PRIMARY STRUCTURE OF THE 'BAIT' REGION FOR PROTEINASES IN α_2-MACROGLOBULIN

Nature of the complex

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1. Introduction

Most proteinase inhibitors in blood plasma, e.g., antithrombin-III, α_1-antitrypsin, inter-α-trypsin inhibitor, are specific towards serine proteinases and form 1:1 complexes engaging the active site and thus completely inhibiting the proteinase activity. α_2-Macroglobulin is a tetrameric glycoprotein, M_r 725 000, consisting of four apparently identical chains. 'Half-molecules', M_r 360 000 can be obtained under non-reductive denaturing conditions [1,2]. Unlike the other inhibitors α_2M can form complexes with proteinases from different classes having different substrate specificities [3–5]. Furthermore, the active site of the complexed proteinase is accessible to smaller molecules as shown by the facts that the α_2M–trypsin can cleave synthetic substrates [6] and be inhibited by the bovine pancreatic trypsin inhibitor (Kunitz) (M_r 6514) [7]. It is not accessible to larger protein substrates or inhibitors, e.g., soy bean trypsin inhibitor, M_r 20 095 [7]. However, neither zymogens such as prothrombin [8] or plasminogen [9], nor active-site-inhibited proteinases such as TLCK–trypsin [3] or DIP–thrombin [8] bind to α_2M. This indicates that the complex formation involves a proteolytic process [3]. Electron microscopy and gel electrophoresis indicate that the quaternary structure of α_2M becomes more compact as a result of proteinase complex formation [5,10–12]. When incubating native α_2M with different serine proteinases (trypsin, thrombin, plasmin, plasma kallikrein or chymotrypsin) the M_r 180 000 subunits were cleaved near the middle of the polypeptide chain to produce 2 fragments of app. M_r 85 000 as seen in reducing SDS–PAGE [13]. In fact binding to α_2M of proteinases from all 4 classes, namely serine, thiol, acid and metallo proteinases (EC 3.4.21–24), is accompanied by cleavage of the α_2M subunits to M_r 85 000 fragments [4]. Recent estimates indicate [11–16] that the α_2M–proteinase complex contains a maximum of 2 mol proteinase/mol α_2M. To provide a structural basis for describing the properties of α_2M its primary structure is currently being investigated. Partial sequence results from these studies [17] indicate that the subunits of α_2M are identical, each being composed of ~1450 amino acid residues and having the C-terminal sequence:

–Asx–Leu–Gly–Asx–Ala

The same conclusion was reached [14] regarding the identity of the subunits of α_2M and the sequence of the N-terminal 13 residues was also reported [14].

At present, 1383 residues from α_2M have been placed in three long, but not yet overlapped stretches and another ~25–50 residues are known as sequences of

Abbreviations: α_2M, α_2-macroglobulin; TLCK, N^{α}-tosyl-L-lysine chloromethyl-ketone; DIP, diisopropylphosphoryl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethanesulfonylfluoride; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography

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smaller peptides, in all accounting for ~1408–1433 residues (L. S., T. M. S., D. M. Rider, P. B. L., T. E. P., S. M., unpublished). Therefore, it appeared reasonable to assume that the site/s cleaved in α_2M during complex formation with proteinases could be identified by comparing the results of direct sequence determination of complexed and of non-complexed α_2M with the already known parts of the α_2M sequence.

Apart from an N-terminal segment of non-complexed α_2M this report describes the sequence of a 35 residue internal stretch of α_2M which contains 2 sites cleaved by elastase and 1 site cleaved by trypsin, plasmin and thrombin.

2. Materials and methods

Human α_2M was prepared by Zn^{2+}-affinity chromatography as in [16].

Human plasminogen was prepared by affinity chromatography using L-lysine Sepharose [18]. Bovine thrombin prepared earlier [19] was used after storage at −20°C. Bovine TPCK-treated trypsin was from Worthington (Freehold NJ). Porcine pancreatic elastase was a gift from Dr D. Shotton (Cambridge). *Staphylococcus aureus* protease [20] was from Miles (Slough). Streptokinase (further purified before use [21]) was a gift from Dr H. Nilehn (Kabi, Stockholm). Sepharose CL-4B and Sephadex G-25 were from Pharmacia (Uppsala). Benzamidine, PMSF, reagents for manual sequence analysis and standard chemicals were from Merck (Darmstadt) or from Fluka (Buchs).

Manual sequence degradation was done [22] using heptane/ethyl acetate 2:1 (v/v) [23] instead of benzene and using diethyl ether [24] to extract the anilinothiazolinones from the dried peptide film or the partly dried protein film after having cleaved with TFA. PTH derivatives were determined by HPLC [25]. Sequenator analysis of proteinase-treated α_2M was performed in a Beckman 890C instrument and the resulting PTHs identified by HPLC and TLC [26]. The peptides described were isolated from enzymic digests of CNBr-fragments from α_2M or of reduced, carboxymethylated α_2M (citraconylated or not) by column chromatography and electrophoresis or chromatography on paper [17,27]. Amino acid compositions of peptides were determined after hydrolysis in 6 M HCl, 0.1% phenol at 110°C for 16–24 h using a Beckman 121MB [28]. An $E^{1\%}_{280\,nm} = 9.1$ [29] was used for α_2M.

3. Results

Solutions of freeze-dried α_2M in 0.05 M Tris–HCl, 0.1 M NaCl (pH 8.0) clarified by centrifugation, contained 3.4–4.3 mg α_2M/ml. Assuming that the trypsin, elastase and thrombin preparations contain 50–60% active enzyme and streptokinase-activated plasminogen [21] 80% active plasmin, an amount of proteinase was added to 15–20 ml α_2M which would give a 2-fold molar excess of active enzyme over α_2M. After incubating with trypsin for 1 min, plasmin for 2 min or thrombin for 60 min, 1 M benzamidine was added to a final concentration of 50–60 mM. Elastase and α_2M were incubated for 1 min and then 0.1 M PMSF was added to 20 mM. Resulting α_2M–proteinase complexes were precipitated by adding $(NH_4)_2SO_4$ to 60% saturation and centrifuging, then redissolved in 6 M urea, 0.1 M NaCl, 0.5 M HCOOH (pH 2.9) and chromatographed as shown in fig.1 for α_2M–trypsin complex.

Incubation of α_2M with trypsin or elastase as described above led to the almost complete cleavage of the 4 M_r 180 000 subunits into M_r 85 000 fragments as revealed by SDS–PAGE under reducing con-

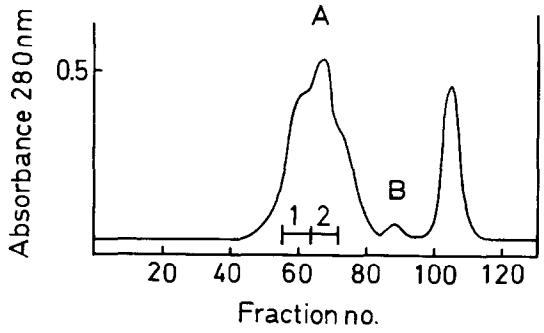

Fig.1. Gel filtration of 51 mg α_2M that had been incubated for 60 s with 5 mg TPCK-treated trypsin. The $(NH_4)_2SO_4$ precipitate was redissolved in 6 M urea, 0.1 M NaCl, 0.5 M HCOOH (pH 2.9) and applied to a column (2.5 × 90 cm) of Sepharose CL-4B, equilibrated and eluted with the same buffer at a flow rate of 20 ml/h (5 ml fractions). The effluent was collected in 2 pools (1,2) as indicated. After desalting on a column (5 × 20 cm) of Sephadex G-25 in 0.1 M HCOOH, the solutions were freeze-dried. Both pools 1,2, together constituting the irregular main peak (A), fractions 59–82, were found to contain α_2M half-molecules (M_r 360 000) and aggregates complexed with trypsin. The small peak (B) fractions 83–92, probably contain free trypsin. On an absorbance basis it would correspond to 15% of the added trypsin. The peak at fractions 100–110 contained residual benzamidine.

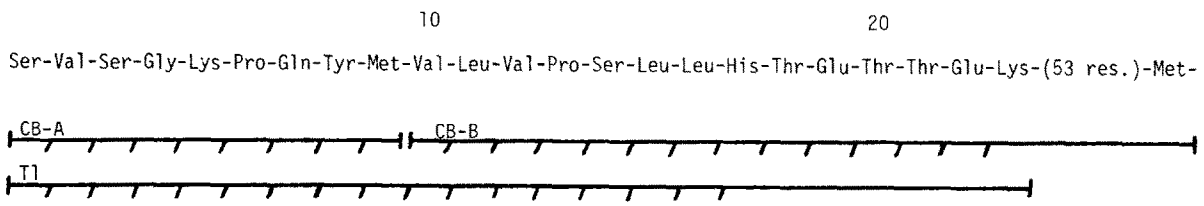

Fig.2. Amino acid sequence of residues 1–23 of human α_2M. Peptide nomenclature: CB-A, CB-B, CNBr-fragments; T1, tryptic peptide from reduced, carboxymethylated α_2M. Solid lines correspond to amino acid compositions, half arrows to manual sequence results.

ditions. However, the extent of cleavage by plasmin or thrombin was only ~50%.

The separation patterns obtained for α_2M incubated with the other 3 proteinases were very similar to that shown in fig.1. In particular, the amount of material eluting in the corresponding peak B could account for only 10–15% of the added amount of proteinase, indicating that the complexed proteinases remained bound to α_2M even under these strongly denaturing conditions.

Direct manual sequence determination through 6 steps of human α_2M gave the sequence:

Ser–Val–Ser–Gly–Lys–Pro– (fig.2)

From this, and the structures of T1, CB-A and CB-B, the sequence of the first 23 residues of α_2M was determined (fig.2). These results agree with and extend data [14] on residues 1–13 of α_2M. Both α_2M–trypsin and α_2M–elastase yielded a mixed sequence with two major residues released in each cycle of Edman degradation, one from the original N-terminus of α_2M (common to both sets of data) and one from the cleavage site in α_2M (tables 1,2). These two major sequences could be discriminated confidently through 12 steps using the sequenator procedure (tables 1,2).

Table 1
Major[a] N-terminal sequences of proteinase-treated α_2M

Position	Known sequences (fig.2,3)		Residues detected (nmol)[b] after proteolytic cleavage of α_2M with					
	New N-terminal	Original N-terminal	Trypsin		Plasmin		Thrombin	
1	Leu	Ser	Leu(25)	Ser(>10)	(Leu)	(Ser)	(Leu)	(Ser)
2	Val	Val	Val(50)		(Val)		(Val)	
3	His	Ser	His(20)	Ser(>10)	–	(Ser)	–	(Ser)
4	Val	Gly	Val(25)	Gly(>10)	(Val)	(Gly)	–	–
5	Glu	Lys	Glu(>10)	Lys(>10)	(Glu)	(Lys)	(Glu)	(Lys)
6	Glu	Pro	Glu(>10)	Pro(>10)	(Glu)	(Pro)	(Glu)	–
7	Pro	Gln	Pro(20)	Gln(15)	(Pro)	(Gln)	–	(Gln)
8	His	Tyr	His(10)	Tyr(15)	–	(Tyr)	–	(Tyr)
9	Thr	Met	Thr(10)	Met(10)	(Thr)	(Met)	–	(Met)
10	Glu	Val	(Glu)	(Val)				
11	Thr	Leu	(Thr)	(Leu)				
12	Val	Val	(Val)					

[a] Additional background could be interpreted as being derived from the known N-terminal sequences of the respective proteinase as discussed in the text

[b] PTH amino acids were analysed and quantitated by HPLC

Positions not properly identified are shown by dashes. Two preparations of α_2M–trypsin complex were analysed with similar results, using material from pools 1 and 2 (fig.1). About 10 nmol was used for each degradation. One preparation each of α_2M–plasmin and α_2M–thrombin complex, respectively, (pools 1 + 2 material, 12 nmol) were analysed with similar results (not quantitated). Brackets indicate residues identified but not quantitated

Table 2
Major[a] N-terminal sequences of elastase-treated α_2M

Position	Known sequences (fig.2,3)		Residues detected (nmol)[b] after proteolytic cleavage of α_2M with elastase	
	New N-terminal	Original N-terminal		
1	Gly	Ser	Gly(>10) Phe(>10)	Ser(>10)
2	Phe	Val	Phe(20) Tyr(<10)	Val(20)
3	Tyr	Ser	Tyr(15)	Ser(>10)
4	Glu	Gly	Glu(>10)	Gly(>10)
5	Ser	Lys	Ser(<10)	Lys(20)
6	Asp	Pro	–	–
7	Val	Gln	Val(10)	Gln(10)
8	Met	Tyr	Met(10)	Tyr(15)
9	Gly	Met	Gly(10)	Met(15)
10	Arg	Val	–	(Val)
11	Gly	Leu	(Gly)	(Leu)
12	His	Val	–	(Val)

[a] Additional background could be interpreted as being derived from the known N-terminal sequence of elastase
[b] PTH amino acids were analysed and quantitated by HPLC

Positions not properly identified are shown by dashes; 10 nmol of pools 1 and 2 material from one preparation of α_2M–elastase complex was degraded with similar results. Brackets indicate residues identified but not quantitated

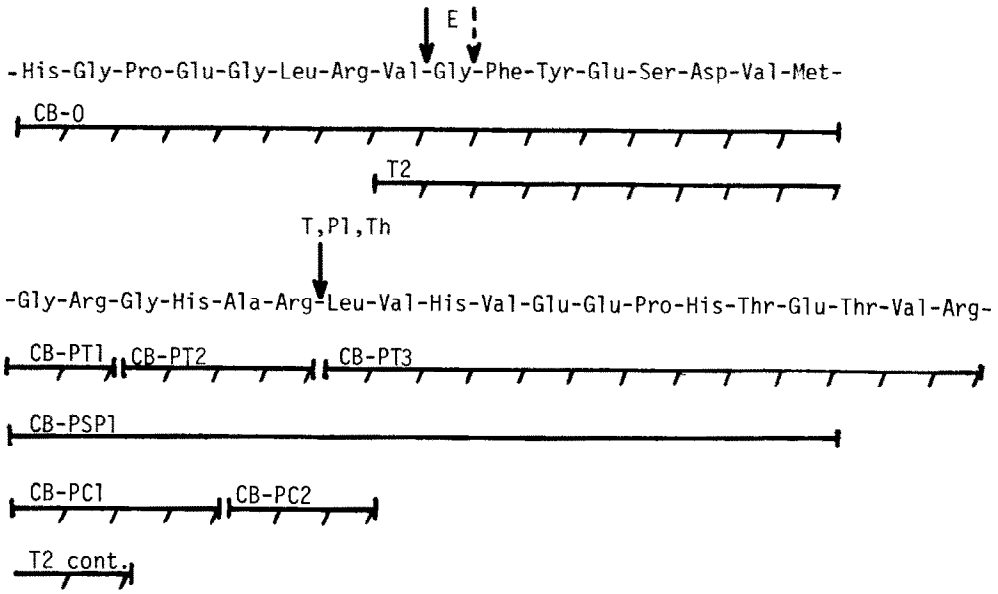

Fig.3. Amino acid sequence of a 35 residue stretch from the middle of the α_2M subunits containing the cleavage sites for trypsin (plasmin, thrombin) and for elastase, respectively. Peptide nomenclature: CB-O and CB-P, CNBr fragments from α_2M; T2, tryptic peptide from reduced, carboxymethylated, citraconylated α_2M; CB-PT1-3, tryptic peptides from CB-P; CB-PSP1, peptide from maleylated CB-P obtained after digestion with a protease from *Staphylococcus aureus* strain V8; CB-PC1-2 chymotryptic peptides from CB-P. Because its N-terminal was blocked the sequence of CB-PSP1 was deduced from the sequences of its three tryptic subpeptides corresponding to CB-PT1-2 and residues 1–10 of CB-PT3: (↓) the cleavage site for trypsin (plasmin, thrombin) and the major cleavage site for elastase; (↓̊) minor cleavage site for elastase; T, trypsin; Pl, Plasmin; Th, thrombin; E, elastase.

The new N-terminal sequence revealed at the trypsin cleavage site was deduced as:

Leu–Val–His–Val–Glu–Glu–Pro–His–Thr–
Glu–Thr–Val–

The new N-terminal sequence deduced for the elastase cleavage site was:

Gly–Phe–Tyr–Glu–Ser–X–Val–Met–Gly–X–
Gly–X

The results (table 2) also indicate the presence of an additional minor new N-terminal α_2M-sequence in the α_2M–elastase, namely Phe–Tyr–. By manual sequence determination, however, 4 residues of the latter sequence could be deduced extending it to:

Phe–Tyr–Glu–Ser–

The 3 sequences thus identified form part of a 298 residue stretch in the part of α_2M already sequenced. Fig.3 shows the relative positions of these cleavage sites in a 35 residue segment of this 298 residue stretch. The sequence of this segment had been deduced by combining data from amino acid analysis (table 3) and manual sequence determination. The minimal evidence required to prove the sequence is presented in fig.3 (redundant information not included). Despite the fact that plasmin and thrombin cleaved α_2M only to ~50% the characteristic sequence:

Leu–Val–X–X–Glu–Glu (table 1)

could be identified in both cases. Thus, thrombin and plasmin cleaved at the same site as trypsin in the 'bait' region.

In addition to the major sequences derived from α_2M itself part of the known N-terminal sequence of the proteinase, namely Ile-1, Gly-3, Tyr-5, Ala-9 of trypsin in a yield of 0.3 mol/mol α_2M-subunit and Val-1, Gly-3, Ala-7, Asn-10 of elastase in a yield of 0.25 mol/mol α_2M-subunit, was recognized as 'background'. In α_2M–thrombin Glu-3 and Ala-7 from the enzyme and in α_2M–plasmin Val-1, Gly-3, Val-6 and Ala-7 were recognized in lower yield.

Table 3
Amino acid composition, yields and electrophoretic mobilities of peptides used for deducing the sequences of the two stretches from α_2M shown in fig.2,3[a]

	CB-A	CB-B	T1	T2	CB-O	CB-PT1	CB-PT2	CB-PT3	CB-PSP1	CB-PC1	CB-PC2
CmCys		1.6 (2)									
Asx		6.0 (6)		1.0(1)	1.0(1)						
Thr		5.6 (6)	2.9(3)					2.0(2)	0.8(1)		
Ser	1.8(2)	7.7 (8)	2.7(3)	0.8(1)	0.8(1)						
Glx	1.0(1)	8.7 (8)	3.2(3)	1.0(1)	2.3(2)			3.1(3)	3.0(3)		
Pro	0.9(1)	2.3 (2)	2.1(2)		1.1(1)			1.1(1)	1.0(1)		
Gly	1.0(1)	2.3 (2)	1.0(1)	1.8(2)	3.0(3)	1.0(1)	1.0(1)		1.8(2)	2.0(2)	
Ala		4.2 (4)					0.9(1)		0.8(1)		1.0(1)
Val	1.2(1)	11.0(10)	3.3(3)	1.9(2)	2.3(2)			3.1(3)	1.9(2)		
Met[d]	+ (1)	+ (1)	0.9(1)	0.8(1)	+ (1)						
Ile											
GlcN		+									
Leu		10.2(10)	3.1(3)					1.0(1)	1.1(1)		1.0(1)
Tyr	1.1(1)	1.0 (1)	1.0(1)	1.2(1)	0.9(1)						
Phe		2.2 (2)		1.2(1)	0.9(1)						
Lys	1.0(1)	2.2 (2)	2.0(2)			0.8(0)[e]					
His		1.9 (2)	1.0(1)		0.9(1)		1.0(1)	2.0(2)	2.6(3)	1.0(1)	
Arg		2.0 (2)		1.0(1)	0.9(1)	1.0(1)	1.0(1)	1.0(1)	2.1(2)	0.9(1)	1.0(1)
Yield(%)	10	30	12	15	16	10	15	25	14	5	21
m 6.5[b]	−0.27		−0.06	−0.23	0.35	−0.77	−0.68	0	0[f]	−0.68	−0.73
m 1.9[c]	0.95		0.95	0.84	0.92			1.32			

[a] The compositions are listed as molar ratios with the residue used as a basis for the calculation underlined. The number of residues expected from the sequence analysis is listed in brackets. The values are not corrected for incomplete hydrolysis losses
[b] Electrophoretic mobility relative to aspartic acid at pH 6.5; [c] Electrophoretic mobility relative to serine at pH 1.9; [d] Homoserine and homoserine lactone in CNBr fragments were not quantitated; [e] CB-PT1 was contaminated with free lysine; [f] CB-PSP1 was carbamylated

4. Discussion

Assuming an initial coupling yield of 60% the N-terminal sequence of α_2M was recovered in a yield compatible with α_2M being composed of 4 identical subunits of M_r 180 000 [14,17].

Moreover, the yield of the additional N-terminal sequence exposed as a result of the specific cleavage by trypsin or elastase during their complex formation with α_2M was essentially the same as that obtained for the original N-terminal sequence, showing that the sequences of all 4 subunits are identical in this region, and also that complete cleavage of all 4 subunits had occurred at closely spaced sites in the 'bait' region. Trypsin, plasmin and thrombin were found to cleave at the same Arg–Leu bond (fig.3). Whether additional cleavage had occurred at the Arg–Val and Arg–Gly bonds located 4 and 15 residues before the Arg–Leu bond is not known, since the resulting small peptides would have escaped detection in the present experiments. Elastase was found to cleave α_2M in 2 other places, a major cleavage at the Val–Gly bound and a minor cleavage at the following Gly–Phe bond in the 'bait' region sequence (fig.3). The 2 elastase-cleavage sites are located 13 and 14 residues before the common trypsin–plasmin–thrombin cleavage site, showing that the bait region comprises at least 15 residues.

Although it is not known whether cleavage of any peptide bond within the bait region will lead to formation of α_2M–proteinase complexes, these data show that cleavage of α_2M by the proteinases investigated reflects their primary substrate specificity. This indicates that only those proteinases which can cleave (a) specific bond(s) within the bait region can form a complex with α_2M. The trypsin/thrombin/plasmin cleavage site is located ~665–700 residues from the N-terminal.

The stability of α_2M–proteinase complexes during SDS–PAGE under reducing conditions suggests that the proteinase binds covalently to α_2M [30–32]. This conclusion is supported by earlier results [15] and the present finding, that most of the proteinase is not released from α_2M–proteinase complexes during gel filtration under denaturing conditions (fig.1). Furthermore the N-terminal sequences of the proteinases were recognized in the α_2M-complexes. The 4 chains of α_2M [16,33] and the α'-chain part of complement component C3 [34,35] both contain an internal reactive thiol ester, involving the γ-carboxyl group of the Glx-residue and the thiol group of the Cys-residue [33,34] in the identical heptapeptide sequences:

—Gly—Cys—Gly—Glu—Glx—Asn—Met—

in the 2 proteins [33,34,36]. The Glx-residue is located ~256 residues after the tryptic cleavage site in the bait region. Upon activation of α_2M by trypsin or pancreatic elastase [16] and of C3 with trypsin [34,35] their thiol ester group/s is/are rapidly cleaved resulting in the appearance of SH-groups [16,34,35]. On the basis of these results it has been suggested that α_2M and C3, when activated, participate in 'acyl-transfer' reactions, leading to the formation of covalent bonds between α_2M and proteinase [16] or between C3 and specific cell-surface structures [34,35]. Because the 'bait' region cleavage reflects the specificity of the proteinase the 'initial' α_2M–proteinase complex must involve the active site of the proteinase binding to the 'bait' region of α_2M. Because the 'final' complex, which presumably involves the thiol-esterified Glx of α_2M [16], is active against small substrates for the proteinase it *cannot* involve the active site of the proteinase. Thus, α_2M–proteinase complex formation occurs in at least 2 structurally well-defined steps. The 'final' complex is rapidly removed from the circulation [37], being taken up by cells, e.g., macrophages and fibroblasts [38–40]. Therefore, the formation of α_2M–proteinase complexes following cleavage of a single peptide bond in the 'bait' region can now be recognized as another example of a physiological process activated by specific limited proteolysis. Because bait-region cleavage in α_2M and anaphylatoxin release from C3 and C5 now seem to play corresponding roles in the 'activation' of these proteins the sequence preceding the cleavage sites in the bait region of α_2M was

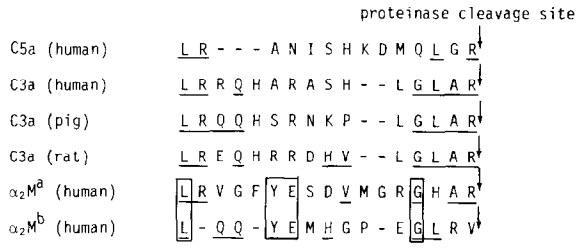

Fig.4. Sequence similarities found by comparing the total sequences of anaphylatoxins C3a and C5a with the 80-odd residue sequence of α_2M preceding the bait region cleavage sites for trypsin/thrombin/plasmin and for elastase. Identities between the complement components on the one hand and one of the α_2M-sites on the other are underlined. Mutually identical residues between the 2 α_2M-sites are boxed. [a] α_2M, the trypsin, thrombin, plasmin-cleavage site; [b] α_2M, the major elastase-cleavage site.

compared with the mutually homologous sequences of C3a and C5a [41] by visual inspection. The sequence similarities found in the last 14–17 residues before the cleavage sites (fig.4) may be the result of analogous/homologous evolution of these parts of α_2M and the 2 complement components.

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