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PROTEOLYTIC CLEAVAGE SITES ON α_2-MACROGLOBULIN RESULTING IN PROTEINASE BINDING ARE DIFFERENT FOR TRYPSIN AND STAPHYLOCOCCUS AUREUS V-8 PROTEINASE

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SUMMARY

The carboxyl and amino terminal sequences of the fragments of the α_2-macroglobulin subunits resulting from the single proteolytic cleavage occurring upon formation of proteinase-α_2-macroglobulin complexes have been compared following reaction with either trypsin or the Staphylococcus aureus V-8 proteinase. Trypsin cleaves a Lys-Leu bond whereas the V-8 proteinase cleaves a Glu-Gly bond. Thus, the cleavage site reflects the specificity of the proteinase rather than the presence of a certain labile bond.

INTRODUCTION

Human α_2M[1] consists of four identical subunits of M_r = 185,000 (1,2). Swenson and Howard (2) have reported a single amino terminal sequence of NH_2-Ser-Val-Ser-Gly-Lys-Pro (etc) and a COOH-terminal sequence of (Ala,Tyr)(Glu,Val) Leu-COOH. Incubation of α_2M with a wide variety of endopeptidases results in a cleavage near the center of the subunit termed the "bait region" which produces two fragments of M_r = 88,000 and 97,000 (3,4). The M_r values of the fragments are independent, within experimental uncertainty, of the proteinase used. Fol-

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1 Abbreviations: α_2M = α_2-macroglobulin
PEG = polyethylene glycol
DPCC = diphenyl carbamyl chloride
SDS = sodium dodecyl sulfate
PAGE = polyacrylamide gel electrophoresis
CPA-DFP = carboxypeptidase A-diisopropylfluorophosphate
CPB-PMSF = carboxypeptidase B-phenylmethylsulfonylfluoride
CPY-R = carboxypeptidase Y-resin bound

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lowing cleavage, a rearrangement of the α_2M tertiary structure occurs which results in the formation of a proteinase-α_2M complex and the appearance of four thio groups per mole of α_2M (5). Sottrup-Jensen et al (5) have proposed that a thio-ester bond is cleaved in α_2M preceding the formation of a γ-glutamyl-proteinase linkage. This bonding site appears to be the same glutamyl residue where methylamine binds (6) and the site of heat cleavage (7,8) which produces two α_2M fragments of M_r = 120,000 and M_r = 60,000.

Whether the proteinase cleavage site involves a specific labile bond or whether proteinases hydrolyze a peptide bond somewhere in the "bait region" according to their specificity is uncertain. The object of this paper is to report the isolation and sequencing of α_2M proteinase cleavage fragments (termed 90 K fragments) following incubation with two proteinases, trypsin and V-8 proteinase, which have markedly different specificities.

MATERIALS AND METHODS

Purified α_2M was obtained from fresh human citrated plasma by a modification of the method of Kurecki et al (9). PEG (Eastman 6000) was used in place of ammonium sulfate for the precipitation steps as described by Barrett et al (10). The 5.5%-12.5% PEG precipitate was dissolved in 0.02 M sodium phosphate-0.15 M NaCl, pH 6.0 and applied to the zinc chelate column. Occasionally, if necessary, further purification was accomplished by gel filtration on a 2.5 x 110 cm Sephacryl S-300 (Pharmacia, Piscataway, NJ) column using a 0.05 M sodium citrate, pH 6.5 buffer. To assure that α_2M samples had not undergone proteolytic cleavage during isolation, they were examined by SDS-PAGE (11) (7.5% total polyacrylamide using 2.6% bis-acrylamide) following reduction as stated below. All samples used showed only the 185 K subunit.

Isolation of 90 K α_2M fragments after incubation with trypsin or V-8 proteinase. The 90 K α_2M fragments were prepared by a 2 min, 20°C incubation of purified α_2M at 10 mg/ml in 0.02 M sodium citrate, pH 6.5 with a 2.0 molar ratio of active site titrated (12), DPCC treated trypsin (Sigma, St. Louis, MO, lot 19C-8085, 49% active) or a 1.5 molar ratio of V-8 proteinase (Miles, Elkhart, IN, lot 0590). The protein was reduced by making the solution 1% in 2-mercaptoethanol, 1% in SDS and immediately placing in a 100°C water bath for 2 min. These reduction conditions produced none of the heat induced α_2M fragments (Fig. 3). The 90 K fragments were isolated by gel filtration on a 2.6 x 90 cm Sephacryl S-300 superfine column using a 0.05 M sodium citrate, pH 6.5, 0.2% SDS, 0.03% NaN_3 buffer. Fractions were examined by slab gel SDS-PAGE (11) and those containing purified 90 K α_2M fragments were pooled, dialyzed against distilled water or 20% acetic acid and freeze-dried.

COOH-terminal analysis of α_2M subunits and 90 K fragments. COOH-terminal analysis was performed by two independent methods, hydrazinolysis (13) and enzymatic hydrolysis with the following carboxypeptidases: (CPA-DFP treated, Sigma), (CPB-PMSF treated, Worthington) by the method of Ambler (14) and Howard et al (7) and (CPY-R, Pierce) according to Hayashi (15). The experimental

TABLE 1

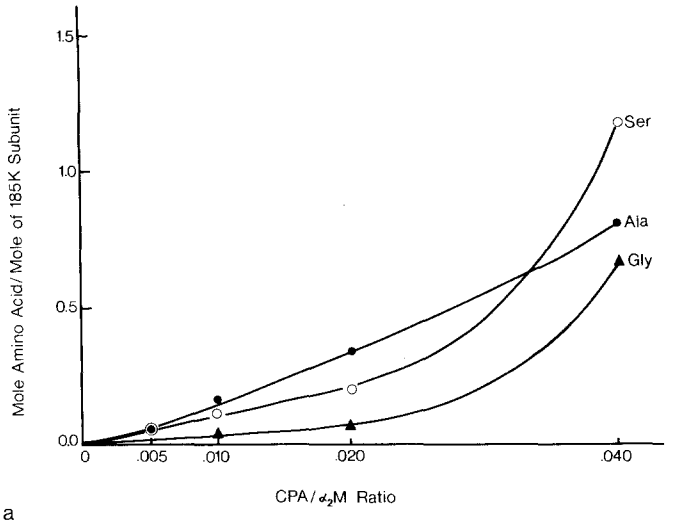
Carboxyl Amino Acids Released From α_2M and Its Fragments by Various Methods

Sample	Method	Moles Amino Acid Released Per Mole of α_2M Subunit							
		Asp	Ser	Glu	Gly	Ala	Leu	Tyr	Lys
Native α_2M	CPA + CPB[a] (22 hr)	0.00	0.20	0.00	0.01	0.58	0.10	0.00	0.00
	Hydrazinolysis[b]	0.00	0.18	0.00	0.68	0.91	0.05	0.00	0.00
	CPY-R[c]	0.00	0.30	0.00	0.00	0.48	0.63	0.00	0.00
185 K subunit	CPA[d] (1 hr)	0.76	1.54	0.24	1.06	0.94	0.28	0.16	0.00
	CPA[e] (15 min)	0.00	0.19	0.00	0.06	0.33	0.03	0.00	-
90 K fragments (S. aureus V-8)	CPA[e] (15 min)	0.00	0.65	0.54	0.11	0.36	0.38	0.53	-
	Hydrazinolysis[b]	0.00	0.18	0.18	0.08	0.90	0.00	0.00	0.00
90 K fragments (Trypsin)	CPA[f] and CPB	0.14	0.13	0.00	0.00	0.07	0.05	0.00	0.66
	Hydrazinolysis[g]	-	-	-	-	-	-	-	0.90

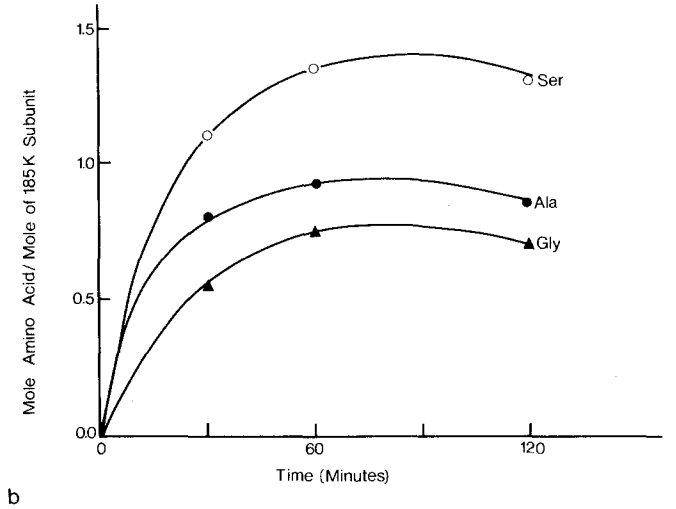
a 6.0 nmoles α_2M at 37°C, 22 hr, pH 9.1, 0.1 M phosphate, 0.72% SDS
b Average of two trials, 11.5 and 25.0 nmoles of α_2M at 80°C for 24 hr
c 4.0 nmoles α_2M at 37°C, 7 hr, 0.1 M N-ethyl morpholine, pH 6.0, 0.5% SDS. Additional amino acids also appearing were Ile (0.38) and Thr (0.25)
d 5.0 nmoles α_2M reduced in 1% 2-mercaptoethanol, 1% SDS at 100°C for 2 minutes in 0.05 M citrate, pH 6.5. Carboxymethylation with recrystallized iodoacetic acid (19). After dialysis against distilled water and 4 M urea, pH 7.0, 0.05 M tris, the sample was incubated with a 1/25 CPA/α_2M ratio for 1 hr at 37°C.
e 10.0 nmoles α_2M with a 1/50 CPA ratio, 15 minutes, 37°C, 4 M urea, pH 7.0, 0.05 M tris
f 9.7 nmoles α_2M. Incubation same as (e). 0.37 moles of Thr per mole of subunit formed in addition to those shown.
g 4.0 nmoles α_2M at 80°C for 24 hr. Short column (basic amino acids) run only.

conditions are provided in Table 1 and Fig. 1. Amino acids were determined with an updated Spinco model 120B (Beckman Instruments, Palo Alto, CA) amino acid analyzer. All values are corrected for zero time and enzyme blanks. Percent recovery, as measured from the yield of added norleucine as an internal standard, exceeded 90%. Samples subjected to hydrazinolysis were vacuum dried 48 hr at 37°C over P_2O_5. Transfers of 2.0 ml of reagent grade hydrazine (anhydrous, 97+%; Matheson, Coleman, and Bell, Norwood, OH) from previously unopened bottles were performed in a N_2 flushed dry box. The free COOH-terminal amino acid produced by incubation of 11.5 to 25.0 nmoles of α_2M with 2.0 ml of hydrazine in a sealed tube for 24 hr at 80°C was isolated from the hydrazides by elution through 1 x 3 cm Amberlite IRC-50 column with 30 ml of 0.1 M ammonium acetate, pH 7. The recovery exceeded 75%.

NH_2-terminal sequence analysis. The NH_2-terminal sequence of the reduced α_2M subunit and the 90 K fragments was performed using a Beckman model 890C sequencer. A 0.1 M Quadrol program (manufacturer No. 030176) was utilized in all analyses. Residues were analyzed by either conversion to phenylthiohydantoins and analysis by high pressure liquid chromatography (16) or by amino acid analysis after back hydrolysis (17). Initial yields ranged from 45-55% for the three sets of sequences.



a



b

FIG. 1: CPA incubation of reduced α_2M under different conditions of enzyme concentration and incubation time. 1a) Reduced α_2M, 10.0 nmoles, incubated 15 min at 37°C, 0.05 M tris-Cl, pH 7.0, 4 M urea with CPA/α_2M weight ratios of 1/200, 1/100, 1/50, and 1/25. The protein was precipitated with acetone at 0°C, the supernatant freeze-dried, and the residue dissolved in 1.0 ml of pH 2.2, 0.2 N sodium citrate and applied to an automatic amino acid analyzer. Values are corrected for enzyme blanks and a zero time blank in which 10.0 nmole of α_2M was precipitated with acetone immediately after adding CPA at a 1/25 ratio. 1b). Reduced α_2M, 44.0 nmoles, incubated at a 1/25 CPA ratio as above. At 0, 30, 60, and 120 min 11.0 nmoles samples were withdrawn and the protein precipitated with acetone.

RESULTS

COOH-terminal analysis of α_2M and its fragments. The release of COOH-terminal amino acids after incubation of reduced α_2M with CPA is shown in

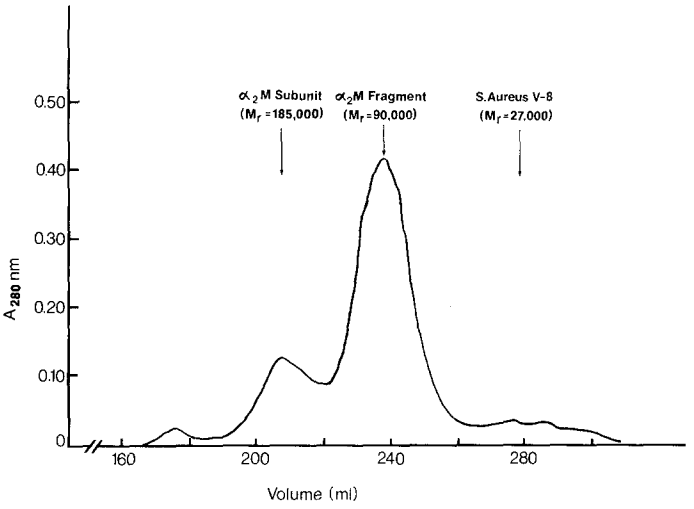


FIG. 2: Isolation of α_2M 90 K fragments. α_2M (15 mg) was incubated 2 min with 0.86 mg of V-8 proteinase in 2.5 ml of 0.05 M sodium citrate, pH 6.5 and reduced at 100°C in 1% 2-mercaptoethanol and 1% SDS for 2 min. After cooling, the sample was applied to a Sephacryl S-300 column (2.6 x 89 cm) and eluted with 0.05 M sodium citrate, pH 6.5, 0.2% SDS at a flow rate of 20 ml/hr.

Fig. 1 as a function of enzyme concentration and time of incubation. At low concentrations of CPA, the principal amino acid released is alanine while at CPA/α_2M ratios greater than 1/50 serine becomes prominant. Since 1.36 moles of serine and 0.92 moles of alanine are present per mole of 185 K α_2M subunit after 1 hr incubation at a 1/25 CPA/α_2M ratio, it appears that two serine residues must be present near the COOH-terminus of the α_2M subunit. Hydrazinolysis further confirms alanine as the COOH-terminal yielding 0.91 moles per mole of α_2M 185 K subunit. In addition to alanine, incubation with CPY yielded leucine as a prominant free amino acid. The source of leucine, however, may not be from the COOH-terminal position of α_2M since Hayashi (15) indicates that leucine or phenylalanine sometimes unexpectedly appear during prolonged incubation of proteins, which are resistant to attack by CPY. Based on these results, the most probable COOH-terminal sequence for the α_2M 185 K subunit is -Ser-Gly-Ser-Ala-COOH. The isolation of the 90 K fragments of α_2M by gel filtration after incubation with trypsin or V-8 proteinase is shown in Fig. 2. A 75+% conversion of the 185 K subunit into the 90 K fragments was obtained using

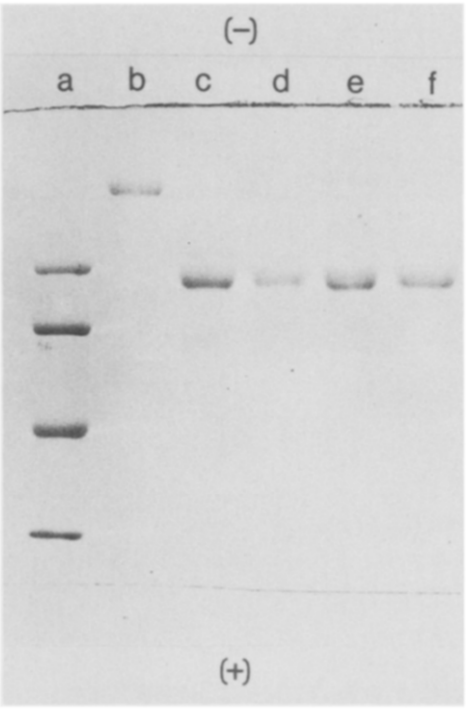


FIG. 3: SDS-PAGE of α_2M 90 K fragments. Reduced α_2M fractions after incubation with proteinases and isolation in the Sephacryl S-300 column (Fig. 2) are examined on a 0.75 mm SDS-7½% polyacrylamide gel (11). Slot (a) contains Pharmacia, Inc. (Piscataway, NJ) high molecular weight standards. The relative molecular weights of the bands in descending order are 94 K, 67 K, 43 K, and 30 K. Slot (b) contains reduced α_2M, slot (c) trypsin treated α_2M and reduced, slot (d) isolated 90 K α_2M fragment, slot (e) V-8 proteinase treated α_2M and reduction, slot (f) isolated 90 K α_2M fragment.

2.0 and 1.5 molar ratios of trypsin and V-8 proteinase, respectively. The final yield of 90 K α_2M fragments was 30%. SDS-PAGE (Fig. 3) of the final products revealed no protein bands other than 90 K. Incubation of the V-8 proteinase derived fragments with CPA yielded glutamic acid, tyrosine, and leucine in addition to the three prominant amino acids (alanine, serine and glycine) released from the 185 K subunit (Table 1). Hydrazinolysis resulted in the release of alanine, glutamic acid, and glycine from the 90 K fragments. These results suggest that the new 90 K COOH-terminal sequence produced by cleavage of the 185 K subunit with V-8 proteinase is -Leu-Tyr-Glu-COOH.

Incubation of the trypsin-produced 90 K fragments with CPB produced 0.66 moles of lysine per mole of 185 K subunit while a control sample of 185 K

TABLE 2

NH_2 Sequence Analysis of α_2M, Trypsin, and V-8 Proteinase Generated Fragments

Sample	Cycle 1	2	3	4	5	6
α_2M subunit	Ser	Val	Ser	Gly	Lys	Pro
90 K fragment (V-8 proteinase)	Ser	Val	Ser	Gly	Lys	Pro
	Gly	Asp	Val	Met	Gly	Ala
90 K fragment (trypsin)	Ser	Val	Ser	Gly	Lys	Pro
	Leu	Val	Ser	Val	Glu	Glu

α_2M yielded no basic amino acids. Hydrazinolysis confirms lysine as an additional COOH-terminal amino acid formed after proteolytic cleavage of α_2M by trypsin. Incubation of the trypsin produced 90 K fragment with CPA yielded alanine, serine, threonine and aspartic acid as the principal acidic and neutral amino acids. Thus, the most plausible new α_2M 90 K COOH-terminal sequence formed by action of trypsin upon α_2M is -Asp-Thr-Lys-COOH.

NH_2-terminal sequence. The NH_2-terminal sequence analysis was performed on 100 nmoles of purified, reduced 185 K α_2M and the two 90 K proteinase produced fragments. The results of six cycles are shown in Table 2. Repetitive yields could not be calculated because of the mixed sequences. However, the amounts of the original amino acid to the new was about 0.6 to 0.4 for the V-8 proteinase-cleaved fragments and 0.55 to 0.45 for the trypsin-cleaved fragments.

DISCUSSION

Both the COOH-terminal and NH_2-terminal results support the conclusion that α_2M is cleaved in a "bait region" according to the specificity of the proteinase rather than at a specific labile bond or "active site". A different, NH_2-terminal sequence appears in the 90 K α_2M fragments isolated after incubation with V-8 proteinase as compared to those isolated after a similar treat-

ment with trypsin. Moreover, the new COOH-terminal amino acid produced by such treatment agrees with the specificity of the proteinase-glutamic acid for the V-8 proteinase and lysine for the trypsin. Based on these results we conclude that the V-8 proteinase cleavage site (marked ↓) occurs in the following sequence of the α_2M 185 K subunit, -Leu-Tyr-Glu↓Gly-Asp-Val-Met-Gly-Ala- whereas the trypsin cleavage site appears between lysine and leucine in the following sequence: -Asp-Thr-Lys↓Leu-Val-Ser-Val-Glu-Pro-. Furthermore, since no overlap appears in the two sequences, the cleavage sites must be separated by a minimum of 10 amino acids and possibly more.

Although the NH_2-terminal sequences we determined for intact α_2M agree with that reported by Swenson and Howard (2), we find alanine to be the COOH-terminal instead of leucine using two independent methods. This result agrees with that suggested by Sottrup-Jensen et al (18).

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