1450 residues in each subunit of α_2M ~1383 residues have now been placed in 3 long stretches of unique sequence (L. S.-J., T. M. Stepanik, D. M. Rider, P. B. Lønblad, T. E. P., S. M., unpublished), namely the N-terminal segment (440 residues), the segment containing the 'bait' region (298 residues) and the C-terminal segment (~645 residues). The Cys- and Glx-residues that form the thiol ester site described here are located as residues ~472 and ~469, respectively, from the C-terminus.

The minor peak of radioactivity in fig.2 (pool 2) was found to be caused by a chymotryptic peptide:
Ser–Phe–Leu–Glu–Asp–Met–Gly–Leu
The Met-residue of this peptide is known to be located 32 residues before the main elastase cleavage site in the 'bait' region (L. S.-J., T. E. P., S. M., H. Jörnvall, unpublished). It appears at present that this site becomes available to alkylation only after the thiol ester has been cleaved.

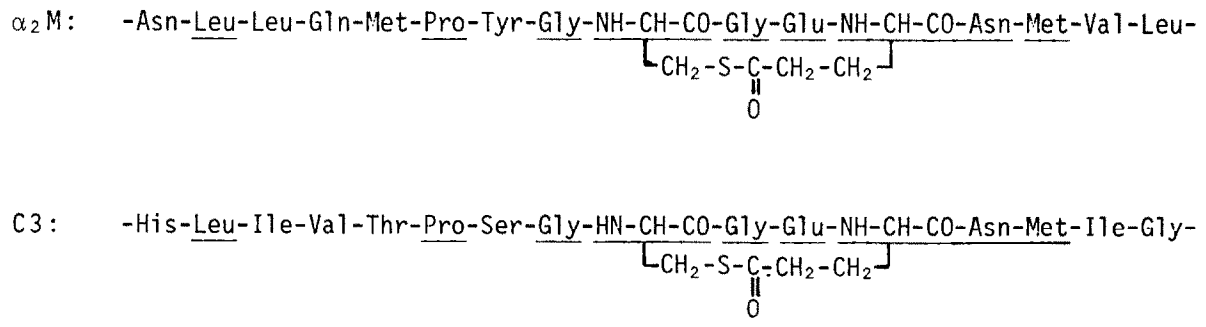

Fig.3. A 16-residue stretch of sequence [1,2,8] from human α_2-macroglobulin containing the methyl amine reactive Glx-residue [1,2,8], which in the native structure forms a thiol ester bond [1] with the Cys-residue. The homologous (9 identities in 13 residues) sequence [6] from human complement component C3 with the methyl amine reactive Glx residue [3,6] in an identical thiol ester structure [6] is shown for comparison. Identical residues are underlined.

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References

[1] Sottrup-Jensen, L., Petersen, T. E. and Magnusson, S. (1980) FEBS Lett. 121, 275–279.
[2] Swenson, R. P. and Howard, J. B. (1979) Proc. Natl. Acad. Sci. USA 76, 4313–4316.
[3] Howard, J. B. (1980) J. Biol. Chem. 255, 7082–7084.
[4] Janatova, J., Lorenz, P. E., Schechter, A. N., Prahl, J. W. and Tack, B. F. (1980) Biochemistry 19, 4471–4478.
[5] Janatova, J., Tack, B. F. and Prahl, J. W. (1980) Biochemistry 19, 4479–4485.
[6] Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L. and Prahl, J. W. (1980) Proc. Natl. Acad. Sci. USA 77, 5764–5768.
[7] Howard, J. B., Vermeulen, M. and Swenson, R. P. (1980) J. Biol. Chem. 255, 3820–3823.
[8] Swenson, R. P. and Howard, J. B. (1980) J. Biol. Chem. 255, 8087–8091.
[9] Sottrup-Jensen, L., Zajdel, M., Claeys, H., Petersen, T. E. and Magnusson, S. (1975) Proc. Natl. Acad. Sci. USA 72, 2577–2581.
[10] Frank, G. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 997–999.
[11] Tarr, G. E., Beecher, J. F., Bell, M. and McKean, D. J. (1978) Anal. Biochem. 84, 622–627.
[12] Hunkapiller, M. W. and Hood, L. E. (1978) Biochemistry 17, 2124–2133.
[13] Sottrup-Jensen, L., Petersen, T. E. and Magnusson, S. (1980) Anal. Biochem. 107, 456–460.
[14] Hirs, C. H. W. (1967) Methods Enzymol. 11, 199–203.
[15] Mendez, E. and Lai, C. Y. (1975) Anal. Biochem. 68, 47–53.